Image-Pro® Plus

Version 7.0 for Windows™

Start-Up Guide

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MAN SU 41N70000 20090630

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Contents

Introducing Image-Pro Plus Version 7.0

Welcome to *Image-Pro Plus®* version 7.0. This version of *Image-Pro Plus* is designed to run under the Microsoft® Windows XP Professional 32-bit (service pack 2),Windows XP Professional 64-bit (service pack 3), and Windows Vista® 32- and 64-bit operating systems. These advanced image processing features are provided through the Microsoft Windows graphical user interface (GUI), delivering powerful imaging capabilities in an easy-to-use tool.

Product Features

Image-Pro Plus gives you state-of-the art imaging and analysis capability for acquiring, enhancing and analyzing your images. This includes the ability to:

- Acquire image data from a camera, microscope, or scanner
- Read and write image data in all the standard image file formats including, TIFF, JPEG, BMP, TGA, and many others.
- Work with gray scale data in 8, 12, 16, or 32-bit floating-point depths. Work with color data in palletized or 24-, 36-, or 48-bit format. Manipulate color data using RGB, HSI, HSV or YIQ models.
- Perform image enhancement using powerful color and contrast filters, including Fast Fourier Transforms (FFT), morphology, field flattening, background subtraction and other spatial and geometric operations.
- Trace and count objects manually or automatically. Measure object attributes such as: area, angle, perimeter, diameter, roundness and aspect ratio. Calibrate your spatial scale to any unit of measure.
- View collected data numerically, statistically or in graphic form (histogram and scattergram). Save the measurement data to disk.
- Sort and classify your measurement data according to your predefined criteria. Color code your objects by class.
- Collect intensity data for an entire image, an area of interest, a single line or a band of lines. Measure intensity using the standard intensity or Optical Density curves supplied by *Image-Pro Plus*, or create your own custom curve. View intensity data in graph or table form. Save the intensity data to disk.

- Extract features with spatial tools that isolate an Area of Interest (AOI) from the rest of the image, or with segmentation tools that extract features by color or intensity value.
- Create composite in-focus images from partially focused source images.
- Create composite images from multiple fluorescent probes.
- Generate reports that can include images, measurement data, text, and pictures.
- Manage your images using Media Cybernetics' *IQbase* product. Create organized collections of image thumbnails that can be easily loaded, located, and retrieved.
- Automate repetitive tasks and customize *Image-Pro Plus* to your particular needs using *Auto-Pro*, the powerful macro language that saves you real time. You can call *Auto-Pro* functions from high-level languages like Visual Basic and Visual C++. You can combine *Auto-Pro* calls with your own programs to create custom user-interface, processing and I/O routines.

What's New in Version 7.0

The following improvements have been made to *Image-Pro Plus v. 7.0:*

- The new features *Live EDF* and *Live Tiling* are available as add-ons to the *Image-Pro Plus program.* When licensed, these features will appear on the *Acquire* menu.
- *Stage-Pro* and *Scope-Pro* have been integrated into *Image-Pro Plus*. These features appear on the *Acquire* menu. The documentation for these products has been incorporated into the *Image-Pro Plus Reference Guid*e.
- *3D Surface Inspector, AFA, SharpStack,* and *3D Constructor* now appear as part of the *Image-Pro Plus* package on the *Advanced* menu if you have licensed them from *Media Cybernetics*. Acquisition speed through AFA has been improved for a selected set of microscopy hardware. In addition, additional shutter control options have been added to AFA.
- *Bayer mask interpolation:* A new post-acquisition feature that allows you to apply Bayer mask interpolation to images that were acquired as raw Bayer data has been added to *IPP 7.0.*
- *Capture improvements: Image-Pro Plus 7.0* contains improvements to capture support and control.
- *Just-in-time loading***.** This feature allows *Image-Pro* to load extremely large multi-frame image sets very quickly as the image data is only loaded when needed (e.g. for

display or processing). Note that not all file formats will be immediately supported with the just-in-time loading feature, but *Image-Pro* sets `(*.ips)`acquired through AFA, and large TIFF or SEQ sequences will be supported.

- *New file format support* : File format support is added for both the Streampix`.SEQ` file format and the MicroManager set format. These new file formats will also support the just-in-time loading mentioned above. This will also include bug fixes for some existing file formats.
- *IQbase 2.6*, the newest version of the Media Cybernetics image information management tool, is included with *Image-Pro Plus 7.0* to provide advanced storage, information management, and reporting ability. This complementary copy of *IQbase* is valid for six months. *Image-Pro Plus 7.0* is compatible with *IQBase* at all levels.
- *Improved menu customization*: For customers who will be using *Image-Pro* in concert with other applications, it is now possible to add menu items and toolbar buttons to invoke other applications. This could be used to invoke Microsoft Office products for documentation and reporting of your experiment, or to invoke StreamPix or MicroManager to acquire experiment data, to give just a few examples.
- Due to size constraints, the *Image-Pro Plus Reference Guide* and *Auto-Pro Reference* are no longer included as part of this package. All the information from those manuals appears in the online help. Printed manuals may be ordered from Media Cybernetics' Customer Service department.
- Many new *Auto-Pro* functions have been developed to support *Bayer Interpolation* and other advanced features of *Image-Pro Plus*.

Package Contents

Before you get started, please check that you have received everything in your *Image-Pro Plus* package:

- 1 *Image-Pro Plus* product CD
- 1 *Image-Pro Plus* CD with additional sample images
- 1 USB port copy-protection plug
- *Image-Pro Plus Start-Up Guide* (this book)
- Registration/warranty card

If any piece is missing, please contact Customer Service at (301) 495-3305.

Important: Updated Capture Drivers may be downloaded from our web site. To check for new drivers, please visit http://support.mediacy.com

System Requirements

To install and work with *Image-Pro Plus 7.0*, you will need the following equipment and software:

- Pentium IV CPU, running at 1.6 Ghz or higher configured with Windows® XP Professional (service pack 2), ,Windows XP Professional 64-bit (service pack 3), or Windows®Vista® 32- or 64-bit Business and Ultimate.
- A minimum of 2 GB of RAM recommended
- 60 GB disk drive with available disk space for installation with additional space to accommodate four times the image size with four bytes/pixel
- An SVGA (or better) high color, 16-bit resolution graphics card with *DirectX 9.0c or above* graphics support, a hardware accelerator, and a minimum of 32Mb memory that supports 2D texturing
- A Windows-compatible mouse, keyboard, and CD-ROM.

The following hardware is optional:

- Digital Camera
- Image Capture Board (frame-grabber)
- Scanner
- Printer.

Upgrading From A Previous Version Of Image-Pro Plus

If you are upgrading from an earlier version of *Image-Pro Plus*, the *Image-Pro Plus* setup will guide you through the process of upgrading from version 5. 0/5.1/6.0 to version 7.0. If you are upgrading from version 5.X or earlier, we recommend that you consider the following before installing version 7.0. (You may have more than one version of *Image-Pro Plus* installed on your computer.)

Image-Pro Plus version 7.0 contains many new and enhanced features. Some of these features may perform differently than similar features in previous versions of *Image-Pro Plus*. To insure a smooth migration to *Image-Pro Plus version 7.0* we suggest that you ***leave*** your previous version of *Image-Pro Plus* installed on your system and install *Image-Pro Plus version 7.0* in a ***separate*** directory.

After installing *Image-Pro Plus version 7.0*, use ***both*** versions and when you are satisfied that your old macros, settings, etc., perform to your satisfaction in *Image-Pro Plus version 7.0,* remove the previous version of *Image-Pro Plus*.

For additional information regarding possible migration issues, please see the *Release Notes*.

Installing Image-Pro Plus

To install *Image-Pro Plus*, follow the steps below.

If you are installing *Image-Pro Plus* as an upgrade to your existing version, please see the section entitled *Upgrading Your Image-Pro Plus Software* later in this chapter.

Note that *Image-Pro Plus 7.0* will not install on versions of *Windows* older than *Windows XP Professional.*

STEP	PROCEDURE
1.	Attach the copy protection plug to an operable USB port. Without this plug, *Image-Pro Plus* **will not run**. More information about the plug appears in the following section.
2.	Place the *Image-Pro Plus* CD in your CD-ROM drive. Within a few seconds, the **Setup** program will start running automatically.
3.	If the CD browser does not start automatically, click the **Start** button on your *Windows* ***desktop,*** and choose **Run.** Use the **Browse** button to find the your *Image-Pro Plus* CD. Select the **Start** program. The path is: **`CD Drive letter:\start.exe`**

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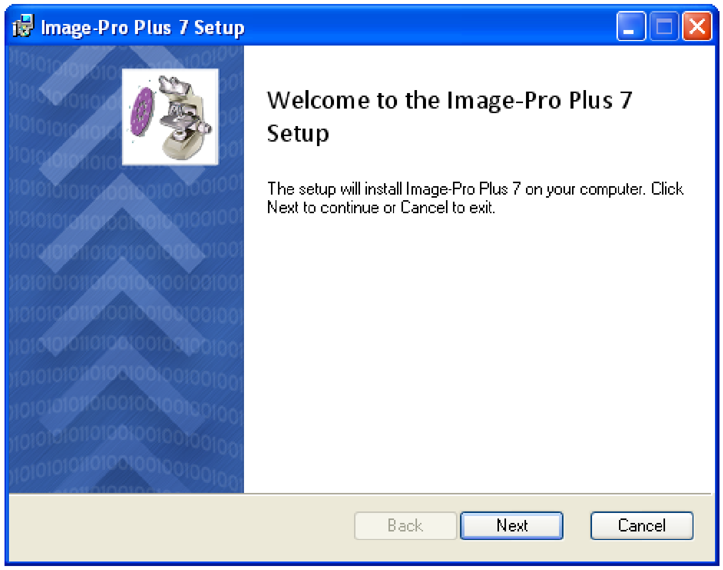
<table>
<tr><th>STEP</th><th>PROCEDURE</th></tr>
<tr><td>4.</td><td>After clicking Install Image-Pro Plus on the Autostart dialog, Image-Pro Plus will ask you to wait for a few minutes to prepare the Install program.


Note that this may take some time, depending on your computer specifications.</td></tr>
</table>

continued on next page

<table>
<tr><th>STEP</th><th>PROCEDURE</th></tr>
<tr><td>5.</td><td>You will see the Image-Pro Plus Welcome screen. After reading it, click Next to continue installing Image-Pro Plus.


Note that you may have only one installation of Image-Pro Plus 7.0 on your computer at any one time.</td></tr>
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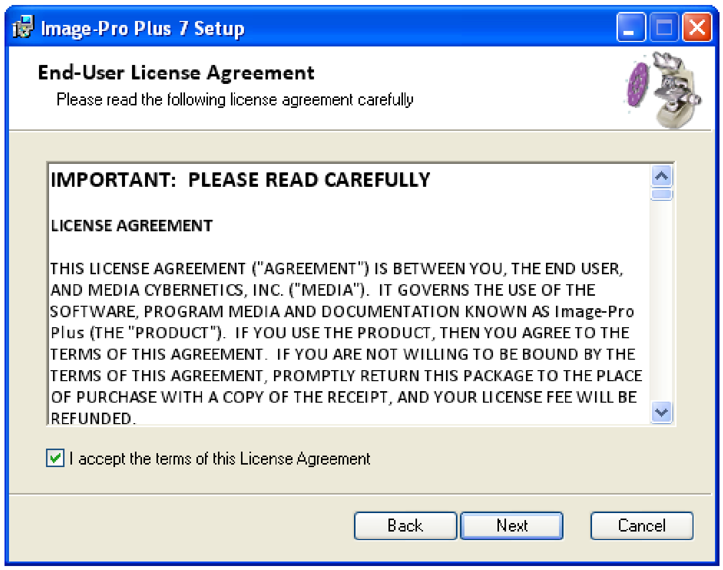
<table>
<tr><th>STEP</th><th>PROCEDURE</th></tr>
<tr><td>6.</td><td>If you have choosen to install or modify Image-Pro Plus, you will see the Image-Pro Plus license agreement. Click Yes to continue the installation process.



</td></tr>
</table>

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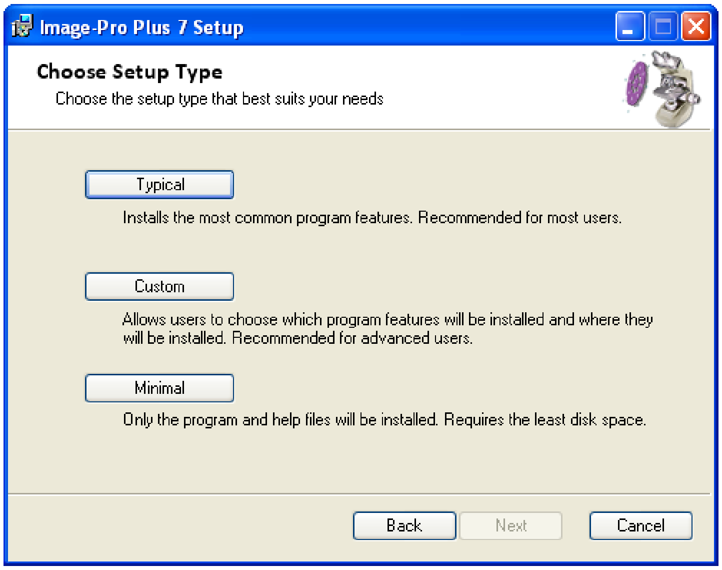
STEP	PROCEDURE
7.	The next screen asks if you want to install, modify, or remove the selected program. This screen will *not* appear if you are installing *Image-Pro Plus 7.0* for the *first* time.  Select one of the options, and click **Next**.

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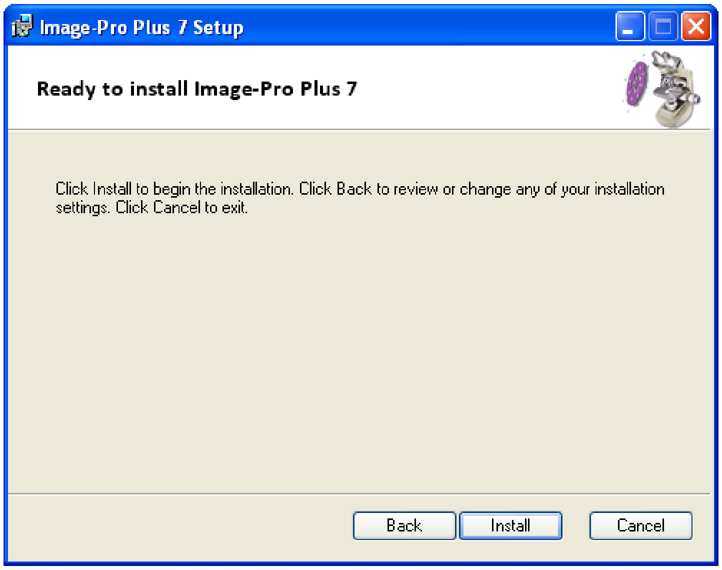
STEP	**PROCEDURE**
8.	Select the type of installation you require from the following choices, as shown on the next screen: 

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STEP	**PROCEDURE**
9a.	Typical is the recommended installation for most computers. Typical gives you all the *Image-Pro Plus* files, including the online help files, as well as the sample images. If you are installing *Image-Pro Plus* for the first time, this is the setup you should choose. If you select Typical, continue the installation process from step 10. If you would like to install additional sample images, click the Install Sample Images button. If you are installing *Image-Pro Plus* to be used with a red network key, choose the Typical installation and then follow the instructions on page 1-15 for installing the network key License Server and Clients. Compact allows you to install a reduced set of files to save space on your hard drive. Custom allows you to choose which Image-Pro Plus components you want to install. If you choose a Custom installation, continue from Step 14. Click on the button for your selected installation.
10.	Select the location for *Image-Pro Plus.* The default destination for *Image-Pro Plus* is the C:\ drive in the directory IPWIN7. You can specify a different location by clicking the Browse button, and selecting or typing in a new name.Click Next to continue the installation process.

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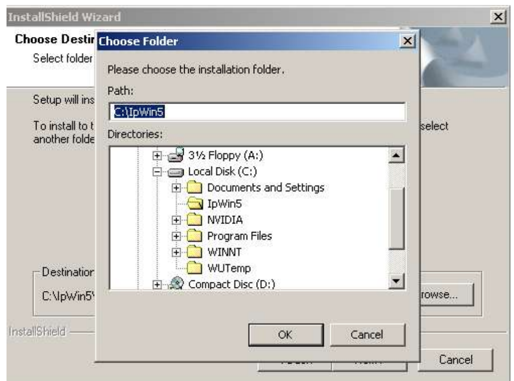
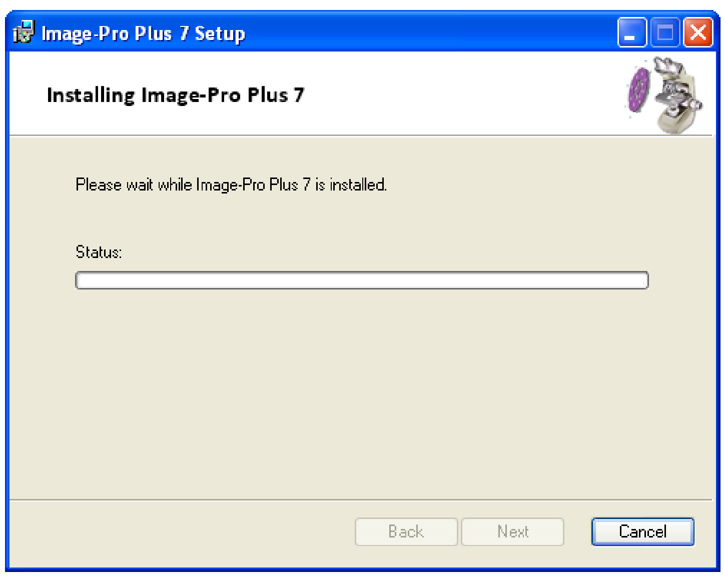
<table>
<tr><th>STEP</th><th>PROCEDURE</th></tr>
<tr><td>10a.</td><td>If you want to install Image-Pro Plus in another folder, use the Browse button to select a folder. Highlight the name of the folder and click OK. Click Next to continue installing Image-Pro Plus.

</td></tr>
<tr><td>11.</td><td>Follow the instructions on the Image-Pro Plus setup screen. The Progress Indicator shows that the Image-Pro Plus files are being loaded onto your computer.

</td></tr>
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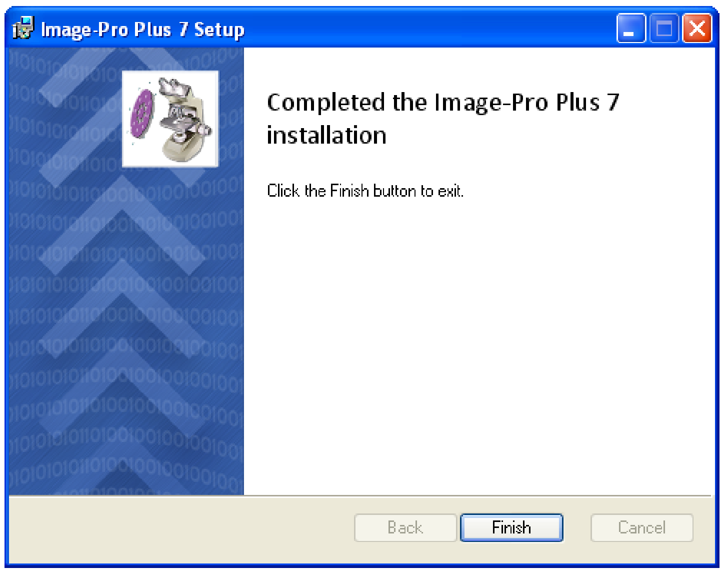
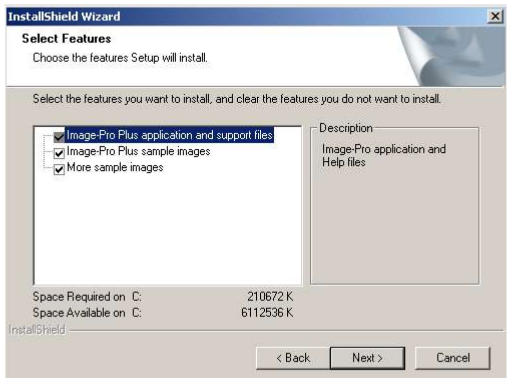
STEP	PROCEDURE
12.	When the installation process is complete *Image-Pro Plus* icon will appear on the Program List when you click the **Start** button. From this screen, you can view the *Release Notes*, start *Image-Pro Plus*, or exit and do something else.  The final installation screen indicates that installing *Image-Pro Plus* has changed your system configuration. It may ask if you want to restart your computer right away, or wait until later. Click the appropriate radio button for your choice.

<table>
<tr><th>STEP</th><th>PROCEDURE</th></tr>
<tr><td>13.</td><td>If you select Custom, you will see the Select Components dialog. This dialog allows the you to selectively install any components desired. This dialog only appears in a Custom installation. Check the components to install, then click Next.


To continue with a custom installation, click Next. (Please go back to step 10.</td></tr>
</table>

Installing the Copy-Protection Plug

Image-Pro Plus is hardware-locked. A special security plug (dongle) is included with *Image-Pro Plus* to “unlock” the program. Only when this plug is connected to the parallel or USB port on your system will *Image-Pro Plus* remain active. If the plug is not installed, or if it is removed while *Image-Pro Plus* is operating, *Image-Pro Plus* will revert to the **demo** mode. To install the plug, simply connect it to any ***operable*** port of the correct type on your system.

Diagnosing Protection Key Problems

If you are having problems with your copy-protection plug, please consider the following:

Download the HASP Diagnostic program (**`HaspDg.exe`**) from the *HASP* section of the **Software Updates** page of the Media Cybernetics Technical Support web page (**`http:\\support.mediacy.com`**). Run this program to see if the key is recognized by the program.

If the HASP program does not recognize your key, please download and run the `HDD32.zip` program from Software Updates page of the Media Cybernetics Technical Support web page `(http:\\support.mediacy.com).` Using this program will reinstall the HASP drivers. Then you can re-run the HASP Diagnostic program (`HaspDg.exe`). If the key still does not work, please call Media Cybernetics customer service at 301-495-3305, and request a replacement key.

Installing *Image-Pro Plus* with a multi-user (red) network key

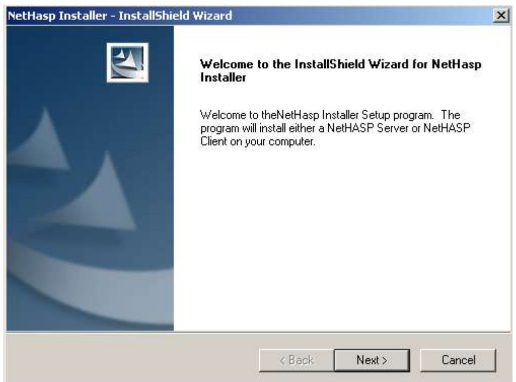
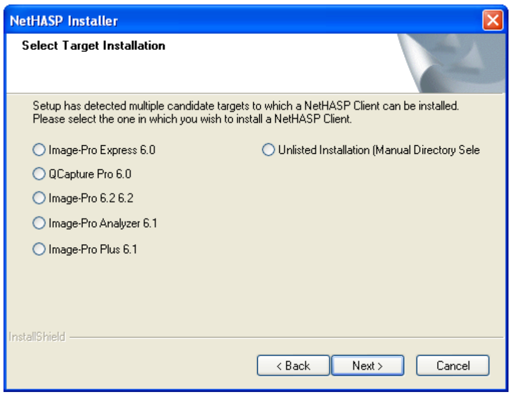
Setting up a multi-user network license is a two-step process. First, the License Server must be installed and configured on the Server computer. Next, the License Client must be installed on every computer that will actually run *Image-Pro Plus* (including the Server, if it will be used to run *Image-Pro Plus*).

NOTE: You MUST be logged on as an Administrator when installing the network key License Server or Client under Windows XP or Windows Vista.

Installing the License Server

NOTE: It is strongly recommended that the License Server be configured with a Static IP Address. If the License Server is using a Dynamic IP Address (i.e. DHCP) you need to reconfigure each Image-Pro Plus client machine every time the License Server’ IP Address changes (e.g. when it is rebooted). If you are not sure whether the License

Server is using a Static or Dynamic IP Address, please contact your System Administrator.

STEP	PROCEDURE
1.	If you will also be running *Image-Pro Plus* on the License Server, do a **Typical** installation of *Image-Pro Plus,* according to the instructions earlier in this chapter. Otherwise, skip to **Step 2**.
2.	Click **Install Network Support**. You will see the **Welcome** screen. 
3.	Click **Next** to advance past the **Welcome** screen. Indicate where you want to install the NetHasp, and then click **Next**. 

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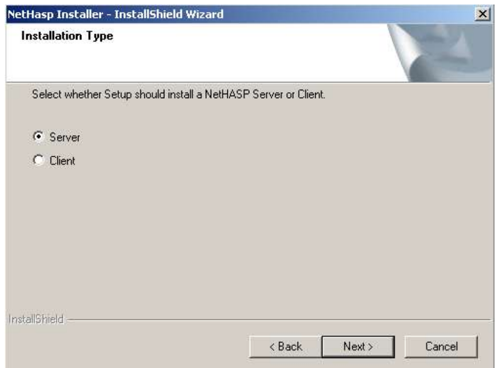
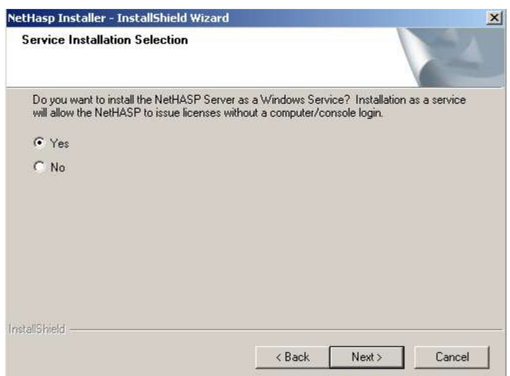
STEP	PROCEDURE
4.	Select a **Server** installation, and click **Next.** 
5.	When you installing the License Server as a service on Windows XP system, this screen asks whether to install the License Server as a service. Doing so is the recommended option, since this allows the License Server to run, even if no one is logged into the system.  Click **Next** after making your selection.

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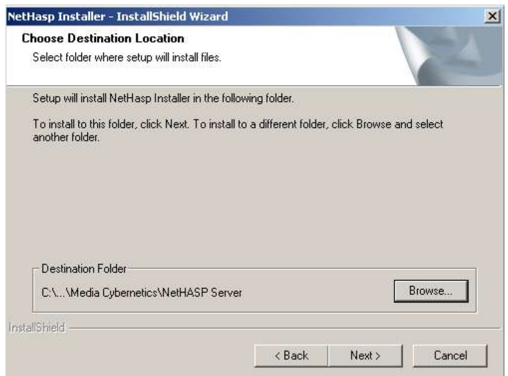
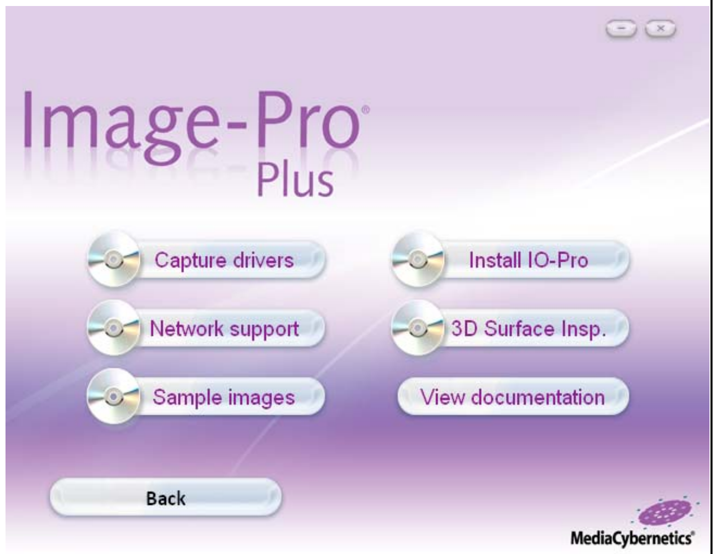
STEP	PROCEDURE
6.	Click **Next** to accept the default directory, and begin copying files, or click **Browse** to change the directory in which the License Server will be installed. 
7.	Make a note of the License Server's IP Address; you will need this during the client installation. If you do not know the License Server's IP Address, start a command prompt (Start: Programs:Accessories:Command Prompt) and type "**`ipconfig`**". This command will produce output like that shown here:

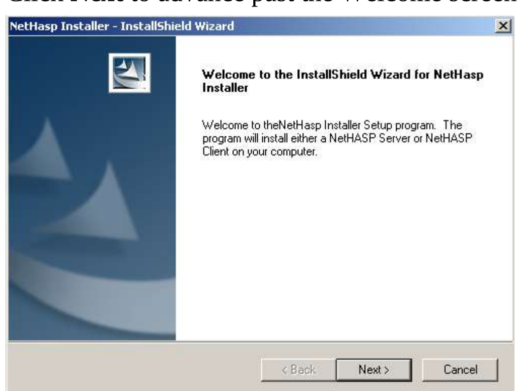
```
Microsoft Windows XP [Version 7.0.2600]
(C) Copyright 1985-2001 Microsoft Corp.

C:\>ipconfig

Windows IP Configuration


Ethernet adapter Local Area Connection:

     Connection-specific DNS Suffix  . :
     IP Address. . . . . . . . . . . . : 192.168.1.100
     Subnet Mask . . . . . . . . . . . : 255.255.257.0
     Default Gateway . . . . . . . . . : 192.168.1.1

C:\>
```

Installing a License Client

<table>
<tr><th>STEP</th><th>PROCEDURE</th></tr>
<tr><td>1.</td><td>Do a Typical installation of Image-Pro Plus, according to the instructions earlier in this chapter.</td></tr>
<tr><td>2.</td><td>From the Install Support screen, choose Network Support.

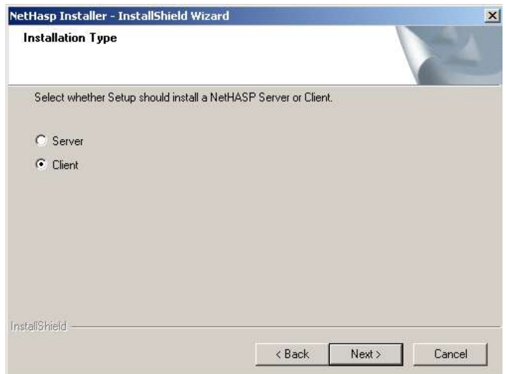
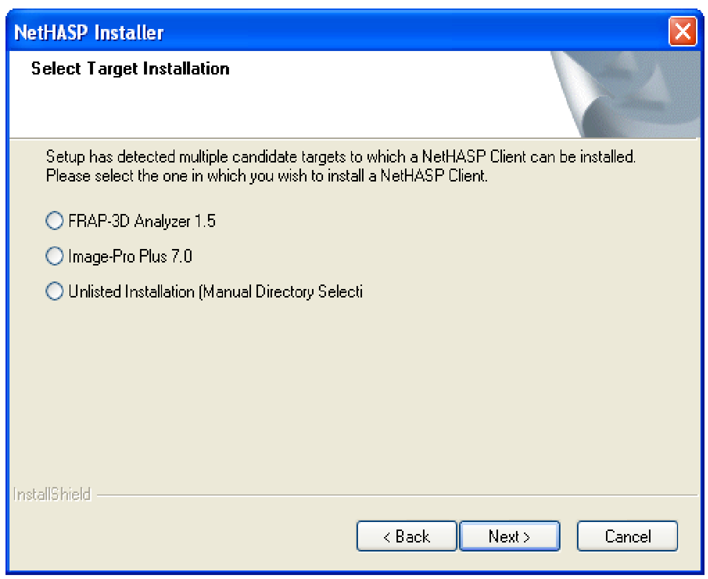


</td></tr>
<tr><td>3.</td><td>Click Next to advance past the Welcome screen.

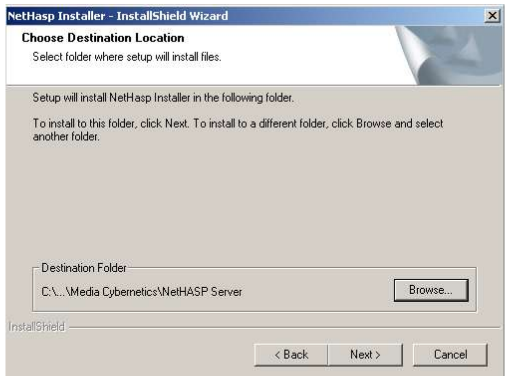
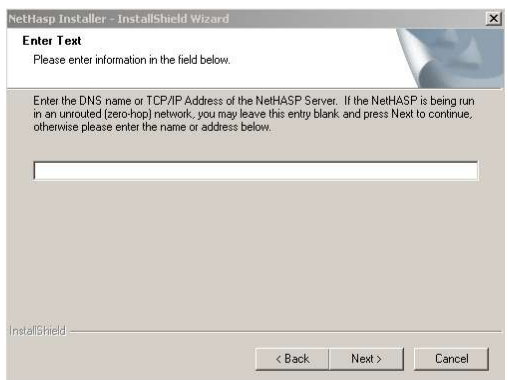


</td></tr>
</table>

continued on next page

STEP	PROCEDURE
4.	Select a **Client** installation, and click **Next**. 
5.	Select the application that will be using NetHASP licensing (*Image-Pro Plus* should be selected by default), and click **Next**. 

continued on next page

STEP	PROCEDURE
6.	Click **Next** to accept the default directory, or click **Browse** to change the directory in which the License Server will be installed. 
7.	You will now be prompted for the IP Address of the License Server (copied down in **Step 7** of the License Server installation).  Click **Next** to continue.
8.	Click **Finish** to begin copying files.

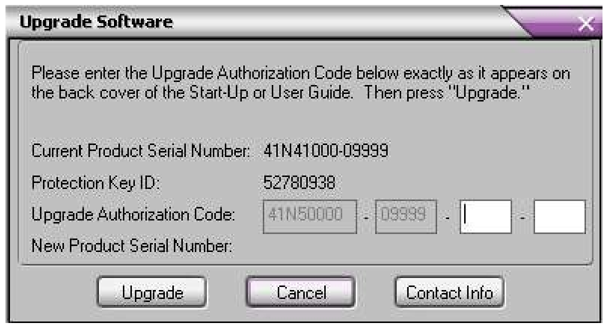
Upgrading Your *Image-Pro Plus* Software

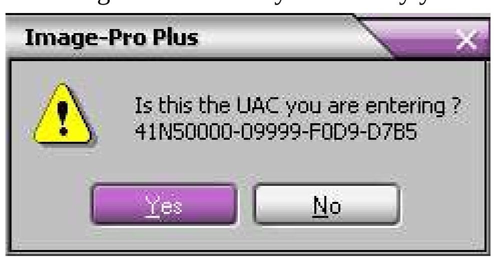
If you are installing *Image-Pro Plus* as an upgrade from versions prior to *Image-Pro Plus* 5.*0*, you will be prompted to enter an **Upgrade Authorization Code** (**UAC**) before starting the program. The code is located on the back outside cover of the *Start-Up Guide* (this manual) and on the sleeve containing the *Image-Pro Plus version 7.0* CD. Follow the steps below:

Note that *Image-Pro Plus 7.0* will not install on versions of *Windows* older than *Windows XP*.

STEP	PROCEDURE
1.	If you know your **Upgrade Authorization Code** (**UAC**), select the radio button for an upgrade.  Press **Continue.**

continued on next page

<table>
<tr><th>STEP</th><th>PROCEDURE</th></tr>
<tr><td>2.</td><td>The next screen asks you to enter your UAC in the spaces provided:


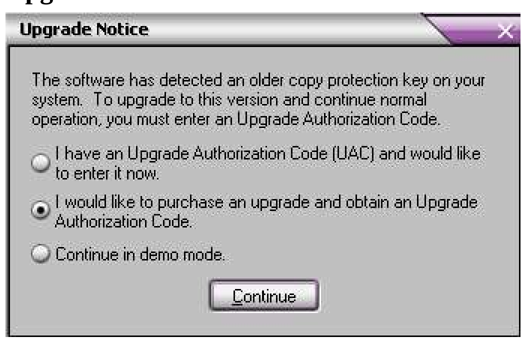
Your serial number will appear automatically. Enter the two 4-digit numbers that appear on the back cover of the Start-Up Guide or on the CD sleeve. The click the Upgrade button.</td></tr>
<tr><td>3.</td><td>A message window asks you to verify your UAC:


Click Yes. Now you can use Image-Pro Plus version 7.0.</td></tr>
</table>

continued on next page

<table>
<tr><th>STEP</th><th>PROCEDURE</th></tr>
<tr><td>4.</td><td>If you don’t have an Upgrade Authorization Code, you can continue in Demo mode for 30 days. Click the radio button for Continue in Demo Mode.

Press Continue to work with Image-Pro Plus version 7.0.</td></tr>
<tr><td>5.</td><td>To receive information about an Upgrade Authorization Code, click the radio button next to I would like to purchase an Upgrade Authorization Code.



Press Continue.</td></tr>
</table>

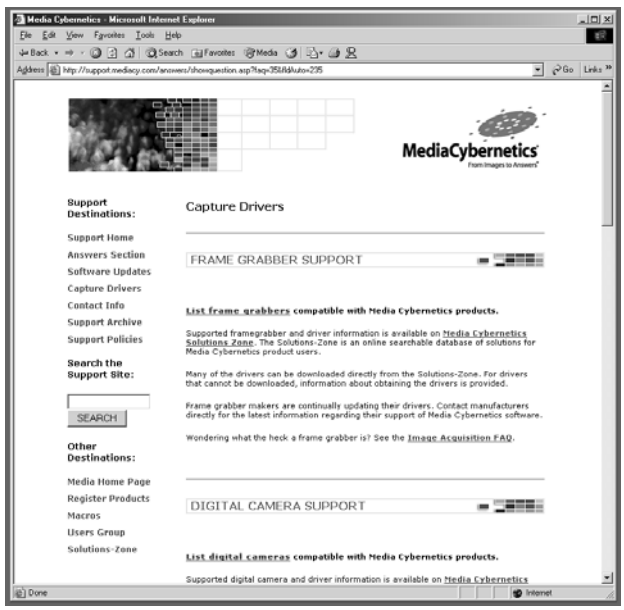
continued on next page

<table>
<tr><th>STEP</th><th>PROCEDURE</th></tr>
<tr><td>6.</td><td>The next screen shows your serial number and copy protection key ID number.


Press Contact Information to see the Media Cybernetics' Customer Support telephone number, fax number, and e-mail address. Contact us for your Upgrade Authorization Code (UAC).</td></tr>
</table>

Installing Image Capture Support

To use a camera or frame-grabber with *Image-Pro Plus*, you will need to install the appropriate capture drivers from the Media Cybernetics web site. You must install the **32-bit version** of the capture drivers to work with *Image-Pro Plus* on 32-bit systems, or the 64-bit version on 64-bit systems.

<table>
<tr><th>STEP</th><th>PROCEDURE</th></tr>
<tr><td>1.</td><td>Go to http://support.mediacy.com</td></tr>
<tr><td>2.</td><td>Select Capture Drivers.</td></tr>
<tr><td>3.</td><td>Choose a driver from the list.

</td></tr>
<tr><td>4.</td><td>Download the zipped file from the web page to your computer.</td></tr>
<tr><td>5.</td><td>Open the zipped file and follow the instructions to install your diver.</td></tr>
</table>

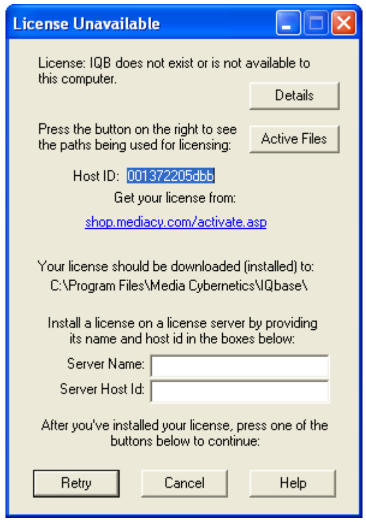
Installing IQbase Support

Image-Pro Plus 7.0 includes a complementary copy of *IQbase 2.6.* You may use this copy free of charge for 6 months. If you already have a copy of *IQbase* running on your computer, you can install *IQbase* support for *Image-Pro Plus 7.0.* This will enable you to manage your images using the advanced features of *IQbase*.

STEP	PROCEDURE
1.	Choose **Install IQbase.** 
2.	The system will ask you to select a target for the database upgrade. Select *Image-Pro Plus 7.0*. Then click **Next.**
3.	Follow the prompt instructions on the screen to continue installing support for **IQbase.**
4.	Click **Finish** to complete the installation.
5.	Once *IQbase* is installed, double click on the *IQbase* desktop icon. The *License Unavailable* dialog will open. Click on the link to go to the Software Activation Page (you must have an Internet connection).

<table>
<tr><th>STEP</th><th>PROCEDURE</th></tr>
<tr><td>6.</td><td>

Your *Image-Pro Plus* package includes a *"Getting Started"* insert. This insert includes your 6-month unlocking code.



Enter your unlocking code and submit your required contact information.

</td></tr>
<tr><td>7.</td><td>

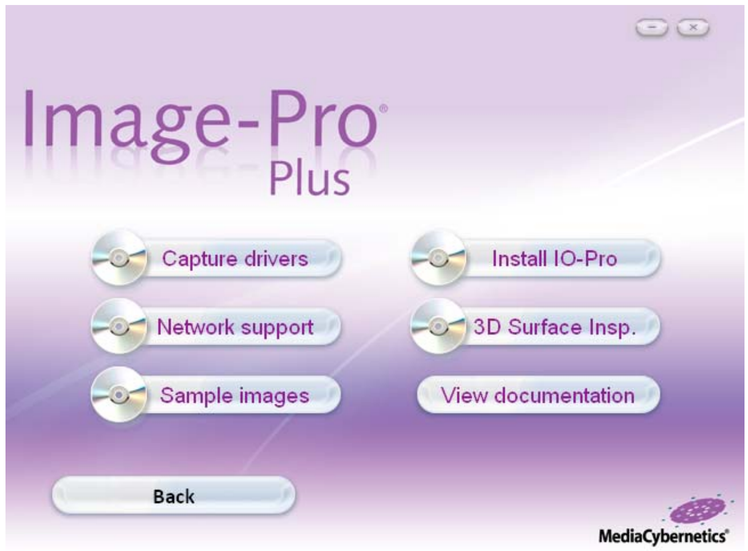
Press **Submit** to retrieve your license. Select **Retry** in the dialog box and start using *IQbase.*

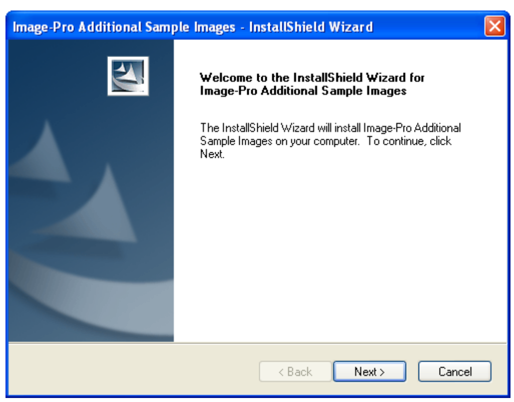
</td></tr>
</table>

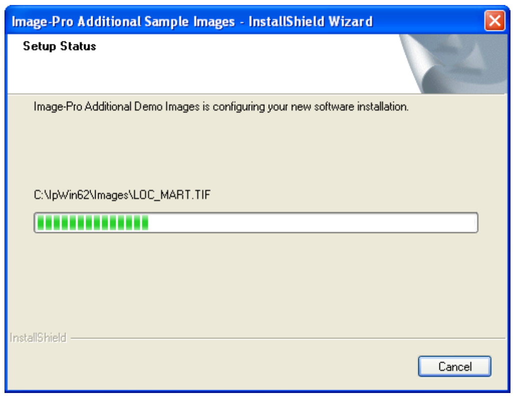
Installing the Sample Images

Image-Pro Plus 7.0 includes an additional CD of sample images. To install and use these images, follow these steps:

STEP	PROCEDURE
1.	Place the *Image-Pro Plus* images CD in your CD-ROM drive. Within a few seconds, the **Setup** program will start running automatically. The *Image-Pro Plus* Selection screen will appear. Choose **Support Files.** 

<table>
<tr><th>STEP</th><th>PROCEDURE</th></tr>
<tr><td>2.</td><td>Image-Pro Plus will ask you to select which support files you want to install:


Choose Sample Images.</td></tr>
<tr><td>3.</td><td>You will see the Image-Pro Plus Welcome screen. After reading it, click Next to continue installing Image-Pro Plus.

</td></tr>
</table>

STEP	PROCEDURE
4.	You will see the images license agreement . Click **Yes** to continue the installation process.
5.	You will see the sample images installation screen. Follow the prompt instructions on the screen to continue installing the images. 
6.	Click **Finish** to complete the installation.

Starting the Image-Pro Plus Program

The *Image-Pro Plus* setup program creates a program folder called-*Pro Plus 7.0* under the **Programs** group in the Windows XP or Vista *Start* menu, and places the *Image-Pro Plus* icon on your desktop. Select this icon to open the *Image-Pro Plus* application window.

*Note: Under Windows XP Professional or Windows Vista you should **remove** Image-Pro Plus using the **Add/Remove Programs** option from the **Control Panel** group under **Settings** on the Start menu if you need to uninstall the software.*

Technical Support On Line

Please refer to the Technical Support page, (accessible from the Media Cybernetics' home page on the World Wide Web at **http://support.mediacy.com**)where valuable technical information is available to you. This information includes:

- Answers to frequently asked questions
- A collection of technical notes, including 'How To...' tips, sample macros, and more.
- Technical information on built-in and third-party video acquisition hardware and software.
- A Technical Support E-mail form

You may obtain additional useful information via e-mail by joining the *Image-Pro Plus* Forum. This service facilitates information exchange among *Image-Pro Plus* users. You will be able to learn how others use *Image-Pro Plus*, and possibly find solutions to your needs. To join the *Image-Pro Plus* Forum, visit our web page at **http://support.mediacy.com.**

Image-Pro on the Web

Automated online tutorials for *Image-Pro Plus* are now available. Clicking this item on the *Help* menu takes you directly to the *Image-Pro Plus* tutorials page of the *Media Cybernetics* web site. Here you can learn more about the features and functions of your *Image-Pro Plus* software.

Media Cybernetics now has a presence on *YouTube*:

http://www.youtube.com/user/MediaCybernetics

We have a new Facebook Group for *Image-Pro Plus Users*. We plan to add Events, Recent News & Videos to this group. Join us! If you don't have a Facebook account, you may not be able to see this group.

Image-Pro Plus Users' Facebook Group:

http://www.facebook.com/home.php?#/group.php?gid=44536513482

Contacting Technical Support

Before you contact Media Cybernetics' Technical Support for further assistance, please be prepared to provide the following information:

- Your registration information: the product serial number (*), your name, phone number, organization name, mailing address, and e-mail address.
- The software version number (*) and system information.
- A description of the problem, and any relevant information regarding prior technical support assistance.

(*) These numbers are displayed on the 'About' screen (open from the *Help* menu)

You may contact Media Cybernetics' Technical Support in either one of the following ways:

- To speak with a technical support representative, please call **301-495-3305**, and select the option for Technical Support. The Technical Support line operates on regular business days, between 9:00 a.m. and 5:00 p.m., EST.
- To use e-mail, you may fill out the e-mail form available from the Technical Support page on the World Wide Web (see above); or e-mail your message directly to: **`techsupport@mediacy.com`**. Please remember to include your *Image-Pro Plus* serial number in your message.
- To send a fax, address your fax to *Image-Pro Plus* Technical Support, and send it to **301-495-5964**. Please remember to include your Image-Pro Plus serial number in your message.

Note: Technical support is available only to **registered** users, during the basic or extended warranty period. Please remember to return your registration card.

Imaging Discussion

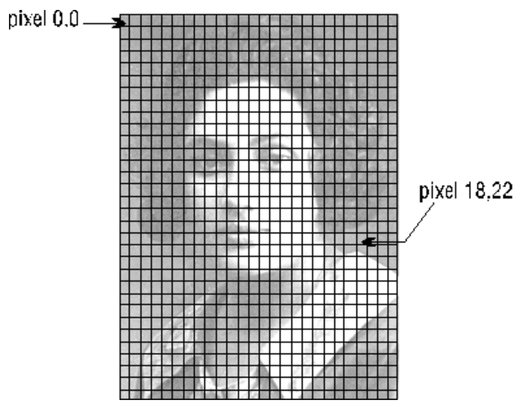
What is Image Processing?

An image is a visual representation of an object or group of objects. Image processing manipulates information within an image to make it more useful. Digital image processing is a specific type of image processing performed with a computer.

You are probably most familiar with photographic images; however, photographic images do not lend themselves to computer analysis because computers work with numerical rather than pictorial information. In order to process an image with a computer, the image must be converted into numeric form. This process is known as image digitization.

Image Digitization

The digitization process divides an image into a horizontal grid, or array, of very small regions called picture elements, or pixels. In the computer the image is represented by this digital grid, or bitmap. Each pixel in the bitmap is identified by its position in the grid, as referenced by its row (x) and column (y) number. By convention, pixels are referenced from the upper-left position of the bitmap, which is considered position 0,0 (row 0, column 0).



pixel bitmap

Note - for illustrative purposes, the pixels in the drawing above are shown much larger than their actual size. A pixel usually represents a very small region within an image, often 1/300th of an inch square, or less.

When a source image, such as a photograph, is digitized, it is examined in grid fashion. That is, each pixel in the image is individually sampled, and its brightness is measured and quantified. This measurement results in a value for the pixel, usually an integer, which represents the brightness or darkness of the image at that point. This value is stored in the corresponding pixel of the computer's image bitmap.

When the image is digitized, the width and height of the array are chosen and fixed. Together, the bitmap's pixel width and height are known as its spatial resolution.

Pixel Depth

Depending upon the capability of the measuring hardware and the complexity of the image, anywhere from 1 to 32 bits might be used to store each pixel value.

Pixel values for line art images, which contain only black and white information, can be easily represented by a single bit: 0=black, 1=white. However, a photographic-like image contains much more information...it takes 24 bits to represent all the possible colors that might occur in a true color image. Given 24 bits, over 16 million colors, far more than the human eye can differentiate, can be represented.

an example of a simple line drawing that can easily be represented with a single bit per pixel

The number of bits used to represent the pixel values in an image is referred to as its pixel depth, or bits-per-pixel (BPP). The number of bits per pixel used to represent each pixel value determines the image's *class.*

Image Class

While the bit depth (BPP) tells us how many unique colors an image can possess, it does not tell us what colors are actually contained within the image. Color interpretation is determined by bit depth and one of several conventions, which Image-Pro refers to as image class. The following classes are supported by Image-Pro:

- Gray Scale 8
- Gray Scale 12
- Gray Scale 16
- Floating Point (Gray Scale 32)
- RGB 24 (True Color)
- RGB 36
- RGB 48
- Palette

Each class is discussed below.

Note - Although Image-Pro supports all the classes listed above, not every operation can be used with every class. For example, FFT(Fast Fourier Transform) operations cannot be performed on a True Color image. The Command Reference in Section 2 of this manual documents such class considerations for each Image-Pro command. Commands that are not supported for a given image class are ghosted (dim) in the menu.

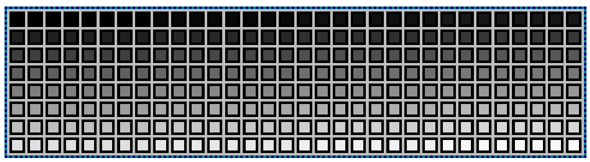
Bilevel

A *Bilevel* image stores image data with 1 bit-per-pixel (1 BPP). Each pixel is interpreted as either completely black (0) or completely white (1). Bilevel images are commonly referred to as either “Black and White” images, “Line Art” or “Halftones.”

Image-Pro Plus no longer actively supports the Bilevel image class. Bilevel images imported into *Image-Pro Plus* will be converted to Gray Scale 8 images.

Gray Scale

Gray Scale pixel values represent a level of grayness or brightness, ranging from completely black to completely white. This class is sometimes referred to as "monochrome." In an 8-bit Gray Scale image, a pixel with a value of 0 is completely black, and a pixel with a value of 255 is completely white. A value of 127 represents a gray color exactly halfway between black and white (medium-gray), and a pixel value of 64 has a gray color halfway between medium-gray and black.



256 shades of gray

Although Gray Scale images with bit depths of 2, 4, 6, 12, 16 and 32 exist, 8 BPP Gray Scale images are the most common. This is for two reasons: 1) its 1-byte-per-pixel size makes it easy to manipulate with a computer, and 2) it can faithfully represent any gray scale image because it provides 256 distinct levels of gray (the human eye can distinguish less than 200 gray levels).

Image-Pro supports the following gray scale bit depths:

NAME	BPP	DESCRIPTION
Gray Scale	8	The most common gray scale format in use. Used by most popular monochrome image formats (e.g., TIFF, PCX). Intensity values are represented with 8-bit integers. Provides 256 (0 - 255) levels of gray.
Gray Scale 12	12	A gray scale format generated by many specialized imaging systems. Intensity values are represented with 12-bit integers. Provides 4096 (0 - 4095) levels of gray.

NAME	BPP	DESCRIPTION
Gray Scale 16	16	A gray scale format generated by many specialized imaging systems. Intensity values are represented with 16-bit integers. Provides 65,536 (0 - 65,535) levels of gray.
Floating Point	32	A gray scale format that is not native to any device or image format, but is useful for certain arithmetic and filtering operations. Intensity values are represented with 32-bit floating point values. Provides a virtually unlimited number of gray levels.

The *Gray Scale 12, Gray Scale 16*, and *Floating Point* classes are used for specialized operations and applications. Gray Scale 12 and Gray Scale 16 images are often generated by specialized imaging equipment such as infrared cameras and medical imaging devices.

A *Floating Point* image is not native to any application or device. Unlike all other image classes, a *Floating Point* image does not have a fixed intensity range. The lowest value in the image is displayed as black, and the highest value in the image as white.

A *Floating Point* image is created within *Image-Pro* using its *Convert To* command. The advantage of a *Floating Point* image is that data are not lost to truncation or clipping when arithmetic and filtering operations are performed upon it (“clipping” refers to the loss of data that occurs when a pixel value falls outside a fixed intensity scale. An example is a negative value that is generated during a filtering or subtractive operation — in a fixed-point image, these negative values get “clipped” to 0). This characteristic makes it especially valuable for image restoration applications.

Although *Image-Pro* supports *Gray Scale 12, Gray Scale 16,* and *Floating Point* images for analysis purposes, most popular file formats do not support these image classes. The TIFF file format does support these image classes, but not all programs that support TIFF files support these variants. These image types can be saved in the *Image-Pro* Workspace (IPW) or flat file formats.

RGB

The RGB image class uses the most straightforward way of representing color images. RGB stands for “Red, Green, Blue,” the three primary colors of light. From the development of color photography and color television we have learned that any color can be represented as a mixture of varying levels of pure red, green, and blue light. RGB 24 is referred to as *True Color*.

In a *True Color* bitmap, each pixel contains a 24-bit value, called an RGB “triplet” or “chunk.” This RGB-triple is made up of three separate 8-bit samples. Each sample represents the level of brightness of its respective color channel: Red, Green, or Blue. These brightness values represent levels within a 256-level scale, just as they do in a Gray Scale image. The first sample is a level of Red, ranging from 0 (black) to 255 (brightest red). The second sample is interpreted as a level of green, and the third sample is the level of blue. Equal levels of Red, Green, and Blue always generate a level of gray.

Due to the increasing popularity of digital cameras, *Image-Pro Plus* now supports 36- and 48-bit color images. The storage for the classes is similar to the method used to store the 24-bit images: triplets of 16-bit words (16-bit red, followed by 16-bit green, and 16-bit blue, followed by the triplet for the next pixel). The two classes are different only in the maximum range for intensity-related values (4095 for RGB-36 versus 65535 for RGB-48). Planar forms of the two new classes will not be supported.

Although *Image-Pro* supports *RGB-36*, and *RGB-48* images for analysis purposes, most popular file formats do not support these image classes. The TIFF file format does support these image classes, but not all programs that support TIFF files support these variants. These image types can be saved in the *Image-Pro* Workspace (IPW) or flat file formats.

Palette

The *Palette* image class uses 8 bits-per-pixel to store color information. *Palette* format is a convenient and efficient way to store images that have fewer than 256 colors. It requires far less storage space than encoding an image in *True Color* (RGB-triple) form.

Unlike all other image classes, the pixel value in a *Palette* image <u>does not</u> represent a brightness value. Instead, the value within the pixel is an index (pointer) to an entry in the image’s “palette.” The palette, which is stored with the image, is a 256-entry table of RGB values. Each entry in the table contains the 24-bit RGB value for that particular index. In a *Palette* image, you could change the color of all pixels with a certain value, say 10, by simply redefining the contents of entry 10 in the palette.

It is important to understand that the values in a *Palette* image have no significance in terms of intensity; i.e., you cannot assume that a pixel value of 0 means black, a value of 255 means white, or that low values are dark and high values are bright. Because of this, *Palette* images should not be used for intensity-driven calculations, such as filtering, or intensity analysis operations such as histogram and line analysis. If you want to perform such operations on a *Palette* image, you should first convert it to a continuous-tone image class such as True Color or Gray Scale.

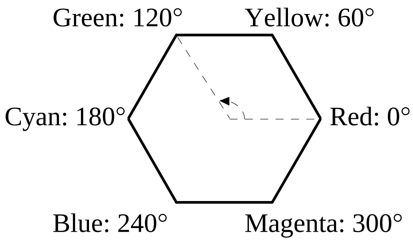
Color Models

Although *Image-Pro* internally maintains a color image in "RGB-chunky" form, it can express a color value in terms of other color models. A color model is simply a standard way to represent color in mathematic terms. Most color models use a 3D-coordinate system. Each point within the system's subspace represents a unique color. The RGB color model, for example, can be visualized as a cube where Red is the X-axis, Blue is the Y-axis and Green is the Z-axis. Each one of the 16.8 million colors is described by a unique point within this cube.

There are many color models in use today: the RGB (Red, Green, Blue), HSI (Hue, Saturation, Intensity) and HSV (Hue, Saturation, Value) models are most frequently used in digital image processing. The CMY (Cyan, Magenta, Yellow) model is a standard used to describe color in the color printing industry, and YIQ (Y-axis, In-phase, Quadrature) is used in broadcast television. *Image-Pro* lets you analyze and modify your image using any of these models.

The RGB (Red, Green, Blue) color model is an especially important one in digital image processing because it is used by most digital imaging devices (e.g., monitors and color cameras). In the RGB model, a color is expressed in terms that define the amounts of Red, Green, and Blue light it contains. In a 24-bit, color image, pure red would be represented as 255/000/000, where 255 represents the highest level of red light possible, untainted by any green (000) or blue (000) light. Various combinations of the Red, Green and Blue values allow us to define 2^{24} (over 16 million) colors.

The HSI (Hue, Saturation, Intensity) color model describes a color in terms of how it is perceived by the human eye. "Hue" is what an artist refers to as "pigment"; it is what we think of as "color" -- yellow, orange, cyan and magenta are examples of different hues. An artist usually starts with a highly saturated (i.e., pure), and intense (i.e., bright) pigment, and then adds some white to reduce its saturation and some black to reduce its intensity. Red and Pink are two different "saturations" of the same hue, Red.



Hue Hexagon

In the HSI model, a hue is specified by its position on a “color hexagon” as measured by its distance, in degrees, from the red axis (e.g., a Hue value of 120 would indicate Green, which is 120º from Red). A color's Saturation and Intensity components are identified using additional coordinates in 3D space (see the HSI glossary entry for details).

The HSI model is useful when processing images to compare two colors, or for changing a color from one to another. For example, changing a value from Cyan to Magenta is more easily accomplished in an HSI model; only the H value needs to be changed (from 180 to 300). Making the same change in an RGB view is less intuitive; since you must know the correct amounts of Red, Green and Blue needed to create Magenta. The HSI model is also a more useful model for evaluating or measuring an object's color characteristics, such as the “redness” of a berry or the “yellowness” of an autumn leaf.

The HSV model is very similar to the HSI model. The main difference between the two is the calculation used to produce the brightness value. In the HSI model, a pixel’s brightness (I) is derived from the mean of its three (R, G and B) color values. In the HSV model, a pixel’s brightness (V) is determined from the mean of the minimum and maximum value of its three color values.

The YIQ model is used in commercial color TV broadcasting. It was designed to take advantage of the human visual system’s greater sensitivity to changes in luminance rather than changes in hue or saturation. YIQ standards call for more bits to be used to represent the Y channel (luminance) and few representing the I and Q channels (hue and saturation).[1]

Within *Image-Pro*, a color model can be specified when performing channel analysis operations with the **Histogram** and **Line Profile Analysis** commands. You can also specify a color model when editing or adding a color in the **Palette** window, or when performing a channel extraction process using the **Extract Channel** tools.

[1] *Digital Image Processing*, Gonzales & Woods, 1992, Addison-Wesley Publishing Co., pg. 228.

Enhancing Your Image

More often than not, an image will require some form of enhancement to improve its appearance for aesthetic reasons, or to improve your ability to extract data from it. Enhancement techniques range from simple operations such as brightness and contrast adjustment, to the sophisticated and complex spatial and morphological filtering operations designed to "tease out" and refine visual information.

There are three basic ways to enhance an image:

- modify the intensity index
- apply a spatial filter, or
- manipulate the image frequencies.

Image-Pro Plus gives you a variety of tools in each category, allowing image enhancement for a variety of purposes and applications. Depending upon your application, you may use some techniques and not others, or you may find that you need to combine several techniques to achieve the results you need.

The following section contains material to help you select the appropriate tool for the task at hand. It describes *Image-Pro Plus's* enhancement features, and gives you some insight into the uses for each.

Modifying the Intensity Index

One way to enhance an image is to change the way intensity values are interpreted. For example, if your image was very dark overall, you could boost all the values by a certain amount. You might boost all values by 20 points, or flatten a range of intensities to a single value (e.g., set all intensities from 75 to 150 to 127).

Image-Pro Plus gives you many tools and controls to manipulate the intensities within your image. Although you will see the effect of an index modification immediately upon your image, pixel changes are not actually written to your image. They are written to an intermediate table called a "Lookup Table" (LUT). This is done so that intensity changes can be executed quickly, and so that they can easily be undone. When you are satisfied with the intensity changes you have made, you must make the changes permanent by either storing your image or explicitly writing the changes to the image bitmap using the *Contrast Enhancement* command.

The following intensity manipulation tools are provided by *Image-Pro Plus.*

Brightness

Brightness is a term used to describe the overall amount of light in an image. In *Image-Pro Plus*, brightness is modified using the “Brightness” () slider control on the BCG Controls. This control affects the overall image. In a color image, the “Brightness” slider control adjusts *luminance*, which is the combined intensity of the RGB channels. Brightness for an individual color channel can be modified using the “Brightness” control in the **Contrast Enhancement** dialog box.

When brightness is increased, you increase the value of every pixel in the image, moving each pixel closer to 255, or white. When brightness is decreased, you reduce the value in each pixel, moving it closer to 0, or black.

Contrast

Contrast is a term used to denote the degree of difference between the brightest and darkest components in an image. An image has poor contrast if it contains only harsh black and white transitions, or contains pixel values within a narrow range (an image whose values ranged from 100 to 140 would have poor contrast). An image has good contrast if it is composed of a wide range of brightness values from black to white. The amount of the intensity scale used by an image is called its “dynamic range.” An image with good contrast will have good dynamic range.

In *Image-Pro Plus*, contrast is modified using the “Contrast” () slider control on the BCG Controls. This control affects the overall image. In a color image, the “Contrast” slider control operates upon the *luminance* channel, which is the combined intensity of the RGB channels. Contrast for an individual color channel can be modified using the “Contrast” control in the **Contrast Enhancement** dialog box.

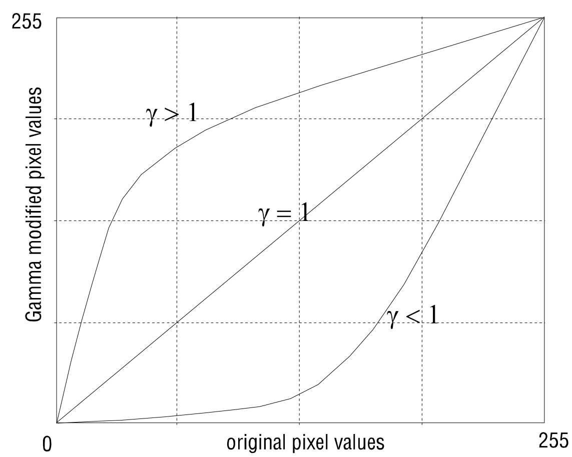
During a contrast operation, each pixel value is scaled by a contrast value, which serves to redistribute the intensities over a wider or narrower range. Increasing the contrast spreads the pixel values across a wider range, while decreasing contrast squeezes the values into a narrower range.

Gamma Correction

Gamma correction is a specialized form of contrast enhancement that is designed to enhance contrast in the very dark or very light areas of an image. It does this by changing the midtone values, particularly those at the low end, without affecting the highlight (255) and shadow (0) points. Gamma correction can be used to improve the appearance of an image, or to compensate for differences in the way different input and output devices respond to an image.

In *Image-Pro Plus,* Gamma adjustments are made using the "Gamma" (γ) slider control on the BCG Controls. In a color image, the "Gamma" slider control operates upon the *luminance* channel, which is the combined intensity of the RGB channels. The Gamma value for an individual color channel can be modified using the "Gamma" control in the **Contrast Enhancement** dialog box.

The Gamma control modifies an image by applying standard, nonlinear gamma curves to the intensity scale. A gamma value of 1 is equivalent to the identity curve, which has no effect on the image. An increase in the gamma value (setting it to a value greater than 1) will generally lighten an image and increase the contrast in its darker areas. A decrease in the gamma value (setting it to a value less than 1) will generally darken the image and emphasize contrast in the lighter areas. The figure below illustrates the effect of the gamma curves on pixel values 0 through 255.



Gamma Correction Curve

Thresholding

Thresholding allows you to reduce your image to just two colors: black and white. This is done by specifying a range of intensities to be emphasized (set to white), and converting all others to black (0). Thresholding is often used to segment an image in order to extract its important features, or to reduce an image to two intensity levels in preparation for a watershed or thinning filtering operation.

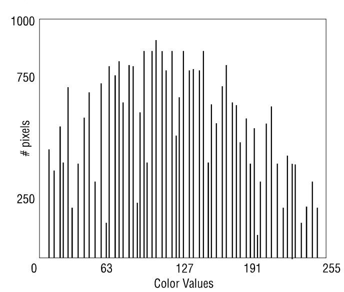
Thresholding is performed using the *Threshold* command on the *Process* menu. When this command is selected, you will be asked to specify the range of values you want emphasized (set to white).

The *Threshold* command operates upon gray scale values, so if you are working with a *True Color* or *Palette* image, it must be converted to *Gray Scale* before it can be thresholded.

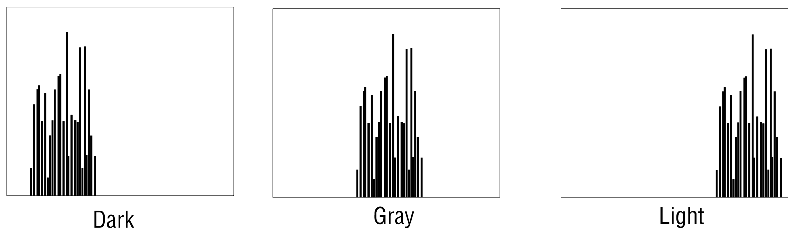
Histograms

Histograms measure and illustrate in graph form, the brightness and contrast characteristics of an image. Histogram data can be created and viewed for data gathering and analytical purposes (discussed in more detail in the *Intensity Analysis* section), or can be manipulated for image enhancement purposes.

A histogram is created using the *Histogram* command on the *Measure* menu. As you can see in the example below, the X-axis in a histogram represents the intensity scale (0 to 255 in this example), and the Y-axis measures the number of pixels in the image possessing that value. When you are working with *Gray Scale 8* images, the x-axis represents gray values 0 through 255. For other *Gray Scale* image types, the x-axis will represent the intensity range (0 to 4095 for *Gray Scale 12*). When working with *True Color* images, you can choose to measure either the image's combined luminosity or its separate color channels (e.g., Red or Green or Blue, Hue or Saturation or Intensity...).



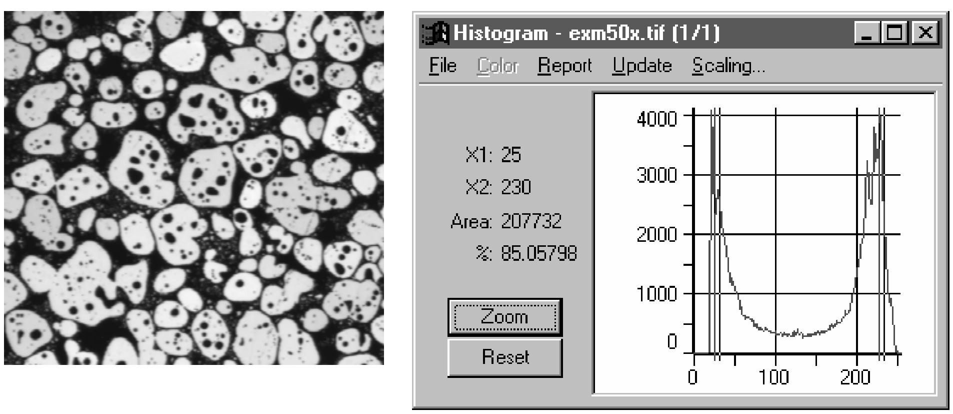
A histogram will show you what kind of brightness/contrast deficiencies exist in an image. Images with low contrast will have histograms that are clustered around a very narrow portion of the color range. The position of the cluster will indicate whether the image is too dark, too light, or simply too gray, as shown in the three examples below.



Poor contrast histograms

Brightness, contrast, and gamma adjustments modify the shape of a histogram. The series of images on the pages that follow illustrate the effect of a Brightness, Contrast, or Gamma adjustment upon the histogram.

This first set of images demonstrates the effect of a brightness change on the histogram.



Original image

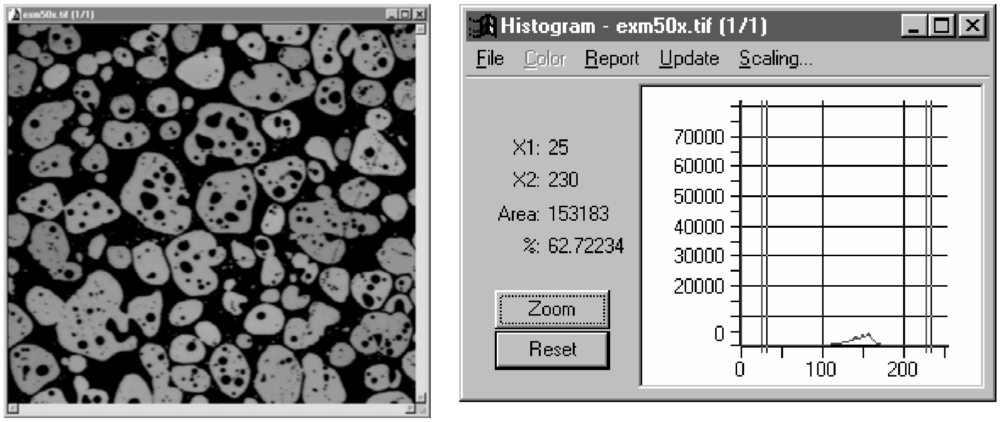


Image with decreased brightness

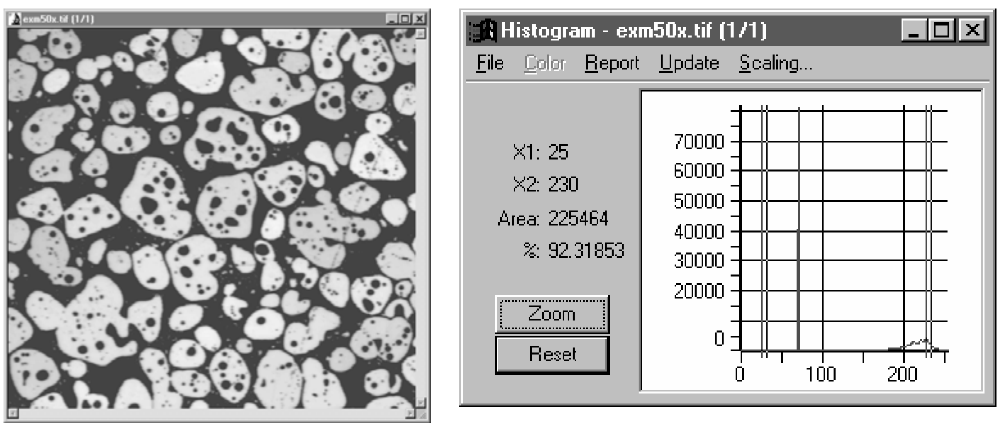
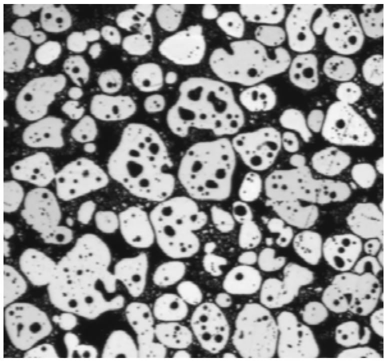
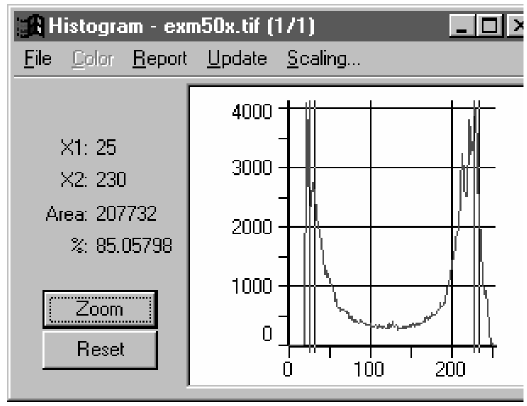


Image with increased brightness

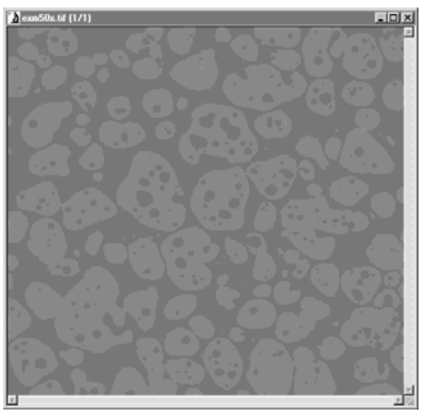
As you can see from the series above, a brightness change affects the position of the histogram on the scale, sliding the entire histogram towards black when it is decreased, and towards white when it is increased.

The following series illustrates the effect of a contrast operation upon an image and its histogram.





Original image



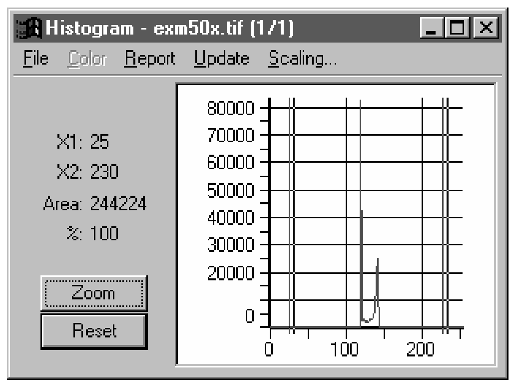


Image with decreased contrast

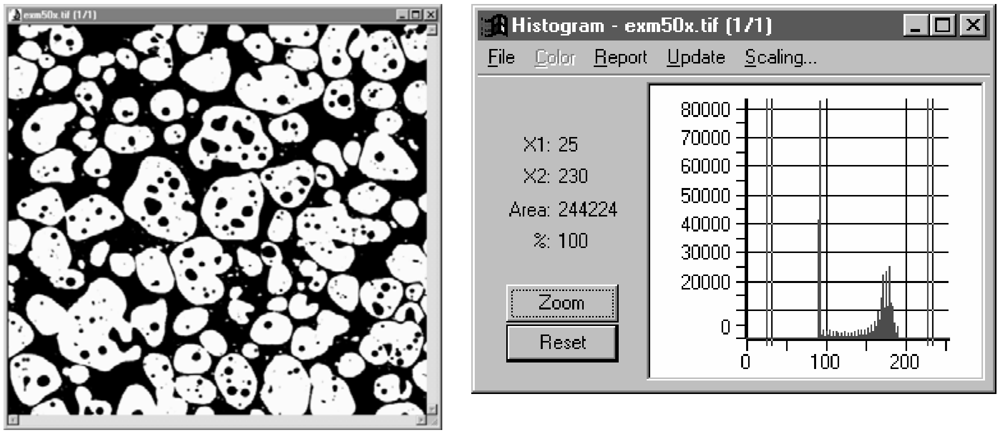
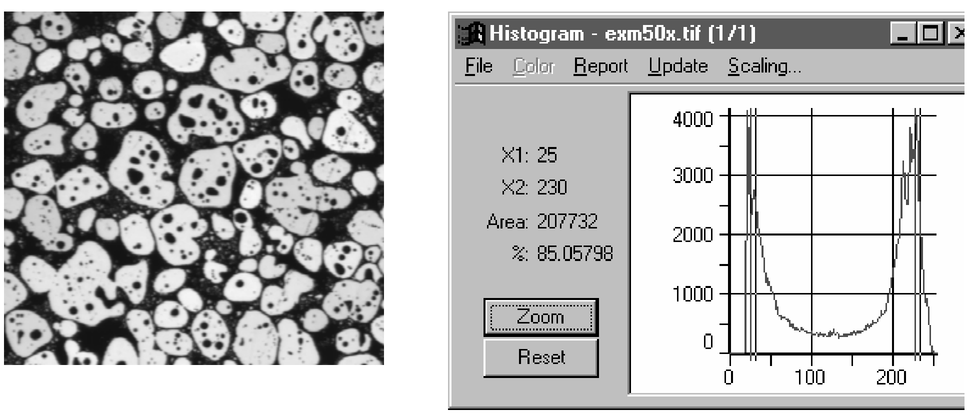


Image with increased contrast

In the image series above, the contrast has been decreased and increased. As you can see, contrast operations affect the width of a histogram; compressing it when it is decreased, and stretching it when it is increased.

The following series illustrates the effect of a gamma operation upon an image and its histogram.



Original image

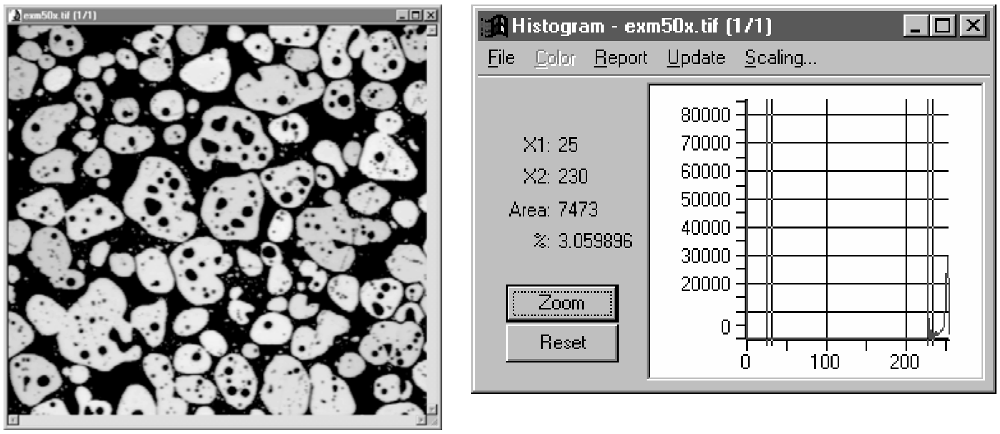


Image with decreased gamma

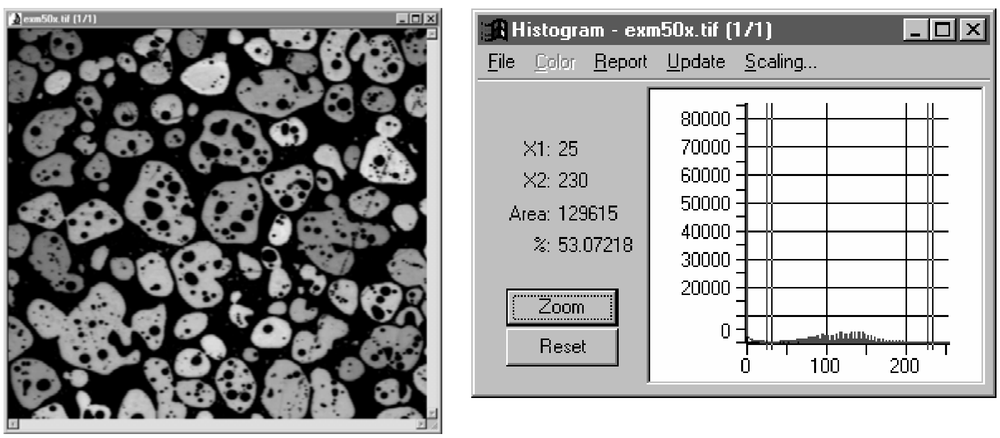


Image with increased gamma

In the image series above, the gamma has been decreased and increased. As you can see, a decrease in gamma brings out features in the lighter area of the image, by stretching the histogram in the upper region. An increase in gamma stretches the lower values, providing increased contrast in the darker areas.

Accumulated Histograms

A cumulative, or *accumulated*, histogram can also be used to assess the brightness and contrast characteristics of an image. An accumulated histogram measures the number of pixels that have a given pixel value *or a lesser value.* The result is an integral of the regular histogram distribution function.

The accumulated histogram indicates the evenness of the intensity distribution. An even distribution will produce a histogram that resembles a linear progression, like the example shown below.

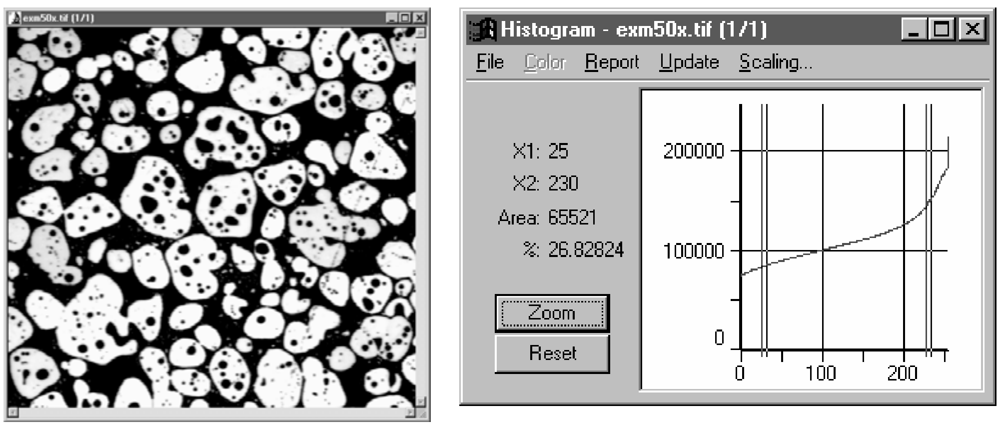
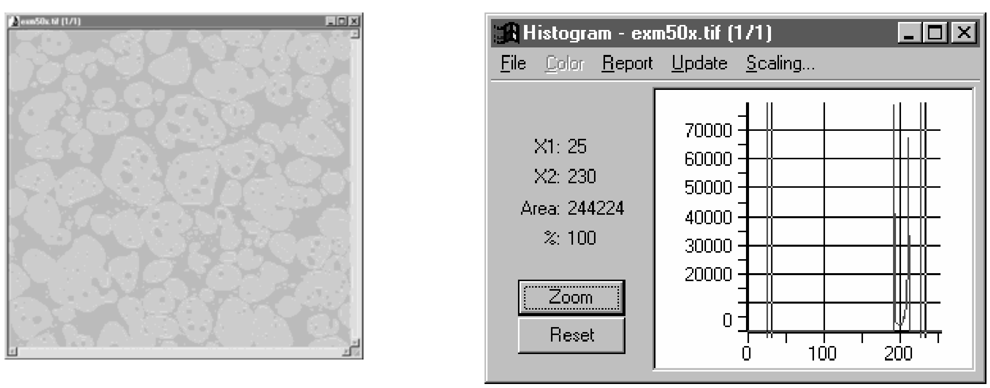
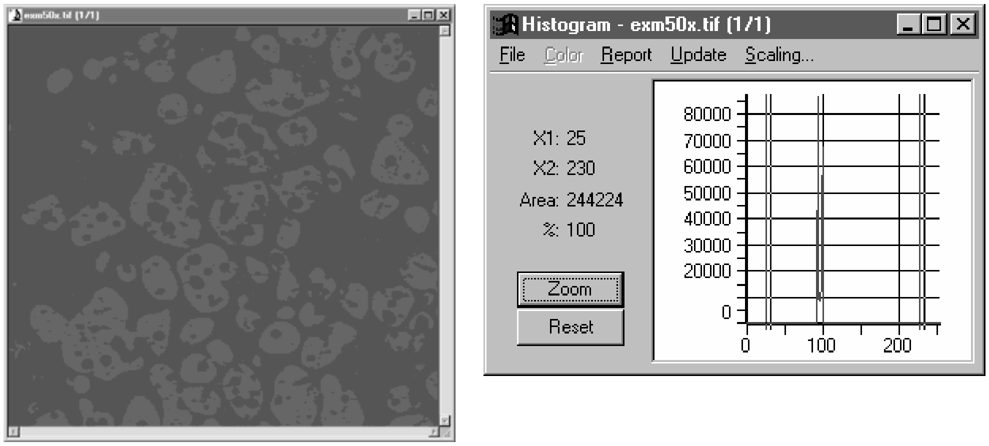


Image with good contrast and dynamic range

As you can see in the very dark and very light examples below, the accumulated histogram is skewed to the dark and light ends of the scale.



Bright image with poor contrast



Dark image with poor contrast

Accumulated histograms are created using the *Accumulated* command on the *Report* menu in the **Histogram** command window.

Histogram Equalization

Image-Pro Plus provides several ways to automatically reshape a histogram. Using the *Contrast Enhancement* command on the *Enhance* menu, you can optimize the brightness and contrast characteristics by directing *Image-Pro Plus* to stretch the histogram to achieve the best possible contrast distribution for the given image.

You can also use one of the specialized distribution functions provided by the *Equalize* command on the *Enhance* menu. These histogram redistribution functions enhance contrast and dynamic range in a nonlinear manner. When you use the *Equalize* command, *Image-Pro Plus* analyzes your image's accumulated histogram and redistributes it to fit the shape you specify:

- **Linear:** Distributes the histogram equally across the intensity scale. This function produces a high contrast image with the highest possible dynamic range.
- **Bell:** Distributes the histogram evenly around the center of the intensity scale. This function produces a high contrast image with less dynamic range than the "Linear" distribution.
- **Logarithmic:** Concentrates the histogram at the low end of the scale. This function produces a high contrast image with little dynamic range. It will tend to darken the image overall. Useful for stretching the contrast in a very light image.

- **Exponential:** Concentrates the histogram at the high end of the scale. This function produces a high contrast image with little dynamic range. It will tend to lighten the image overall. Useful for stretching the contrast in a very dark image.
- **Best Fit**: Use the *Best Fit* command to instruct *Image-Pro Plus* to optimize the values for your particular image. The results are achieved by stretching the histogram to achieve the best possible contrast distribution of pixel values in your image in this way: Best fit assigns the bottom 3% of the values to the shadow point, (0) and the top 3% of the values to the highlight point (255). The rest of the values are distributed evenly across the scale).

Spatial Filtering

Filtering operations reduce or increase the rate of change that occurs in the intensity transitions within an image. Areas in which there are sudden or rapid changes in intensity appear as hard edges in an image. Areas where there are gradual changes produce soft edges. Filtering acts to detect and modify the rate of change at these edges. It can increase the intensity differences in a soft edge to make it appear sharper, or reduce the intensity differences in a hard edge to smooth and soften it.

Filtering operations produce their effect by modifying a pixel's value based upon the values of the pixels that surround it. For example, blurring is accomplished by averaging all of the pixel values in a specified region, and replacing the center pixel with the averaged value. This produces reduced variation among neighboring pixels, which visually softens the image. A sharp black/white edge would be softened with intervening levels of gray.

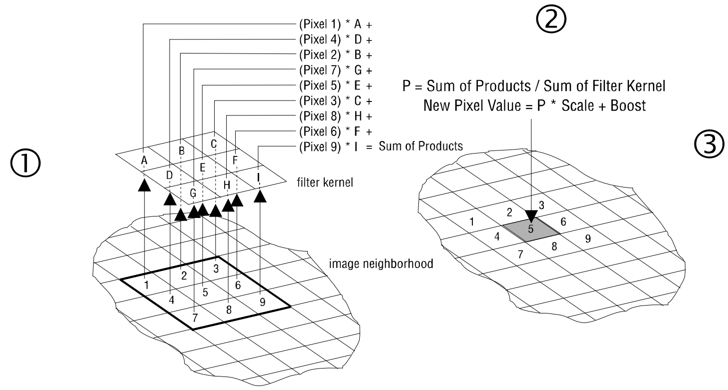
Filtering techniques are divided into two categories: *convolution filters* (linear filters) and *nonconvolution* (nonlinear) filters. Both techniques accomplish their results by examining and processing an image in small regions, called pixel "neighborhoods." A neighborhood is a square region of image pixels, typically 3x3, 5x5, or 7x7 in size.

In *Image-Pro Plus,* spatial filtering is performed using the *Filters* command on the *Process* menu.

Convolution/Enhancement Filters

Enhancement or convolution filters process image neighborhoods by multiplying the values within a neighborhood by a matrix of filtering coefficients (integer values). This matrix is called a "kernel." It is the same size as the neighborhood that it is being applied to. The results of this multiplication are summed and divided by the sum of the filter kernel. The result replaces the center pixel in the image neighborhood.

This process is further illustrated and described on the following page.



The Convolution Filtering Process

THE CONVOLUTION FILTERING PROCESS	
STAGE	**DESCRIPTION**
1.	Each pixel in the image neighborhood is multiplied by the contents of the corresponding element in the filtering kernel.
2.	The results from the multiplication are summed and divided by the sum of the kernel.
3.	The result is scaled and boosted, and used to replace the center pixel in the image neighborhood.

Note - The convolution process always uses a neighborhood's original (unfiltered) pixel values as input. When, in the example above, the kernel's focus is moved to pixel 6, the filtering process will use pixel 5's original value, not the one it was just assigned by the convolution.

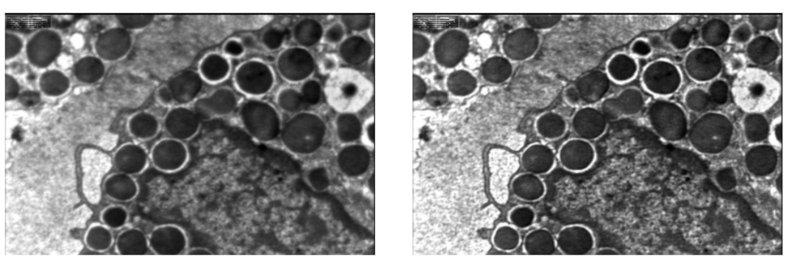
The following Enhancement filters are provided by *Image-Pro Plus*:

- **Lo-Pass**: This filter blurs an image by modifying a pixel value to be more like its neighbors. This eliminates harsh edges by reducing the intensity differences between adjacent pixels. The Lo-Pass filter can be used to blur an image for aesthetic reasons, to eliminate detail in preparation for object segmentation, or to eliminate random image noise (see also *Median filter* in the *Nonconvolution Filters* section).

Use of Low-Pass filter to eliminate contouring in a 32 level (5 BPP) Gray Scale image

- **Hi-Pass**: This filter accentuates intensity changes in an image by modifying a pixel's value to exaggerate its intensity difference from its neighbors. This filter produces an image with harsh intensity transitions, and generally results in an image with only edges of high contrast visible. Fine detail with low contrast is usually lost to the background. This filter can be used when you need to pull out just the elements having high contrast to the image background.

- **Sharpen**: This filter accentuates all edges within an image by significantly enhancing *all* intensity transitions in the image. This is accomplished using a technique called “unsharp masking,” which essentially sharpens an image by subtracting its low-pass results from the original image (although this is the result, it is accomplished using the convolution process, not by actually subtracting a blurred image from the original). Sharpening is used to bring out fine detail in an image, or to re-focus an image that has been blurred.

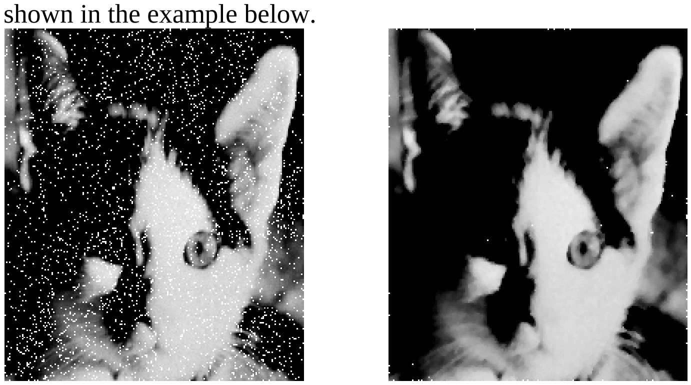


Use of Sharpen Filter to accentuate image elements and enhance fine detail

- **Gauss**: Use this filter to soften an image by eliminating high-frequency information using a Gauss function. This has the effect of blurring sharp edges.

The operation of the Gauss filter is similar to the LoPass filter, but it degrades the image less than the LoPass filter.

- **Higauss**: Use this filter when you want to enhance fine details. Its operation is similar to the unsharp masking technique (see the **Sharpen** filter), but it introduces less noise in the process. It uses a Gaussian curve type of kernel. Available in 7x7 and 9x9 kernel sizes.
- **Local Equalize**: This filter is used to enhance pixel contrast based on the histogram of the local neighborhood.
- **Flatten**: Use the Flatten filter to even out background variations. This is often done to prepare an image for a count/size operation if its objects are difficult to isolate because the background contains pixels of the same intensity as the objects of interest. Flatten reduces the intensity variations in the background pixels.
- **Median**: This filter smoothes an image by modifying pixels that vary significantly from their surroundings. This is accomplished by replacing the center pixel in a neighborhood with the *median* value of the neighborhood. Although median filtering will soften an image, it generally preserves its edges. This filter is particularly effective at removing random, high-impulse noise from an image (e.g., spots or points that vary significantly from the background), as shown in the example below.



Use of Median filter to eliminate impulse noise

- **Rank**: Select this filter if you want to remove impulse noise from an image. The **Rank** filter replaces the center pixel with a ranked pixel value from the kernel when the gray value difference is larger than the threshold value.

Convolution filters can be applied in kernels of 3 x 3, 5 x 5, or 7 x 7. Generally, the smaller the kernel, the more subtle the result. *Appendix B* of the *Reference Guide, File Format Specifications*, identifies the coefficients contained in each of the kernels used by the convolution filters described above.

Edge Filters

- **Roberts**: This filter extracts and enhances fine edges in an image by expressing the differences between neighboring pixels (cross pairs) as an intensity value. In neighborhoods where there is no difference among values in the neighborhood the pixel's intensity is set to 0 (black); where there is the greatest possible difference, the pixel is set to 255 (white). Intermediate levels of gray reflect varying amounts of difference. The result is an image in which edges and contours are highlighted against a dark background.

  Unlike most other filters, which use an odd-sized square neighborhood, the Roberts filter operates upon a 2 x 2 neighborhood. Since this neighborhood has no center, the pixel in the upper left-corner is the one replaced with a new value. Its filtered value is calculated using the following formula:

$$[(A - D)^2 + (C - B)^2]^{1/2}$$

where the neighborhood is arranged as:

A	B
C	D

  The Roberts filter enhances all edges within an image, even those introduced by noise.

  Note - if your image appears black following a Roberts filter, use your brightness, contrast and gamma controls to lighten the image and bring out the edge detail.

- **Sobel**: This filter extracts and enhances edges and contours in an image by expressing intensity differences (gradients) between neighboring pixels as an intensity value. This is done by combining the difference between the top and bottom rows in a neighborhood, with the difference between the left and right columns, using the following formula:

$$(X^2 + Y^2)^{1/2}$$

  where: $X = (C + 2F + I) - (A + 2D + G)$

  $Y = (A + 2B + C) - (G + 2H + I)$

and the neighborhood is arranged as:

A	B	C
D	E	F
G	H	I

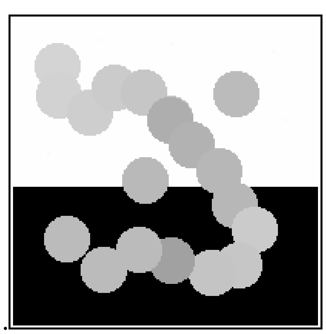
In neighborhoods where there is no difference among values in the neighborhood the pixel's intensity is set to 0 (black); where there is the greatest possible difference, the pixel is set to 255 (white). The results are similar to the Roberts filter: highlighted edges against a dark background, but the Sobel filter is less sensitive to image noise. This generally results in an image with smoother, more pronounced outlines of only the principal edges.

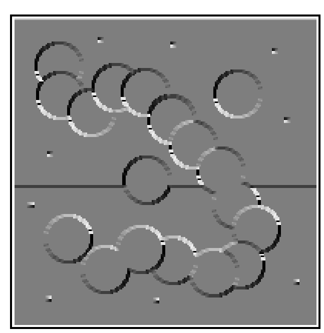
- **Laplacian**: The Laplacian filter is an edge filter that accentuates intensity changes in an image by modifying a pixel's value to exaggerate its intensity difference from its neighbors. Its results are very similar to those of Hi-Pass. It produces an image with harsh intensity transitions, and results in an image with only edges of high contrast visible.

- **Horizontal Edge**: This edge filter accentuates the horizontal edges in an image by highlighting pixels with significant intensity differences from those above and below it. It produces an image with just its horizontal edges visible against a flat background. Horizontal Edge filtering is used when horizontal features need to be extracted from an image.

- **Vertical Edge**: This edge filter accentuates the vertical edges in an image by highlighting pixels with significant intensity differences from those to the left and right of it. It produces an image with just its vertical edges visible against a flat background. Vertical Edge filtering is used when vertical features need to be extracted from an image.

- **Phase**: This filter produces an image that expresses the direction of intensity change (the gradient) in a neighborhood as an intensity value (this filter is a complement of the Sobel filter). The phase filter gives a 3-dimensional relief look to the image -- areas that have no intensity differences are flat, and those with any variation are coded to indicate whether they are brighter or darker than those above it. Generally, the lightest intensities depict vertical transitions to brighter intensities.





Use of the Phase filter to locate edges and indicate direction of intensity changes. Note how minor imperfections also became visible in the filtered image.

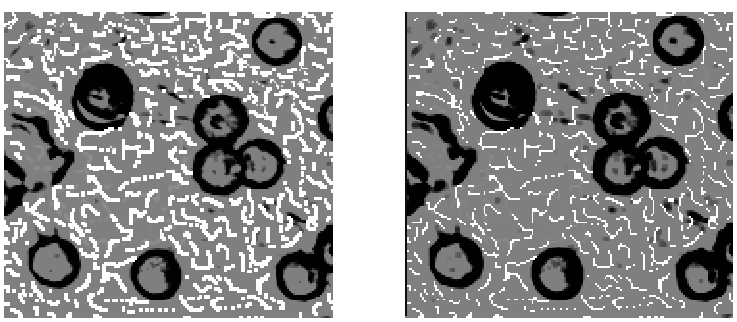
- **Variance**: Select this filter if you want to detect and emphasize edges and textures. The **Variance** filter replaces the standard deviation for its neighborhood.

Nonconvolution/Morphological Filters

Nonconvolution filters also work with pixel neighborhoods; however, unlike convolution filters, they do not multiply the neighborhood values by a kernel of filtering coefficients. Instead, a nonconvolution filter works only with the data in the neighborhood itself, and uses either a statistical method or a mathematic formula to modify the pixel upon which it is focused.

The following nonconvolution filters are provided by *Image-Pro Plus*:

- **Erode**: The Erosion filter is a morphological filter that changes the shape of objects in an image by eroding (reducing) the boundaries of bright objects, and enlarging the boundaries of dark ones. It is often used to reduce, or eliminate, small bright objects.



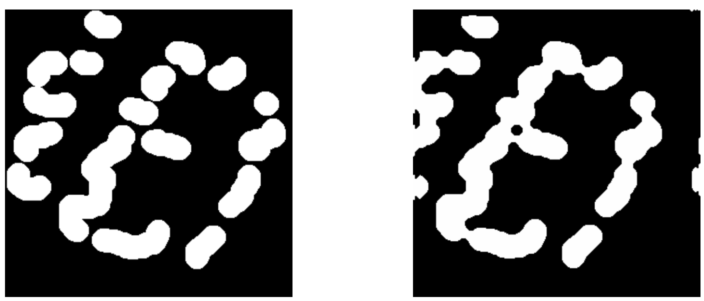
Use of Erosion filter to reduce bright object size

- **Dilate**: The Dilation filter is a morphological filter that changes the shape of objects in an image by dilating (enlarging) the boundaries of bright objects, and reducing the boundaries of dark ones. The dilation filter can be used to increase the size of small bright objects.

- **Open**: The Open filter is a morphological filter that performs an erosion, then a dilation. In images containing bright objects on a dark background, the opening filter smoothes object contours, breaks (opens) narrow connections, eliminates minor protrusions and removes small dark spots. In images with dark objects on a bright background, the opening filter fills narrow gaps between objects.

Use of 5 x 5 Octagonal Opening filter to eliminate connections between bright objects

- **Close**: The Close filter is a morphological filter that performs a dilation followed by an erosion. In images containing dark objects on a bright background, the opening filter smoothes object contours, breaks narrow connections, eliminates minor protrusions and removes small bright spots. In images with bright objects on a dark background, the closing filter fills narrow gaps between objects.



Use of 11 x 11 Octagonal Closing filter to fuse breaks between bright objects

Note - The morphological filters: Erosion, Dilation, Open and Close, are named for their effect on bright objects on a dark field. For example, the erosion filter “erodes” bright objects, the closing filter closes gaps between bright objects, and so forth. To obtain a morphological effect upon dark objects on a bright field, use the opposite morphological filter -- e.g., use the dilation filter to do an erosion, use the opening filter to close gaps. etc.

- **Tophat**: Use this filter to detect and emphasize points, or grains, that are brighter than the background. Available in 3 kernel sizes; click the radio button indicating the kernel size that most closely matches the size of the grains you want to detect.

- **Well**: Use this filter to detect and emphasize points, or grains, that are darker than the background. Available in 3 kernel sizes; click the radio button indicating the kernel size that most closely matches the size of the grains you want to detect.

- **Watershed**: Select this filter to separate objects that are touching. The **Watershed** filter erodes objects until they disappear, then dilates them again, but will not allow them to touch. The **Watershed** filter will not operate upon *True Color* images. If you want to separate objects in a *True Color* image, you must first convert it to *Gray Scale.*
- **Thinning**: This filter reduces an image to its skeleton. It operates on a binary basis; pixels are part of an object if their intensity is greater than 127, otherwise, they belong to the background. To identify what objects the thinning filter will operate upon, it is best to threshold the image before applying the thinning filter.
- **Pruning**: Select this filter to eliminate projecting arms from an object.
- **Reduce**: Select this filter to reduce the objects in an image to a single point or group of points.
- **Branch/Endpoints**: Use this filter to identify morphological branch and endpoints in an image.

Special Filters

- **Sculpt**: Use this filter to apply a sculpted effect to the image
- **Background:** Select this filter to extract the background from an image. The Background filter works by filtering out objects using a very large filtering kernel. The background image, with objects removed, is placed into a new, untitled image window.
- **Distance**: The distance filter is used for showing the distances of pixels within blobs to the outer boundaries of those blobs. After applying the distance filter, the background will be black (i.e., pixels with value 0). Only the area within the blobs will have non-zero values (will be white). The values of each pixel within the blob will be a count of the shortest distance from that pixel to the edge of the blob. Thus, all pixels along the blob's border will have a value of 1 (since they are one pixel away from the edge of the blob), pixels that are a distance of 2 from the border will have the value 2, and so on. This creates a distance map of the image.
- **Phase:** Select this filter if you want to enhance edges in a manner that also indicates the direction of the intensity change.

Frequency Filtering

Image interference that presents itself as a regular pattern across an image can be especially difficult to remove using a spatial approach. The best way to eliminate such *periodic* or *coherent noise* is by converting the image to a set of frequencies, and editing out the frequencies causing the problem.

Converting an image to its frequency domain is called a Fourier Transform (named for its inventor, Joseph Fourier), and is performed in *Image-Pro Plus* using the *FFT* (Fast Fourier Transform) command on the *Process* menu. Using the **Forward** button within the *FFT* command window, you can express the image's frequency domain as a symmetrically centered cloud of points, where brightness represents amplitude of the waveform, and the position represents the frequency of the waveform.

In a normal image, the spectrum will appear as a roughly circular cloud that is brighter and denser near its center. Images containing a regular pattern will reflect pattern-like effects in their spectra. It is this characteristic that can be manipulated to remove coherent noise. Coherent noise usually manifests itself as bright points outside of the central cloud. Removing these points eliminates the frequency causing the noise.

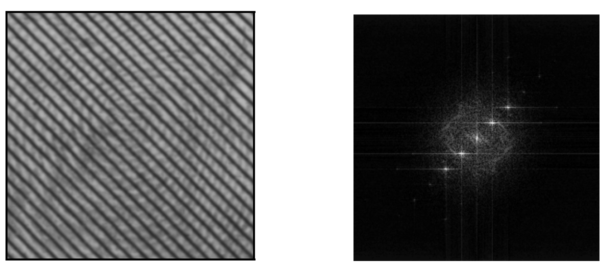


Image with coherent noise with its Fourier spectrum

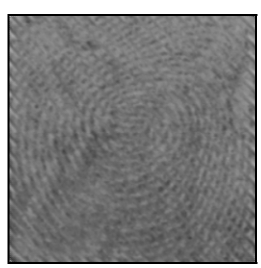


Image after removing frequency spikes

In *Image-Pro Plus*, frequency spikes are removed by defining an AOI around the spike, then selecting the Spike Cut option on the *FFT* command window. The frequencies within the AOI are either removed (set to zero), or attenuated depending upon the Transition value you have specified in the Inverse/Filter Options dialog box.

Just as frequencies can be removed or reduced, they can also be boosted. You might want to do this to accentuate a periodic pattern in an image.

Fourier transformations can also be used to perform image sharpening and blurring. In fact, the Lo-Pass, Hi-Pass and Sharpen convolution filters, and their Lo-Pass and Hi-Pass counterparts on the *FFT* command window, serve exactly the same purpose -- they both allow only the specified frequencies (low or high) to remain in an image. When you use these filtering options from the FFT command window, you define an AOI in the spectrum, within which, or outside of which, frequencies will be eliminated or attenuated according to the Transition value you have specified in the **Inverse/Filter Options** dialog box.

Intensity Analysis

Intensity analysis operations let you collect data from your image based upon the intensity values it contains. *Image-Pro Plus* provides the following intensity analysis tools on the *Measure* menu:

- Histogram Analysis
- Line Profile Analysis
- Bitmap Analysis.

Histogram Analysis lets you create a histogram of an image or AOI. Line Profile Analysis lets you plot the intensity values along a given line. Bitmap Analysis allows you to display the values of individual pixels in a bitmapped image. Using the *Save* commands on the *Histogram*, *Line Profile* , and *Bitmap Analysis: File* menus, you can store the results of your analysis to a file for use with other applications.

Although the analysis tools actually measure the intensity levels within your image, you can calibrate *Image-Pro Plus* to express these values in units relevant to your application. For example, by setting *Image-Pro Plus's Standard Optical Density* option on the **Intensity Calibration** dialog box, the intensity scale can be calibrated to standard optical density values used by most transmitted light experiments. You could also calibrate the intensity scale to indicate temperature, or protein content, for example.

Intensity calibration is performed using the *Calibration* command on the *Measure* menu.

Optical Density

Optical Density (OD) analysis is a common image processing application. Optical Density analysis determines the amount of matter in a material by measuring the amount of light it transmits (lets pass through it). Because OD analysis measures the amount of light passing *through* a material, OD measurements are meaningful only in the analysis of images that have been captured with the light source radiating from *behind* them. Some examples are: images captured from a microscopic slide (where light passes through the sample from underneath the slide) or gel samples captured in a light box (where light passes through the gel from the back of the box). Optical density measurement is not useful in the analysis of images captured under reflected light.

The **Standard Optical Density** option in the **Intensity Calibration** dialog box, uses the following standard light transmission formula for calculating OD from intensity. This formula assumes an exponential decay of light inside the transmitting material:

OPTICAL DENSITY(x,y) = -log[(INTENSITY(x,y) - BLACK) / (INCIDENT - BLACK)]

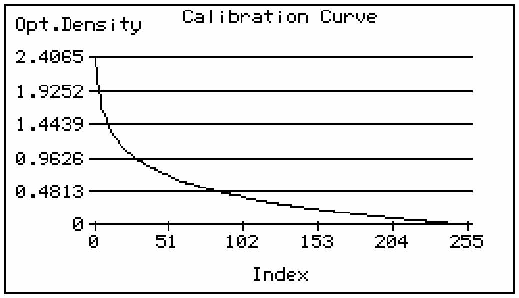
where:

INTENSITY(x,y) is the intensity at pixel (x,y)

BLACK is the intensity generated when no light goes through the material,

INCIDENT is the intensity of the incident light.

A material through which no light is transmitted has an infinite optical density, and one through which all light is transmitted, has zero optical density. As you will see by viewing the OD curve, the OD scale is inversely related to the intensity scale — that is, on an optical density scale, dark pixels produce high values, and light pixels produce low values.



Standard Optical Density Curve
X-axis depicts intensity, Y-axis depicts density

When working with optical density, it is important to establish the intensity values representing the “black” and “incident” levels in your image. By default, *Image-Pro Plus* assumes these values are 0 and 255, respectively; however, due to camera noise and other interference, these pure values are rarely appropriate for real-life images. The black and incident values should always be calibrated to your camera or image. For accuracy, this calibration should be performed for each experiment or image.

To calibrate the black level to your camera you must acquire an image while your camera’s lens is shut. Then, use the Black Level **Image** button in the **Optical Density Calibration** dialog box to select a pixel from this image (you should select a pixel located near the center of the image). The value of this pixel will indicate the black level for your camera. It represents the value that your camera registers when no light at all is transmitted to its lens.

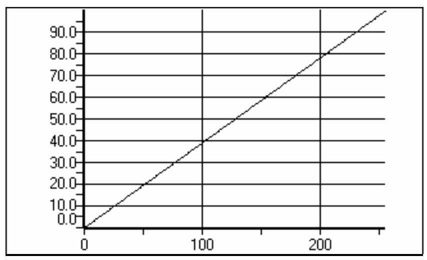
You must similarly set the incident level to your camera. To calculate incident light, you must acquire an image with nothing between the camera's lens and the light source. Then, use the Incident Light **Image** button to measure the intensity of a pixel near the center of the empty image.

Hint - With the proper preparation, you can also obtain black and incident levels from within an image itself. If a solid material (such as a penny) can be affixed to your sample, your image will contain an area through which absolutely no light is passed. You can then select a pixel in this area to calibrate the black level. Similarly, you can set the incident level if your image contains an area where there is no absorbing material present.

Custom Calibration

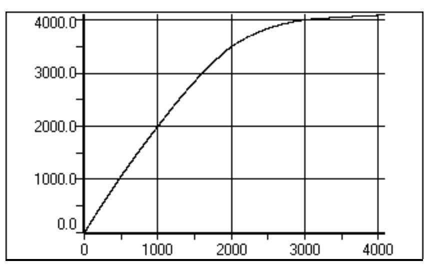
If *Image-Pro Plus's* intensity or Optical Density scales do not fit your application, you can define a scale that meets your needs. This is done using the **Free Form** option in the **Intensity Calibration** dialog box. Within this dialog box, you can define the name of your unit (e.g., degrees, luminance, disintegration/minute) and calibrate the scale.

Custom calibration is accomplished by specifying reference points that define how your scale relates to the intensity scale associated with the image. Reference points are specified in the **Free Form Intensity Calibration** dialog box. If your scale is linear, only two points on the scale need to be identified. In the example below, intensity has been linearly mapped to temperature. This curve was established by setting: 0 = 0 and
255 = 100.



Linear Intensity Scale
X-axis depicts actual intensity, Y-axis depicts calibrated intensity

If your scale is nonlinear, more than two reference points must be used to define your scale (many medical imaging devices have nonlinear scales). The scale below is an example of a nonlinear intensity relationship.



Nonlinear Scale
Intensity scale on bottom; your scale on left

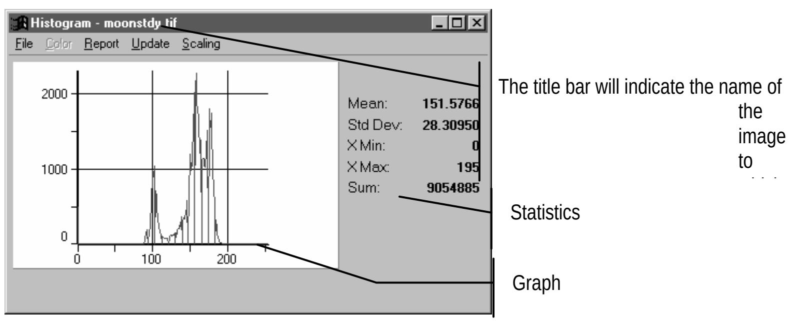
Generally, test strips, with documented reference points, can be purchased for calibrating a non-linear curve. By capturing an image containing this strip, you can calibrate *Image-Pro Plus* to the values it represents (for optical density analysis, this strip is referred to as a density "wedge").

If the reference values are not readily available, a test image, made up of various index levels and known intensity values, should be created.

Intensity Analysis Tools

The intensity analysis commands, *Histogram*, *Line Profile*, and *Bitmap Analysis*, are located on the *Measure* menu. If you have calibrated an intensity and/or spatial scale of your own, these tools will express their results in your units of measure.

When one of the intensity analysis commands is selected, a command window is presented. This window contains a graph of the intensity data and, optionally, a list of statistics. This command window is specifically linked to the image under analysis. The name of the image to which an analysis window is linked is displayed in the title bar.



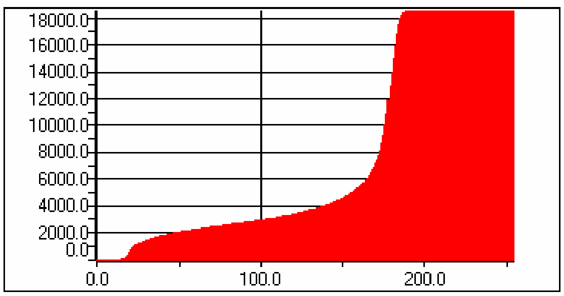
The graph and statistics are obtained from the intensity values contained in the image's bitmap. They *do not* reflect intensity adjustments contained in the Lookup Table (LUT). If you want your analysis to measure the pixel values as modified by the Lookup Table, you must first apply the table values using the *Apply Contrast* command on the *Enhance* menu.

Histogram Analysis

As discussed earlier in this manual, histograms measure and illustrate in graph form, the intensity characteristics of an image. They can be used to manipulate the image for enhancement purposes (as discussed under *Enhancing Your Image*), or can be used for data gathering purposes to measure the area associated with a specific intensity value.

In a histogram, the X-axis represents the intensity scale (as defined by the current intensity calibration), and the Y-axis measures the spatial value (as defined by the current spatial calibration). A satellite image that had been calibrated to express intensity as temperature, would show temperature along the X-axis, and area (perhaps in square miles) along the Y-axis. The axes may be scaled using the *Scaling* menu in the command window.

You can produce a cumulative histogram by selecting the *Accumulated* option on the *Histogram* window's *Report* menu. An accumulated histogram measures the number of pixels (in terms of the current spatial unit) that have the measured value, *or a lesser value*.



Accumulated Histogram

Histogram data can be viewed in graphic or numerical form using one of the graphing options on the *Report* menu in the Histogram command window.

When an accumulated histogram is selected, the numerical data will reflect the accumulated index values at each point along the X-axis.

Histogram data (the numerical data) can be saved into a file using the *Data to File* command on the *Histogram* window's *File* menu. Data and statistics are stored in an ASCII file (assigned the extension .HST), which contains descriptive information followed by the data points. An excerpt from a *.HST file is shown below. You can learn more about .HST files in *Appendix B of the Reference Guide, File Format Specifications.*

```
# Image-Pro Plus Histogram Data

# Time: Mon Jun 12 17:16:55 2003
# Image: newmoon.tif
# Spatial Unit: Pixels
# Intensity Unit: Pixels
# Number of Data Sets: 1
```

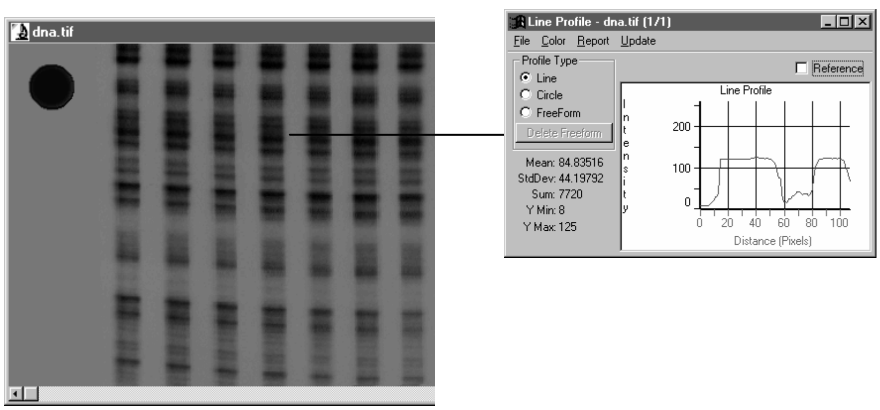
```
# Set Size: 256

#      Mean        Min        Max    Std.Dev

   175.3897        4.0      254.0   69.91221

# Gray Level      Pixels

         0.0         0.0
         1.0         0.0
         2.0         0.0
         3.0         0.0
         4.0       874.0
         7.0        38.0
         7.0        19.0
         7.0        22.0
         8.0         0.0
         9.0        24.0
        10.0         0.0
        11.0      1404.0
        12.0         0.0
        13.0       560.0
               .
               .
               .
```

Line Profile Analysis

Line profile analysis allows you to collect the actual index values along a line that you define. A profile plot shows the pixel positions of the line along the X-axis, and, on the Y-axis, measures the index value for each position along the line.

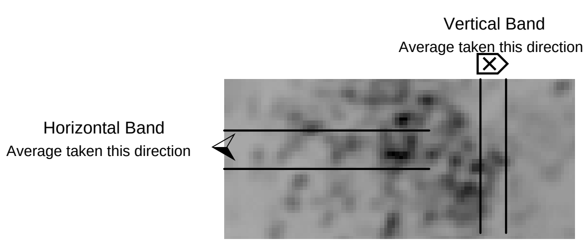
In a line profile, the X-axis represents the spatial scale (as defined by the spatial calibration), and the Y-axis measures the intensity value (as defined by the current intensity calibration). A DNA gel that had been calibrated to express intensity as density, would show density along the Y-axis.

The profile below illustrates the intensity changes along a line drawn horizontally across the image.



Line profile of DNA image

Image-Pro Plus lets you obtain data for a single line (as shown above), or for a band of lines. A profile for a band of lines is called a thick profile.



A thick profile plots the average (or standard deviation) for each row in a vertical band, or each column in a horizontal band.

Both single-line and thick-line profiles are created using the *Line Profile* command on the *Measure* menu. By default, a single line profile is created when this command is initially invoked. You can create a band profile by selecting the *Thick Horz* or *Thick Vert* options on the *Report* menu. A line profile can be of any length and position. While the line for a "Normal" line profile can be of any orientation, a thick profile can only be obtained for a horizontal or vertical band of pixels. If the pixels you want to measure are at an angle, you will need to rotate your image until they are aligned vertically or horizontally, before defining your band.

Profile data can be written to a file using the *Data to File* command on the *File* menu in the command window. Like histogram data, line profile data is stored into an ASCII file, which is assigned a *.HST extension. An excerpt from a line profile .HST file is shown below. You can learn more about .HST files in *Appendix B of the Reference Guide, File Format Specifications.*

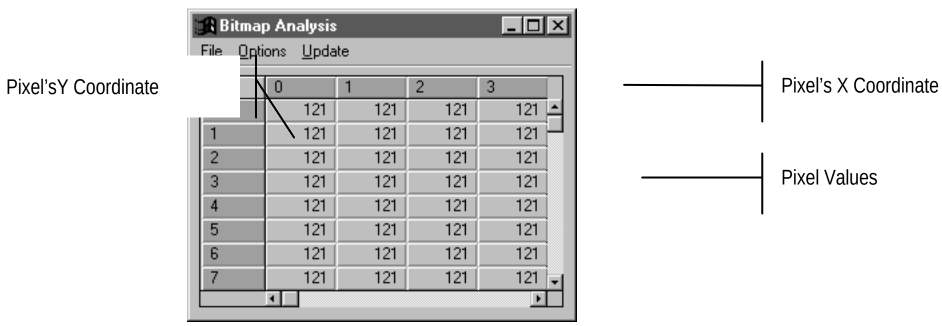
```
# Image-Pro Plus Line Profile Data

# Time: Mon Jun 12 17:53:35 1998
# Image: stripes.tif
# Spatial Unit: Pixels
# Intensity Unit: Pixels
# Number of Data Sets: 1
# Set Size: 148

#      Mean        Min        Max    Std.Dev

   153.7973       33.0      231.0   80.95961
           1       231.0
           2       231.0
           3       231.0
           4       231.0
           5       231.0
           6       231.0
           7       231.0
           8       231.0
           9       231.0
          10       231.0
          11       231.0
```

Bitmap Analysis

Bitmap Analysis command on the *Measure* menu is used to view the pixel values of the active window (or AOI) in numeric format. These values can be saved to an ASCII file for later use with an external program, or copied to the Clipboard and pasted into another application (a 3D plotting package, for example).

Pixel values can be displayed as they actually exist in the image or in their calibrated form. They are not interpreted through the display LUT however.

When the **Bitmap Analysis** command is selected, the **Bitmap Analysis** window is opened and the pixel values associated with the active image or AOI are displayed.



Bitmap analysis of a Gray Scale image

Bitmap data can be written to a file using the *Data to File* command on the *File* menu in the command window. Like histogram data, bitmap data is stored into an ASCII file, which is assigned a *.BIT extension. The bitmap data are saved in a spreadsheet format, as shown above. You can learn more about .BIT files in *Appendix B,of the Reference Guide, File Format Specifications.*

Measuring and Counting

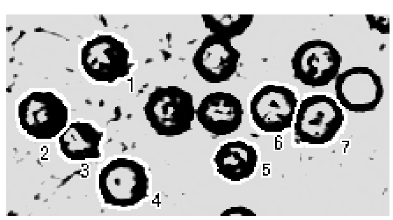
One of *Image-Pro Plus's* most powerful capabilities is its ability to let you perform spatial measurements upon your image, manually or automatically. There are two basic ways to perform measurements within *Image-Pro Plus*:

- **Manually measuring single objects**. Using the *Measurements* command in the *Measure* menu, you can measure the length of lines or polylines that you define, the area of polygons that you define, and the angles of arcs that you define. The *Measurements* command also lets you automatically trace and measure the edge of an object or feature in your image. You can measure distances between any two features, and perform tolerance testing using the new features of *Image-Pro Plus*.

- **Automatically counting and measuring multiple objects**. Using the tools on the *Measure* menu, you can collect multiple measurements of multiple objects within a single image. If your objective is to count the number of cells in a sample, and measure the area, roundness or perimeter of these cells, this is the tool to use. Once the objects have been counted and measured, you can use the *Count/Size* window's *Measure* menu options to automatically sort and classify the objects by any of the measured characteristics. You can also visualize the classified data by plotting it on a scattergram, or pseudo-coloring the counted objects by class.

- **Manually counting and measuring multiple objects**. Using the **Manual Tag** selection on the *Measure* menu, you can collect multiple measurements of multiple objects within a single image. You may select the number of objects in a class (up to 256) and the number of classes in a single image (up to 16), as well as the color, symbol, and name to identify each class.

When a measurement is made, *Image-Pro Plus* highlights the measuring outline and assigns a reference number to it as shown in the object trace example below.



By default, *Image-Pro Plus* displays its measurement outlines in yellow, and reference numbers in blue, but if you prefer other colors, you can assign them using the **Options** button in the *Count/Size* or *Measurements* window. Measurements remain on the screen until you close the image or explicitly delete the measurements using the **Delete Measurements** button.

When you have taken automatic measurements using the *Count/Size* command, the measurement values are recorded on a data sheet. You can view measurement information by simply double-clicking the measured object within the image. You can view all current measurements by selecting the *Measurement Data* command on the *Count/Size* window's *View* menu. This will open the *Measurement Data* window, into which the data sheet for the active AOI or image will be displayed. Measurement data can be saved using the *Data* to File command on the *Measurement Data* window's *File* menu. Measurements are stored in ASCII form (*.cnt). The format for a measurement file is defined in *Appendix B of the Reference Guide, File Format Specifications.*

Before Measuring, Calibrate Your Image

Measurement operations are performed in terms of image pixel positions, e.g., the length of a measurement is determined by the number of pixels along the line, the area of an outlined object is determined by the number of pixels within the outline, and so forth.

Note - When Image-Pro Plus performs a measurement, the pixels included in the outline are included in the measurement. For example, when you are measuring the area of an object you have outlined, the pixels making up the outline are included in the area calculation.

Image-Pro Plus's pixel-level measurements can be scaled to fit any coordinate system. This allows you to obtain measurements which are reported in terms meaningful to your application. For example, you can calibrate the measurement scale so that one pixel is equal to one foot, or so that five pixels are equal to one foot (or inch, or mile, or micron). Fractional values are allowed. *Image-Pro Plus* will express your measurements in terms of that unit. Additionally, if your image contains a measurable object of the unit length, you can calibrate your scale directly from that object, using the **Image** button in the **Spatial Calibration** dialog box.

Important *- Calibrate your scale* <u>*before*</u> *taking your measurements. Once measurements are recorded, their values are not affected by a subsequent change in spatial calibration. For example, if after taking a line measurement of 50 pixels where 1 unit equals 1 pixel, you changed calibration to 1 unit equal to 10 pixels, the first measurement you recorded would remain at 50; Image-Pro Plus will not automatically re-scale it to 5 to after the calibration is changed. You must take the measurement* ***again*** *in order for the new calibration to be reflected.*

Aspect Ratio

With respect to spatial calibration, the aspect ratio reflects the relationship between the length of the vertical and horizontal axes in your image, and is expressed as X/Y. Aspect Ratio is also calibrated using the *Calibration* command. If your camera's aspect ratio is not equal to your video card's aspect ratio, an image of a square object will not appear square when viewed on your display. To correct for this inconsistency, you can calibrate the aspect ratio from a reference square within an image.

Note - Camera/video card inconsistencies are not the only reason a square might not appear square on your display. It might simply be a misadjustment of your monitor's Vertical Size control.

Taking Manual Measurements

The manual measuring tools are accessed using the *Measurements* command on the *Measure* menu. The manual tools are useful for obtaining individual measurements, and they are the only way to obtain straight-line length and thickness measurements. You can define features that have measurements of their own (the length of a line, for example) or take measurements between two existing features (i.e. distance, angle, or thickness).

The following tools are used to create the primary features:

 **Selection:** This button is used to select existing features and/or measurements, i.e. to move or delete them. You may select more than one feature by holding down the **<Ctrl>** key while you use the mouse to click on the feature.

 **Remove:** This button is used to remove all of the currently selected features or measurements from the image. All related measurements in the **Measurement** tab and the corresponding row(s) in the **Feature** tab will be removed.

 **Point:** This button is used to create a point feature on the current image.

 **Straight Line:** This button is used to create a straight line feature on the current image. (This is the equivalent of the Length tool in previous versions of *Image-Pro Plus*.)

 **Best Fit Line:** This button is used to create a straight line that best fits several points on the current image. Click on the image to add up to 1000 points, and double-click to end the list of points. A setting on the **Options** page determines a user-defined limit to the number of points and the feature will be added when this limit is reached. For instance, to draw a two-point line (with two clicks) this option

could be set to a user limit of 2. The end points of the line will be the point where the line is perpendicular to the first and last points.

Circle: This button is used to create a circle feature on the current image.

Best Fit Circle: This button is used to create a circle that best fits several points on the current image. Click on the image to add up to 20 points, and double-click to end the list of points, creating the best-fit circle. A setting on the **Options** page determines a user-defined limit to the number of points and the feature will be added when this limit is reached.

Best Fit Arc: This button is used to create an arc that best fits several points on the current image. Click on the image to add up to 20 points, and double-click to end the list of points. The points of the arc will need to be ordered, i.e. clicked in order from the beginning to the end of the arc. A setting on the **Options** page determines a user-defined limit to the number of points and the feature will be added when this limit is reached.

Rectangle: This button is used to create a rectangle feature on the current image.

Polygon: This button is used to create a polygon feature on the current image. This will be created using the current trace/wand tool, and will always yield a closed figure. (This is the equivalent of the Area tool in previous versions of *Image-Pro Plus*.)

Trace: This button is used to create a trace on the current image. This will be created using the current trace tool, and will always yield a open figure. (This is the equivalent of the Trace tool in previous versions of *Image-Pro Plus*.)

The automatic tracing tool works by following an edge of significant contrast. When you start a trace, select the point at which you want the trace to begin by clicking your mouse button. Then, click a second point along the edge to indicate the direction that *Image-Pro Plus* should begin following the edge. If *Image-Pro Plus* gets lost during its trace (loses the edge), it will stop. If it has wandered beyond the edge of your object, you can press Backspace to back up the trace to the edge, then manually lead the trace by moving the cursor to the next significant point along the boundary and clicking the left mouse button.

Successful tracing is dependent primarily on how well differentiated your object is from the background. If there is good contrast between it and the pixels that surround it, your automatic traces will perform very well. Edges that have low contrast from the background, or have lots of competing edges intersecting them, will be interrupted more frequently. These controls let you fine-tune *Image-Pro Plus's* edge detection mechanism, used by the **Trace** and **Freeform** tools.

- **Magic Wand:** The magic wand tool makes it easier to outline an irregular AOI. Simply place the wand cursor inside the area that you want to trace, and click the left mouse button once. The magic wand will automatically trace the outline of the object(s) based on the color similarities or difference of intensity ranges between the pixel under your cursor, plus or minus a specified tolerance interval.

- **Threshold:** this value (from 1 - 10) describes the level of contrast distinguishing the edge you want to track from its surrounding elements. If there is low contrast, this value should be set to a low number.
- **Smooth:** this value (0-9) specifies the amount of post-filtering of the outline you want. 0 = no smoothing, 9 = high degree of smoothing. .
- **Noise:** this value (1 - 6) specifies the number of pixels *Image-Pro Plus* advances when moving to the next edge position. A larger value here results in a smoother outline (it won't follow every nook and cranny) and faster trace, but may cause it to get lost more frequently. Specifying values over 3 or 4 generally causes the Trace to overshoot curves along your edge.
- **Speed:** this value (0 - 5) specifies the rate at which the outline will be drawn. A small value here will slow down the trace function. You might do this to make it easier for you to watch the trace as it progresses

The following measurement tools are available to take measurements between features created with the feature tools:

Distance Measurement: This button creates a measurement of the distance between two user-selected features.

Angle Measurement: This button is used to create two lines and do an angle measurement between these two lines. Note that this tool is different from the five other measurement tools in that rather than selecting two features for the measurement, two features are created and then the measurement derived from them.

Angle Measurement: This button creates a measurement of the angle between two user-selected line features. (This is the equivalent of the Angle tool in previous versions of *Image-Pro Plus*.)

Horizontal Thickness: This button creates a measurement of the horizontal minimum or maximum distance between two user-selected traces.

Vertical Thickness: This button creates a measurement of the vertical minimum or maximum distance between two user-selected traces.

Curve Thickness: This button creates a measurement of the minimum or maximum distance between two user-selected traces in any direction

Automatic Counting and Sizing

Using the *Count/Size* command on the *Measure* menu you can count and measure multiple objects automatically. Once the objects have been counted and measured, you can use the *Count/Size* window's *Measure* menu to automatically classify them by any of the measured characteristics. You can also visualize the data by plotting it using the *Count/Size* window's *View* menu options, or color the counted objects by class using the *Auto Classification* command on the *Count/Size: Measure* menu.

A new feature of *Image-Pro Plus* version 7.0 allows you to sort your counted objects according to area or other measurements. Use the **Sort Objects** selection on the *Measure* menu to generate a new image of your objects in sorted order.

Identifying Objects to be Counted

For counting purposes, objects are identified by their intensity (monochrome images) or color (*True Color*-class images). Therefore, it is best to begin with an image that contains objects that are clearly distinguishable from the background, and have an intensity range/color different from other elements in the image. Because many images do not initially fit such ideal conditions, there are a number of "pre-processing" steps you can take to correct such deficiencies (pre-processing is discussed a little later in this section).

The first step in performing a count is to identify the objects you want to include in your count. This is done by specifying the range of intensities or colors that uniquely identifies your objects. The way in which you do this first step depends upon whether your image is *Gray Scale* or *True Color*.

Note - If your image is a Palette-class image, it must first be converted before the ***Count/Size*** *command can be used with it.*

- **If you are working with a gray scale image,** selecting your object's intensity range in a *Gray Scale* image can be done manually, or automatically. To manually specify object intensity, use the **Manual** option and the **Select Range** button. With your image on the screen, move the range markers until all your objects are highlighted. If you highlight a few extraneous objects in addition to the ones you want, don't worry — they can be manually removed from the object group after the count using the *Hide* option in the *Object Window* command. However, if you find that your objects can't be highlighted without including numerous unwanted elements, you will need to use one of the pre-processing tools to increase object differentiation or eliminate unwanted noise. See *Pre-Processing* below.

To have *Image-Pro Plus* automatically set the intensity range that defines your objects, you can use its **Automatic** option. When a count is performed with the **Automatic** option, *Image-Pro Plus* will analyze your image, calculate the intensities distinguishing objects from background, and establish the intensity range based upon the objects that it finds. When running a count with the **Automatic** option, you must be sure to set the **Bright Objects** or **Dark Objects** option to describe your objects.

- **If you are working with a True Color Image,** selecting your object's color range is done by identifying an AOI that encompasses the colors of your objects. Selecting a color for counting purposes is essentially performed in the same way as extracting a set of colors using the Segmentation tool -- that is, you select a set of colors that separates certain features (in this case, objects to be counted) from the rest of your image. To confirm that your color selection is extracting just the objects you want, use the **Create Image** button to view your image with all but the selected colors grayed out. Refine your selection by adjusting the color ranges, until just the objects you want to count are visible.

Pre-Processing

If you are having difficulty separating your objects from other elements within your image, a number of pre-processing techniques are offered to help you enhance object definition.

- **Contrast Enhancement:** Often, simply increasing the contrast in your image will improve your ability to extract objects more successfully.

- **Background Flattening:** The *Flatten Background* command on the *Count/Size* window's *Image* menu is used to even out intensity variations in the background of a *Gray Scale* image (flattening is not applicable to *True Color* images). When flattening the background, you will be required to describe your object size and color in the **Flatten Background** dialog box. If your image has lots of objects, or your objects are very large, leaving only small patches of background showing, you may need to set the **Background** option to specify the color of your objects, rather than the background.

  You can also perform background flattening using *Image-Pro Plus's Filter* command.

- **Background Subtraction:** The *Background Correction* command on the *Process* menu can be used to produce a flat background, and compensate for nonuniform lighting, nonuniform camera response or minor optic artifacts (such as dust specks that mar the background of images captured from a microscope). To use the *Background Correction* tools, you must have, or be able to capture, an image of the background light (to produce a background image, capture an image with the slide removed from the stage, or with the optics completely defocused). When the background image is subtracted from your image, areas that are similar to the background will be replaced with values close to the mean background intensity.

*Note - within the **Background Correction** dialog box, are two correction options: **Background Correction** and **Background Subtraction**. If the purpose of the correction is to facilitate object segmentation, use **Background Subtraction.** If the purpose of the correction is to obtain accurate density measurements, use **Background Correction** instead.*

Selecting Your Measurements

Once you have defined your objects, you must choose the measurements you want to take. This is done in the *Select Measurements* command on the *Count/Size* window's *Measure* menu. *Image-Pro Plus* gives you the following measurement options, and you can select as many as you need. All spatial measurements are reported in the current spatial unit; all intensity measurements are reported in terms of the current intensity calibration.

- **Angle:** Reports the angle between the vertical axis and the major axis of the ellipse equivalent to the object (i.e., an ellipse with the same area, first and second degree moments), where 0° ≤ Angle° ≤ 180°. The vertical angle is 0°, unless an offset has been set with the Calibration command.
- **Area:** Reports the area of each object (minus any holes). The area comprised of pixels having intensity values within the selected range is reported unless the Fill Holes option has been enabled. If Fill Holes is enabled, all pixels within the object perimeter are included in the area measurement.
- **Area/Box:** Reports the ratio between the area of each object, and the area of its imaginary bounding box, as determined by Area of Object / Area of Box.
- **Area (Polygon):** Reports the area of the polygon that defines the object's outline.
- **Aspect:** Reports the ratio between the major axis and the minor axis of the ellipse equivalent to the object (i.e., an ellipse with the same area, first and second degree moments), as determined by Major Axis/Minor Axis. Aspect is always ≥ 1.
- **Average Diameter:** Reports the average length of diameters measured at 5° intervals around the centroid of each object.
- **Box Height:** Reports the height of the bounding box along the major axis (i.e., the bounding box is the smallest rectangle that completely encompasses the whole object).

- **Box Length:** Reports the length of the bounding box along the major axis (i.e., the bounding box is the smallest rectangle that completely encompasses the whole object).
- **Box Width:** Reports the width of the bounding box along the major axis (i.e., the bounding box is the smallest rectangle that completely encompasses the whole object).
- **Box X/Y:** Reports the ratio between the width (X) and height (Y) of each object's imaginary bounding box, as determined by Box Width / Box Height.
- **Centroid X**: Reports the X-coordinate position of the centroid of the object from the left side of the image.
- **Centroid Y:** Reports the Y coordinate position of the centroid pixel of the object from the top of the image.
- **Center Mass-X:** Reports the X-coordinate position of the centroid of the object based on intensity measurements.
- **Center Mass-Y:** Reports the Y coordinate position of the centroid pixel based on intensity measurements.
- **Class:** Reports the class number to which the object belongs. This value will only be reported if the objects have been previously classified using the Single Variable Class command. If they have not, a Class measurement will not appear on the Measurements data sheet.
- **Cluster:** A cluster is a group of objects defined by an AOI. Cluster reports the number of individual objects contained within the outline.
- **Clumpiness:** Derived from Heterogeneity measurement; The fractions of heterogeneous pixels remaining in an object after an erosion process. It reflects the object texture.
- **Count (adjusted):** Reports the size-weighted object count. Only works when “clean border” flag is turned on.
- **Dendrites/Dendritic Length**: Reports the number of dendrites (1-pixel thick open branches) and the total length of all the dendrites. Measurements made of dendrite number and dendrite length should be performed on objects which have been skeletonized, or “thinned” using the **Thinning** filter in *Filters: Morphological*. Dendrite measurements performed on non-thinned objects may return unacceptable results.
- **Density Blue:** Reports the mean blue value for the measured object in a true color image.
- **Density Green:** Reports the mean Green value for the measured object in a true color image.
- **Density Red:** Reports the mean red value for the measured object in a true color image.

- **Density Sum:** Reports the sum of the intensity values of all the pixels of a counted object.
- **Diameter (max):** Reports the length of the longest line joining two outline points and passing through the centroid.
- **Diameter (mean):** Reports the average length of the diameters measured at two degree intervals joining two outline points and passing through the centroid.
- **Diameter (min):** Reports the length of the shortest line joining two outline points and passing through the centroid.
- **Feret (max):** Reports the longest caliper (feret) length.
- **Feret (mean):** Reports the shortest caliper (feret) length.
- **Feret (min):** Reports the average caliper (feret) length.
- **Fractal Dimension:** Reports the fractal dimension of the object's outline.[2]
- **Heterogenity:** Reports the fraction of pixels that vary more than 10% from the average intensity of the object.
- **Holes:** Reports the number of holes inside an object. A "hole" is defined as any contiguous set of pixels within an object that have intensity values outside the selected range for objects. If the Fill Holes option is set, this value will be 0.
- **Hole Area:** Reports the area of holes within an object. A "hole" is defined as any contiguous set of pixels within an object that have intensity values outside the selected range for objects. If the Fill Holes option is set, this value will be 0.
- **Hole Ratio**: Reports the ratio of the object area excluding holes, to the total area of the object, as determined by Area / (Area + Holes Area). Remember,

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[2] IPP implements a variation on the hand and divider method (first introduced by Richardson). IPP accounts for variations in the perimeter measurement as a result of different starting points along the outline (i.e. depending on the where you first put the ruler to start measuring the outline, the perimeter is going to vary slightly even if the ruler stays the same). The original method doesn't compensate, which is one of its main drawbacks.

The fractal dimension is defined as the slope of the linear part of the function that relates the log of the outline length to the log of the stride length, where the stride length is how long a ruler we attempt to lay along the perimeter of the object. Perimeters change with different size rulers, as the rulers bridge various small wriggles in the outline.

We calculate the fractal dimension as 1 minus the slope of the regression line obtained when plotting the log of the perimeter (using a particular stride) against the log of the stride length, as calculated with multiple starting points in the outline for the strides. The stride length limits can be set using BLBCMD_SETFRACTDIM (see the SDK for details on how to use this), although we stop when the stride length is close to the minimum diameter of the object in any case.

when a Hole measurement is selected, Area is the area of the object less the area of the holes. If the Fill Holes option is set, this value will be 1.

- **Integrated Optical Density (IOD):** Reports the average intensity/density of each object. This value will be expressed in terms of the current intensity/density mode and calibration.
- **Major Axis:** Reports the length of the main axis of the ellipse equivalent to the object (i.e., an ellipse with the same area, first and second degree moments).[3]
- **Minor Axis:** Reports the length of the minor axis of the ellipse equivalent to the object (i.e., an ellipse with the same area, first and second degree moments)3
- **Max Diameter:** Reports the length of the longest line that can be drawn to pass through the centroid position and join two points on each object's perimeter.
- **Min Diameter**: Reports the length of the shortest line that can be drawn to pass through the centroid position and join two points on each object's perimeter.
- **Max Radius:** Reports the maximum distance between each object's centroid pixel position and its perimeter.
- **Min Radius:** Reports the minimum distance between each object's centroid pixel position and its perimeter.
- **Max Density:** Reports the maximum intensity or density inside the object.
- **Min Density:** Reports the minimum intensity or density inside the object.
- **Margination:** Reports the distribution of intensity between the center of an object and the edge of the object.[4]
- **Perimeter:** Measurement to report the length of the outline of each object using a polygonal outline. The perimeter of interior holes are not included in this measurement.
- **Perimeter2:** Old measurement from version 3.0. Faster but less accurate than current perimeter measurement**.** Reports the length of the outline of each object. When holes are outlined, the perimeters of the holes are added to the perimeter of the object.

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3 The Equivalent Ellipse is an ellipse with the same moments of inertia (firstand second moments) of the object. Note that this will in general NOT have thesame area (the '0' moment) of an object, as two objects with similar areas but different shapes can have different moments of inertia. As a conceptual example,
consider the moment of inertia of a barbell around its center of gravity with the weights at the ends of the bar, compared to the same barbell with the weights slid to the center of the bar. Both have the same weight, but the second example is much easier to rotate, since its weight is concentrated at the center of gravity. In two dimensional terms, a long capital letter "I" will have the same equivalent ellipse as a much larger filled circle, even though the filled circle will have a larger area.

4 Margination algorithm courtesy of Professor Ian T. Young, Pattern Recognition Group Delft University of Technology Department of Applied Physics Lorentzweg 1 NL-2628 CJ Delft The Netherlands

- **Perimeter3:** New measurement in *Image-Pro Plus* 7.0. Reports a corrected chain code length of the object perimeter, not including holes. Much less biased than Perimeter2.[5]
- **Perimeter (Convex):** Reports the perimeter of the convex outline of each object.
- **Perimeter (Ellipse):** Reports the perimeter of the ellipse surrounding the outline of each object.
- **Perimeter (Ratio):** Reports the ratio of the convex perimeter to the perimeter of the outline of each object.
- **Perimeter Length:** Reports the length of the object as estimated from its perimeter, most accurate for single-pixel wide objects, equivalent to the perimeter divided by 2.
- **PerArea:** Reports the ratio between the area of the counted object to that of the entire area. The ratio is determined by *(Object Area/Total Area)* where *Total Area* is the area of the active image or AOI (whichever was in effect when the measurement was taken).
- **Radius Ratio:** Reports the ratio between Max Radius and Min Radius for each object, as determined by *Max Radius / Min Radius.*
- **Roundness:** Reports the roundness of each object, as determined by the following formula: $(perimeter^2) / (4 * \text{pi} * area)$. Circular objects will have a roundness = 1; other shapes will have a roundness > 1.
- **Size Count:** Reports the count of multiple objects of varying sizes in multiple frames.
- **Size (length):** Reports the feret diameter (caliper length) along a major axis of the object.
- **Size (width):** Reports the feret diameter (caliper length) along a minor axis of the object.
- **Std. Dev. Density:** Reports the standard deviation of density or intensity inside the object.

[5] Kulpa, Z., Area and perimeter measurement of blobs in discrete binary pictures. Computer Vision, Graphics and Image Processing, 1977. 6: p. 434-454.

Selecting Your Ranges

Once you have identified the measurements you want to take, you can set measurement criteria that will determine which objects *Image-Pro Plus* includes in the count. For example, you can instruct *Image-Pro Plus* to include only objects larger than a certain size. This is done by establishing a valid range (i.e., specifying minimum and maximum values) for each measurement using the *Set Ranges* command in the *Measure* menu. If the object's measurement is within this range (min and max, inclusive), it will be included in the count. Objects with values outside the range are ignored.

Selecting Other Measurement Options

Image-Pro Plus offers several additional options that let you tailor your count to your needs, including how partial objects will be treated, and how objects will be labeled. These miscellaneous options are selected using the **Options** button in the **Count/Size** window:

- **Smoothing:** This option lets you set a factor that determines whether or not the perimeters of the counted objects will be smoothed. A factor of 0 indicates that no smoothing should be done. Values greater than 0 turn smoothing on, and indicate the degree of smoothing that should take place. If perimeter and roundness are critical measurements to your analysis, you may want to use this option. Bear in mind that smoothing will lengthen the time it takes to perform your count, so you will want to use this option only when it is truly necessary.

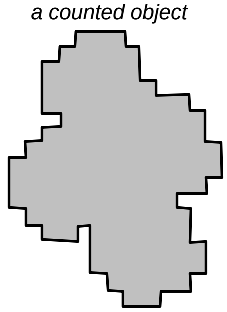
- **Clean Borders:** This option determines whether partial objects that lie at the border on an image should be included in the count (partial objects can skew your results). If Clean Borders is selected, these partial objects will be ignored.

- **Outlines:** This option determines whether outlines of the counted objects will be shown or not. If objects are to be outlined you can select one of several outline formats: perimeter, elliptical, filled or class. When **Outline** is selected, the object's perimeter is outlined. When **Ellipse** is selected, the object's equivalent ellipse (an ellipse with the same area, first and second degree moments as the object) is displayed. The **Ellipse** option is particularly meaningful in grain-counting experiments, because it identifies an object's ideal domain, from which binding sites can be identified. The **Filled** option shows counted objects in solid color, and the **Class** option colors the objects by category (see *Visualizing Measurement Data* below). This option also allows you to select the color in which you want the outlines displayed. **Dot** marks each counted object with a small cross hair positioned in the center of each object's bounding box.

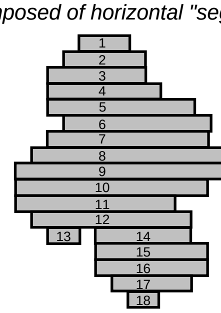
- **Label:** This option determines whether an object's unique numeric label should be displayed following the counting process, and what color should be used to display it. The displayed labels allow you to visually match objects with your results, and should rarely be turned off. The only times you may want to turn labeling off are to eliminate screen clutter or inspect the details of a particular outline.

- **Object Limits:** Up to 16,000 objects can be included in a single count. If your image contains more than 16,000 objects, consider using AOIs to process the count in a series of small batches. *Image-Pro Plus* also has an internal limit of 250,000 object “segments.” A counted object is built of segments, which are horizontal, single-pixel “slices” of the object. Each segment is represented internally by its beginning and endpoint coordinates.

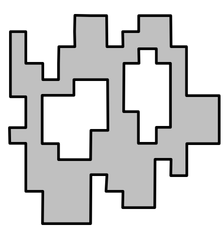
a counted object



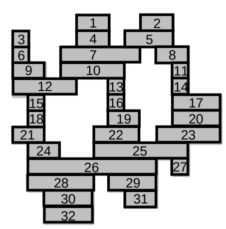
is composed of horizontal "segments"



a counted object with holes and/or fissures



will be composed of many, many segments



Objects containing lots of fissures or holes will require many segments to describe their shape. If you have many such objects, the internal limit of 250,000 segments could be exceeded. Consider using AOIs to process the image in a series of small batches if this limit is encountered.

The *Outline* option in the **Count/Size Options** dialog box can outline object perimeters comprised of up to 4096 vertices. If your object has a very, very large, irregular perimeter, you may exceed this limit and its outline will not be displayed correctly (all measurements taken for the object are correctly reported, however). To display an outline of a very, very large object, select either the **Filled** or **With Holes** outline styles.

Performing the Count

Once you have identified your objects, selected your measurements and tailored the count with the options you need, you are ready to initiate the count itself. To do this, click the **Count** button.

Image-Pro Plus will analyze your image, and will outline and measure the objects it finds meeting your selection criteria. When it is done measuring, it will display the object outlines, and write all the requested measurements to the measurement data sheet. You can view the measurements for an individual object by double-clicking the object in the image. This produces a pop-up window that contains the measurements for the selected object. The measurement data sheet, which is a table of all the measurements, can be viewed by selecting the *Measurement Data* command in the *View* menu.

Editing the Count

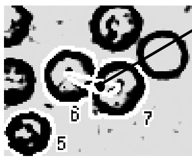
Before working with the data sheet, you should take a moment to review the display to make sure you are satisfied with the objects that have been extracted — you may want to eliminate extraneous objects, separate clusters that have been counted as a single object, or combine separate outlines into one. On the *Edit* menu, *Image-Pro Plus* gives you a number of tools that let you edit and finalize your count.

- **Draw/Merge Objects:** This option lets you manually outline an object that was not included in the count, or combine two objects so that they are counted as one.

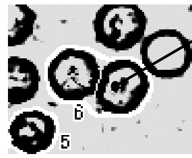
  To manually include an outline, select Draw/Merge then draw a polygon that traces the object, using any of the standard AOI tools: rectangular, elliptical or freeform (you can even use the Freeform tool's **Auto Trace** feature to automatically trace an edge: See *AOI Tools.*) When you have finished drawing the polygon, the specified measurements for that shape will also be added to the data sheet.

  Note - You can draw a polygon that defines an object that doesn't visibly exist in your image. This is usually done to define a region from which population density can be calculated.

  To combine two closely positioned objects into a single object, simply draw a narrow polygon that *connects* (not outlines) the two existing objects (see example below).



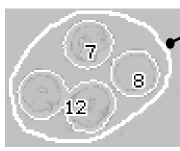
polygon drawn to connect objects 6 & 7



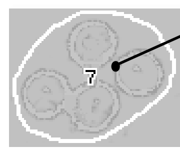
single object produced by merging objects 6 & 7

Once connected, the measurements of the two objects will be outlined and considered as a single object. In the data sheet, the combined measurement will replace the measurements of one of the original objects and the other's measurements will be deleted.

You can also merge more than two objects into a single object. The objects needn't be close together, either. As shown in the drawing below, you can merge multiple objects into a group by drawing a polygon around them, or by drawing a polygon with its vertices inside the individual objects.

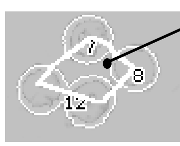


polygon drawn around 3 objects

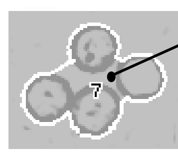


single object it produced

or



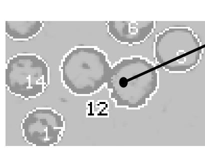
polygon drawn inside the 3 objects



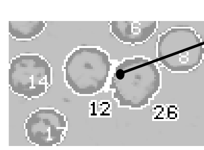
single object it produced

Merging many objects in such a manner might be done to develop area outlines for population density analysis (see *Population Density Analysis* later in this section).

◆ **Split Objects:** This option lets you manually divide clusters into single objects. Dividing is done drawing a division line between the objects you want to separate (see example below).



clustered objects



line drawn to separate the cluster

After separation, the measurement data sheet will be updated to reflect the additional object, and the measurements of the original object will be adjusted to reflect the remaining object.

- **Auto Split:** You can also use the *Auto Split* command to instruct *Image-Pro Plus* to analyze all existing outlines and automatically split any clustered objects it finds. Of course, not all clustered objects can be separated with *Auto Split*; in general, circular objects with minimal overlap work best. Use the following criteria for determining whether your objects are clustered in a way that can be easily separated by the *Auto Split* operation:

  - Individual objects within the cluster should be convex-shaped. An object is considered convex if all lines joining two points of the outline fall within the object.
  - Objects should not overlap each other by more than 30%.
  - The intersection of two overlapping objects should be identifiable by two concave points.
  - Object outlines should be smooth enough to readily distinguish a concavity created by an overlap from a concavity that is simply part of the object.

  Note - Image-Pro Plus splits objects with a straight line. It does not attempt to reconstruct the missing or hidden portions of an object.

  If the *Auto Split* command does not yield satisfactory results, try using the *Watershed Split*. This method does not rely on object concavities to determine lines of separation. Instead it erodes an object until it disappears, then dilates it back to its initial size, but does not allow it to touch its neighbors.

  - **Watershed Split:** Use the *Watershed Split* to automatically analyze every counted object and split clusters using the *Watershed* separation technique. The *Watershed* method erodes objects until they disappear, then dilates them again such that they do not touch.

    The *Watershed Split* command may be more effective than the *Auto-Split* method if your objects are not convex.

You may remove an extraneous object from your count by using the *Toggle Objects On/Off* feature from the *Count/Size:Edit* menu. When an object is hidden, its outline and label are removed from the display and its measurements are eliminated from the data sheet. Deactivated measurements are also excluded from global statistic, classification and visualization operations. To permanently remove hidden objects, choose *Delete Hidden Objects* from the *Count/Size:Edit* menu.

Manual Tagging

Another option to identify objects in your image is to use the **Manual Tag** option from the *Measure* menu. **Manual Tag** allows you to select points in an image, and assign them to a specific class. You can define up to 16 classes of objects for a single image, and assign each class a name, color, and identifying symbol. *Image-Pro Plus* automatically keeps track of the points as you select them. You can display information about each class including minimum, maximum, mean, standard deviation, and total by using the *Manual Tag : View: Class Stats* menu.

Manual Tag : Options allows you to see the **x** and **y** position, intensity, and class of each tagged object in the image.

Manual Tag measurement information can be stored using the *Data to File* command on the *Manual Tag : File* menu. Data and statistics are stored in an ASCII file (assigned the extension .CNT), which contains location information about the different markers. A portion of a .CNT file appears below.

Marker	Class	X	Y	Intensity
1	Class 3	78	88	169
2	Class 3	178	88	180
3	Class 3	181	179	185
4	Class 3	79	177	168

A Manual Tag file is created when you store your class and/or tag marker information in the *Manual Tag* selection from the *Measure* menu. A *.TAG file is created using the **Save Points** command on the *File* menu in the **Manual Tag** dialog box.

A *.TAG file is an *Image-Pro Plus* file that contains the class and marker information that is to be applied to the objects in an image. This data is not in ASCII format. Users should not attempt to modify a *.TAG file

Using the Outlines

There may be situations where you will need to generate your outlines from one image, then superimpose the outlines onto another image to identify object position, or to obtain an average intensity measurement. Such is necessary in Auto-Radiography applications, where cell position and shape is captured in visible light, but cell contents are captured with a different apparatus which does not produce a cell outline. To measure the intensity within a cell, the outlines are generated by the first image, then superimposed upon the second image. Then, an intensity measurement is performed using the **Population Density** measurement in the *Count/Size* command's *Measure* menu.

Object outlines can also be stored using the *Save Outlines* command on the **Count/Size** window's *File* menu. Outlines are stored to *Image-Pro Plus's* outline files (.OUT). An outline file contains a list of all the polygonal shapes comprising your outlines. The format of this file is described in *Appendix B of the Reference Guide, File Format Specifications*.

Stored outlines can be used as input to an external program, or can be reloaded into *Image-Pro Plus.* A stored outline can be subsequently loaded into *Image-Pro Plus* for positioning purposes or for additional measuring. Outline files are also an essential component to the population density process (see *Population Density Analysis* later in this section).

Viewing Measurement Data

When a count operation is performed, the requested measurements (those selected with the *Select Measurements* command from the *Count/Size:Measure* menu) for all counted objects are written to the measurement data sheet. This data sheet can be viewed by selecting the *Measurement Data* command from the *Count/Size:View* menu.

The data sheet contains only values for the requested measurements; if you want to incorporate additional measurements in your data sheet you will need to select them, then re-measure the outlines by clicking the **Measure** button (keep the original measurements selected too, if you want them to appear in the data sheet).

Statistics, such as minimum, maximum, mean and standard deviation can be viewed using the *Statistics* command on the *View* menu. A set of these statistics is produced for each measurement you have selected.

Measurement data can be stored to a file using the *Data to File* command on the **Count/Size** window's *File* menu, or on the **Measurement** window's *File* menu. Data can be exported to an *Excel* spreadsheet..

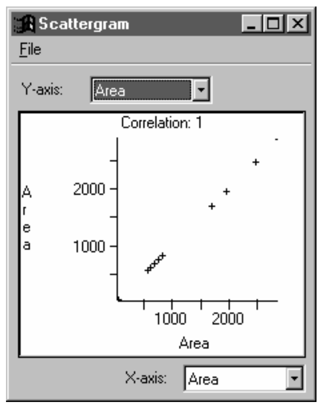
Visualizing Your Measurement Data

Two plotting devices, *scattergram* and *histogram*, are provided to let you visualize your data in chart form. You can also render your objects using color to represent the measurement values they possess.

Scattergrams

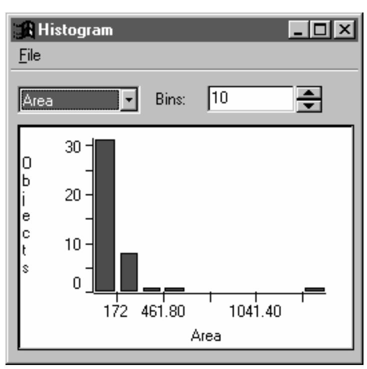
A scattergram plots two measurements against each other. Scattergrams can be used to illustrate a correlation between two measurements. For example, a scattergram can graphically show you whether or not there is any relationship between two measurements, or whether there is any clustering of the data points.

A scattergram is created by selecting the *Scattergram* command from the *Count/Size*:*View* menu. *Image-Pro Plus* displays the scattergram along with its correlation factor and a best fit line drawn through the points.



To select the measurements you want to plot on each axis, use the list boxes provided in the **Scattergram** window.

Measurement histograms (not to be confused with intensity histograms) provide another way to plot your measurement data. Measurement histograms illustrate the distribution of your object population over a measurement range. The histogram's X-scale identifies the measurement's range (from its minimum value to its maximum value), and the Y-scale measures the number of objects within each measured interval.

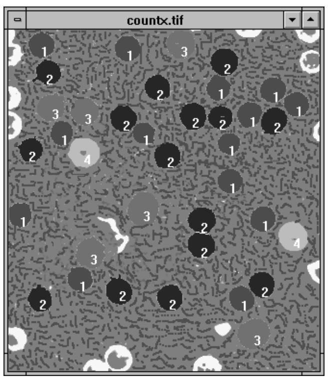


Measurement histograms are created using the *Histogram* command on the **Count/Size** window's *View* menu. Within this menu, you must select both the measurement you want to plot, and the number of intervals (from 2 - 100, inclusive), into which you want your X-axis segmented.

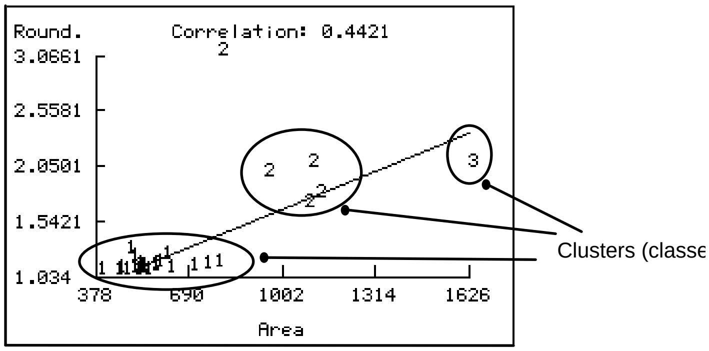
Scattergrams and histograms can be captured and saved into an image file using *Image-Pro Plus's Screen Capture* facility. The captured image file can be printed in *Image-Pro Plus*, or imported into your word processing program, or included in a report (*see The Report Generator*).

You can also visualize your measurement data with color, using the classification commands on the **Count/Size** window's *Measure* menu and coloring the objects by class. This lets you distinguish the objects with a color that represent their measurement values. Objects may be classified using one of two methods. The *Single Variable Class.* command lets you divide the results of a single measurement type into the number of categories that you define. If you then select the **Class** outline options in the *Count/Size* options dialog box, your objects will be colored according to the class to which they belong.

In the example below, the spores have been classified and colored according their Area measurement.



You may also classify your measurement results using the *Auto Classification* command. This lets you categorize data into groups of objects with similar measurement characteristics. In the **Auto-Classification** dialog box, you must define the number of groups you want your data sorted into and the measurements you want used as classifiers. *Image-Pro Plus* will look for clusters of data points that are created when the selected measurements are plotted against each other (see figure below). Data clusters (classes) are isolated using a cluster-seeking technique.



Clusters identified during automatic classification by Area and Roundness

Although *Image-Pro Plus* does not display a plot like the one above during its automatic classification process (it would be impossible to produce for more than 3 classifiers), you can check for clusters yourself by generating a Scattergram for each measurement pair in your classifier selection list (see *Scattergram* discussion earlier in this section). Creating a scattergram is a good way to determine whether or not you have selected meaningful classifiers - if your scattergram yields a correlation close to 1, you only need to use one of the plotted measurements as a classifier because the values of the two are so similar.

After your measurements have been classified, you can select the **Class** outline options in the **Count/Size** options dialog box, and your objects will be colored according to the class in which they belong.

Saving Your Counting Environment

You may spend a considerable amount of time tailoring the counting options, intensity ranges and classifiers to a particular image. Instead of manually resetting these parameters next time you work with a similar image, you can save all these settings, then reload them when they are needed. This is done using the *Save Settings* command on the **Count/Size** window's *File* menu.

Environment parameters are stored in .ENV files. A counting environment can be restored by loading the appropriate .ENV file using the **Load Settings** option.

Population Density Analysis

Population Density is the measurement of the number of objects per unit of area. It is often used to determine the density of a cell sample.

Calculating population density is performed using the *Population Density* command on the **Count/Size** window's *Measure* menu. However, there are two very important steps that must be performed before the *Population Density* command is selected. The three steps required to produce a population density analysis are:

Step 1 - The outlines of the sites to be measured must be created and saved to a file.

Step 2 - The individual objects within the areas to be measured must be counted.

Step 3 - The *Population Density* command must be called.

Each step is discussed below. You may also want to refer to the *Population Density* tutorial in the *Image-Pro Plus Installation Guide* for additional background.

Step 1 - Defining the Areas to be Measured

Defining the sites to be measured can be done in two basic ways:

- ◆ If your image contains small objects (cells or nuclei, for example) inside of a visibly defined larger object (site), you can use the normal object counting process to measure and outline the large objects (sites).

- ◆ If your small objects are not contained within any visible entity, you can use **Count/Size** window's *Draw/Merge* command or the **Measurements** window's **Area** tool to manually draw outlines of the sites to be measured.

Regardless of which method you use to define the outlines of the sites, once you are satisfied with the outlines on the screen, they must be saved to an outline file using the *Save Outlines* command on the window's *File* menu. This file will be requested when you perform the *Population Density* command in Step 3.

Step 2 - Counting the Individual Objects

Next, you must count the individual objects (grains, nuclei, or whatever the case may be). If the site outlines and measurements from the previous step are still active, be sure to clear them before you begin. Then, count (and measure, if needed) all the small objects using any the normal counting procedure.

It is important that you have an accurate count of the number of *individual* small objects in your image. If some of your small objects are overlapping and clumped into groups, the objects within the groups will need to be measured. This is done by selecting the **Clusters** measurement when you perform the count (select **Cluster** in the **Select Measurements** dialog box). A cluster measurement calculates and records the number of objects contained in each counted outline. This value is written to the measurement data sheet.

Step 3 - Calculating Density

Once you have the small-objects outlined and count on the screen, you are ready to begin the density analysis. To do this, select the *Population Density* command from the **Count/Size** window's *Measure* menu and select the file that contains the site outlines created in Step 1. The site outlines will be superimposed over the small objects you've just counted, and the object density within the outlines will be calculated. The results of the density calculations are displayed in the *Population Density* window. The following data is reported:

- **Site Number:** This number identifies a site outline within the image. Each site is given a unique numeric label, which is displayed in brackets, [ ], to distinguish it from the small object labels.

- **Object Count:** This number identifies the number of objects within the site. An object is considered within a site if its center of gravity lies within the site outline.

- **Site Area:** This number reports the area of the site. It will be reported in terms of the current spatial unit.

- **Density (raw):** This number reports the gross density for the site as calculated by dividing **Object Count** by **Site Area**.

- **Density (corrected):** This number reports an adjusted density for the site as calculated by subtracting the AOI's background density from the Site's raw density. This gives a value that represents the density of the site independent from the background population.

- **Sites (mean):** This row of number reports the average object count, area density statistics for all sites within the AOI.

- **Sites (sum):** This row reports the total number of objects and the total area for all sites within the AOI.

- **Background:** This row reports the number of objects that fell outside of the site outlines, the area of the AOI that fell outside the site outlines (the background area), and the density of the background calculated by dividing the **Background Site Area** value by the **Background Object Count** value. The background density value is used to create a **Corrected Density** value for each site.

- **Total:** This row reports the total number of objects within the AOI (those within the sites *and* those within the background), the total area of the AOI and the population density of the entire AOI.

Customizing the Command Menus and Buttons

Image-Pro Plus adds new capabilities for customizing the user's interaction with the application. While previous versions maintained a single `*.MNU` file that defined the application's menu and toolbar, *Image-Pro Plus* supports multiple menu files and user selection of the menu file to be used. *Image-Pro Plus* ships with 4 menu files, which you can modify to customize the application for your purposes, but you can also use one of these menus as the source for creating a new menu file of your own that is added to the list shipped with *Image-Pro*. This is a very powerful addition to the customization capabilities of *Image-Pro*. The rest of this section will discuss the contents of an MNU file, and while the examples will refer to the *Image-Pro Plus* reference MNU file, the discussion is relevant to the other standard MNU files as well as any new ones that you create.

Note - You can completely customize Image-Pro using Image-Pro's Software Development Kit (SDK). The SDK allows you to incorporate your own, custom-designed DLL's and toolbar icons, letting you create powerful, vertical-market applications based upon Image-Pro. For more information about the Image-Pro SDK, contact Media Cybernetics or your Image-Pro dealer.

The selected `MNU` file in the application's resources folder defines the main menu and tool bar. By editing this file (using any plain text editor) you can add your plug-in to a menu or attach it to a button on the tool bar. When you add it to the tool bar, you can use one of the icons provided with *Image-Pro* or design one of your own.

Along with the ability to select a different menu and toolbar arrangement, *Image-Pro Plus 7.0* also adds the ability for the MNU file to give the application hints about the intended purpose of the specialized menu. Individual features of *Image-Pro* may customize their appearance or tool set based on the selected menu's hints.

Note - You may need to close any open dialogs before switching menus to see changes in the feature set.

You may therefore wish to define new *Image-Pro* menus for a number of different reasons:

- To change the set of features available to your users to customize the product for their experience and workflow, and/or
- To change the menu hints

An MNU file is made up of a series of definition statements. The first set of statements defines any application hints. The second set of statements defines the menu structure. The third set of statements is a portion of .MNU which defines the tool bar buttons.

While editing a `.MNU` file, the following rules must be followed:

- Keywords must appear *entirely in lower case* (i.e., **`progitem`**, not `PROGITEM` or `ProgItem`)
- The first parameter must be separated from its keyword with a space. Subsequent parameters must be separated from one another with a comma.
- You may use spaces or tabs to indent statements (usually done to improve readability).
- The # character is the “comment” character. It can be used to designate a statement that should not be processed. This is usually done to include lines of descriptive information, or to “comment out” a statement that is to be excluded from the file. *The # character must appear in the first position of the line.*

Note: Although you may edit the `MNU file` while your application is active, your changes will not take effect until the program is restarted, or until you re-select the menu from the Select Menu item in Image-Pro’s Window menu. Also, be sure to close the `MNU file` in your editor before starting the application, otherwise it may not load properly.

The presentation of the menus and buttons is defined by the current *.MNU file. This file is located in your *Image-Pro* resources directory. By editing the contents of this file using any ASCII editor (the Window's “Write” could be used, for example), you can modify the appearance of your menus and command buttons.

*Note - although you may edit the *.MNU file while Image-Pro is active, your changes will not take effect until Image-Pro is restarted, or the menu is reloaded using the Select Menu item on the Windows menu.. Also, be sure to close the *.MNU file in your editor before starting Image-Pro; otherwise Image-Pro may not load properly.*

***Important** - always make a copy of your *.MNU file before making any changes to it. Mistakes in this file can easily affect the operation of Image-Pro, and render* *commands inoperable. A back-up file will ensure that you can revert to a previous, good, version of this file in the event of problems with your edits.*

Customizing the Menus

The first section of the *.MNU file defines the contents of the command bar. You can edit this section to remove, rename or re-organize the *Image-Pro* main menu and commands therein.

Menu statements

The example below defines a *File* menu (though not the *Image-Pro Plus* standard File menu). The menu begins with a **columnbegin** statement and ends with an **endcolumn** statement.

```
columnbegin &File, 0
   progitem &New...,Make document.,0,101            Menu items
   progitem &Open..., Open image file.,0,102        (commands)
   progitem &Reload, Reload image file.,0,112
   separator                                         Separating line
   group &LUT
      item &Load LUT. . ., Load LUT file.,iplut32.DLL,14,1        A sub-menu
      item &Save LUT. . ., Save LUT to file.,iplut32.DLL,14,2
   endgroup
   item &Convert..., Convert image on disk.,ipconv32.dll,32,0
endcolumn
```

Each statement in the MNU file is made up of a keyword followed by several parameters. A complete, syntactical description of the allowed statements is documented at the end of this chapter.

Important Note: When adding columns to the menu, (which adds a new menu to the application's command bar) new menus must be added after the *File* column and before the *Window* column.

*Note - *.MNU provides the controls for modifying the* <u>*main*</u> *Image-Pro menu. Menus that are presented within command windows, such as* ***Count/Size*** *and* ***Histogram****, are not defined by the *.MNU file and are not modifiable.*

Before editing this file, you should familiarize yourself with the elements of its structure. The following table introduces you to the basic statements that are used to create a menu in *Image-Pro*. A complete, syntactical description of each of these statements is documented at the end of this section under **.MNU Syntax*.

STATEMENT	**DESCRIPTION**
`columnbegin`	Marks the beginning of a menu
`endcolumn`	Marks the end of a menu
`progitem`	Defines a menu item — i.e., a "command" listed in the menu. Differs from `item`, below, in that it invokes a procedure, or macro, that is resident in the main *Image-Pro* program.
`item`	Defines a menu item — i.e., a "command" listed in the menu. Differs from `progitem`, above, in that it invokes a procedure that resides in an *Image-Pro* DLL.
`group`	Marks the beginning of a sub-menu within the current menu (i.e., a "pop-out" menu).
`endgroup`	Marks the end of a sub-menu within a menu.
`separator`	Draws a line between items in a menu.

The example below shows a portion of the *Image-Pro File* menu description, and points out the menu components within it.

```
columnbegin &File                    File Menu Title                              Menu Items
       progitem &New...\tCtrl+N,Make new document.,0x0000,101
       progitem &Open...\tCtrl+O,Open document from disk.,0x0000,102
       item Image &Database...,Open document from image database.,ipgali32,
0x0480, 0
       %item Database Stub,Start database.,ipgali32,0x0480,20
       progitem &Reload,Reload document from disk.,0x6B0F,112
       progitem &Close...,Close current document.,0x6B0F,103
       progitem &Save...\tCtrl+S,Save current document to disk.,0x6B0F,104
       progitem Save &As...,Save current document to disk with new
name.,0x6B0F,105
       item Arc&hive...,Store image in the image
database.,ipgali32.dll,0x6B0F,10
       item Con&vert...,Convert file format.,ipfile32,0x0000,0
       separator                                                          Separating Line
       item Send &Mail...,Send Mail.,ipmail32,0x0480,0
       group Remote File &Transfer
              item &Open Remote File...,Open file on remote FTP           Submenus
server.,ipftp32,0x0480,0
              item &Save Remote File...,Save file on remote FTP
server.,ipftp32,0x6B0F,1
              item &Personal File Server...,Configure personal FTP file
server.,ipftp32,0,2
       endgroup
```

Removing a Menu Item

You can remove an item from a menu by placing the "#" character in the first position of the `item` or `progitem` line. The "#" character tells *Image-Pro* to ignore that particular statement. In the following example, the three color-processing commands have been removed from the *Process* menu.

```
columnbegin &Process
    item &Background Correction...,Perform Background
Ops.,ipflt32,0x6BAF,101
    item Restricted &Dilation...,Perform masked image
dilation.,ipflt32,0x6BAF,103
    item &Threshold..., Binarize Image.,ipseg32,0x0B82,100
    item &FFT...,Fast Fourier Transform operations.,ipfft32,0x0080, 100
    item &Stretch..., Slide/Stretch.,iplut32,0x6B02,111
    item Fi&lters...,Perform Spatial or Morphological
Filters.,ipflt32,0x6B8F, 100
    item &Operations...,Perform arithmetic & Logical
Operations.,ipflt32,0x6BAF,102
#   item Color C&hannel...,Color channel processing.,ipcolr32,0x6BAF,100
#   item &Segmentation...,Perform Segmentation.,ipseg32,0x6BAE,100
#   item &Pseudo-Color..., Colorize Image.,iplut32,0x0B02,201
    item &Registration...,Register two images.,ipreg32,0x698A,100
    progitem Image Overla&y...,Overlay an image with another.,0x6B2E,3008
    item &Mosaic Image...,Combine several images into a new mosaic image.,
IPMos32, 0x6BAE, 1
    item Trace Ob&jects...,Draw overlay tracing
objects.,iptrce32,0x698A,100
    item &Grid Mask..., Creates grid mask, ipgrid32, 0x0080, 1
endcolumn
```

Moving a Menu Item

You can move a command from one menu to another by simply moving its `item` (or `progitem`) statement to another menu. Be sure you move the *entire* statement, including the carriage-return character that terminates it.
Note - be sure to consider the documentation impact when modifying your menus. Commands in the Image-Pro Reference Manual are documented in the order in which they appear on the default menus. Rearranging commands will reduce your user's ability to "thumb-through" the manual to locate a command.

Renaming a Menu Item

You can rename a command on the menu by changing the contents of the *Title* parameter in its `item` or `progitem` statement (see **.MNU Syntax* for the position and content of the *Title* parameter).
Within the *Title* parameter, the "&" character must precede the character designated as the "access" key (sometimes called "accelerator" key); the access character

specifies the key that can be used to select the command once the menu is opened — it appears as an underscored character when the menu is displayed. Try to choose a character that is not in use as an access key by any other command in the menu. If you want an ellipsis (i.e., ...) to appear after your command name, be sure to include it as part of your command name in the *Title* parameter (by convention, an ellipsis is used to denote commands that produce a dialog box when selected — e.g., *Print...*).

Note - keep in mind that if you rename a command, your users may have difficulty locating information about it in the Image-Pro Reference Manual and on-line help system.

Adding a Macro to a Menu

Macros can be added to the tool bar by creating a **macrobutton** statement for it in the `MNU` file. The **macrobutton** statement identifies the icon that is to be displayed on the tool bar, and specifies the macro that is to be executed when it is clicked (including the script file that contains the macro). See the `MNU` statements at the end of this chapter for a complete description of the **macrobutton** statement.

The following example shows a **macrobutton** statement defined for a macro called `Histogram` in `the C:\IPWIN7\DEFAULT.IPM` script file. In this example, the icon called `USER1` is assigned to the tool bar.

```
macrobutton USER1,Macro: Histogram,C:\IPWIN7\DEFAULT.IPM
,0,Histogram
```

You may use any of the predefined icons for the macro button, but the application will set it back to `USERx` if the buttons are recreated.

.

An *ID* of...

`5000` identifies the first macro in the selected script file,
`5001` identifies the second macro in the selected script file,
`5002` identifies the third macro in the selected script file,

and so forth.

The following example shows a `progitem` statement defined for the 4th macro (#5003) in a script file.

```
progitem &Sub-Sample Sequence...,Create sub-sampled sequence.,0x6B0F,5003
```

Important - *a macro is always invoked from the currently selected script file. If you change script files mid-session, a menu item will invoke the specified macro number from* *the new script file. To ensure that the macro you intended is always the one invoked by a menu command, do not change script files during your Image-Pro session.*

Advanced Menu Editing

By following the syntax outlined under **.MNU Syntax* at the end of this section, you can structure your *.MNU file such that it creates, deletes or renames entire menus. You can also regroup the existing menu commands so that they appear in any order you'd like.

Customizing Command Buttons

The second section in the *.MNU file contains the statements that determine which command buttons are shown on the tool bar. You can even assign a macro that you have developed to a command button on the toolbar. This allows you to customize your tool bar with just the tools you want to have close at hand.

A command button is defined by one of the following statements in the *.MNU file:

STATEMENT	DESCRIPTION
`progbutton`	Defines a command button that is to be displayed on the Toolbar. Differs from `button,` below, in that it invokes a command that is resident in the main *Image-Pro* program. The `progbutton` statement is also used to assign a user-defined macro to a command button.
`button`	Defines a command button that is to be displayed on the Toolbar. Differs from `progbutton`, above, in that it invokes a command that is resident in an *Image-Pro* DLL.

The *.MNU file contains definitions for all of the *Image-Pro* commands that can be assigned to the Toolbar. By default, only a few of these statements are active. You can selectively activate/deactivate buttons by including or removing the # character (the “comment” character) at the beginning of each statement. Button statements that begin with the “#” character are ignored by *Image-Pro,* and the buttons for such statements are not shown on the Toolbar.

The example below shows an excerpt from the *.MNU file. In this example, the first 3 statements are “active” — the command buttons for these will be displayed. The last 3 statements are commented out — these buttons will not be displayed.

```
button FFT,Fast Fourier Transform,ipfft32,0x0080, 100
button FILTER, Spatial filtering,ipflt32,0x6B8F, 100
```

```
button OPER,Arithmetic and logical perations,ipflt32,0x6BAF,102
#button TRANSFORM,Color channel processing,ipcolr32,0x608A,100
#button SEGMENT,Perform segmentation,ipseg32,0x6B8E,100
#button PSEUDO, Colorize gray scale images,iplut32,0x6B02,201
```

See the next section (**.MNU Syntax*) for syntactical descriptions of the `button` and `progbutton` statements.
*Note - approximately 20 command buttons can be shown simultaneously, however, the exact number will depend upon the resolution of your monitor. You will need to experiment with the button statements in the *.MNU file to determine just how many buttons will fit on your Toolbar*

Assigning an Image-Pro Command to a Button

You may assign any of the menu commands to a command button by following the procedure below:

1. **Copy the `item` or `progitem` statement** from the menu definition section at the top of the *.MNU file to the button description section (do not delete the statement from the menu definition section unless you deliberately intend to remove it from the menu).
2. **Change the statement's keyword as follows:**

IF THE KEYWORD IS...	CHANGE IT TO...
`progitem`	`progbutton`
`item`	`button`

3. **Change the contents of the *Bitmap* parameter** to the name of one of the pre-defined button bitmaps. See the `progbutton` or `button` definition under **.MNU Syntax* for a description of the *Bitmap* parameter.

The following example shows how a copy of the "Video Average" `item` statement would be modified to assign it to the **Camera** button. The underscoring denotes the parts of the statement that would be changed.

This `item` statement...

```
progitem &Next Image \tF2,Activate next document.,0x6B0F,657
```

would be changed to...

```
progbutton NEXT,Activate next document.,0x6B0F,657
```

Assigning a Macro to a Command Button

Macros can be added to the tool bar by creating a **macrobutton** statement for it in the `MNU` file. The **macrobutton** statement identifies the icon that is to be displayed on the tool bar, and specifies the macro that is to be executed when it is clicked (including the script file that contains the macro). See the `MNU` statements at the end of this chapter for a complete description of the **macrobutton** statement.

The following example shows a **macrobutton** statement defined for a macro called `Histogram` in `the C:\IPWIN7\DEFAULT.IPM` script file. In this example, the icon called `USER1` is assigned to the tool bar.

```
macrobutton USER1,Macro: Histogram,C:\IPWIN7\DEFAULT.IPM
,0,Histogram
```

You may use any of the predefined icons for the macro button, but the application will set it back to `USERx` if the buttons are recreated.

You can assign a macro to a command button by creating a `progbutton` statement for it in the MNU file (the `progbutton` statement, not the `button` statement, *must* be used when assigning a macro to a button).

Like the `progbutton` statement for a built-in feature, the `progbutton` statement for a macro consists of the *Bitmap, Descrip, BitFlags* and *ID* parameters (see **.MNU Syntax* for more about the position and content of these fields). For a macro, however, the *ID* parameter, which is the last parameter in the statement, *must* contain a value in the 5000 to 5999 range. This value notifies *Image-Pro* that a macro is to be invoked, and specifies the macro's position in the current script file, as follows.

An *ID* of...

`5000` identifies the first macro in the selected script file,

`5001` identifies the second macro in the selected script file,

`5002` identifies the third macro in the selected script file,

and so forth.

The following example shows a `progbutton` statement for the 4th macro (#5003) in a script file.

```
progbutton REPORT,Launch Report Generator, 0x0000, 5003
```

Important - *a macro is always invoked from the currently selected script file. If you change script files mid-session, a button will invoke the specified macro number from the* *new script file. To ensure that the macro you intended is always the one invoked by a button, do not change script files during your Image-Pro session*

The *Bitmap* field specifies the name of one of the predefined bitmaps that can be used as button icons. Any of the icons listed under *Pre-defined Button Bitmaps* at the end of this manual can be used.

Note: You can also assign a macro to a button on the toolbar through the **Toolbar** tab in the *Edit:Preferences* menu. This is described in the *Preferences* section under the *Edit* menu later in this manual.

*.MNU Syntax

The *.MNU file contains the description of *Image-Pro's* main menu and sub-menus, as well as descriptions for the command buttons that appear on the Toolbar. Each statement in *.MNU is made up of a keyword followed by several parameters. Within the *.MNU file, the following rules must be followed:

- Keywords must appear *entirely in lower case* (i.e., `progitem`, not `PROGITEM` or `ProgItem`)
- The first parameter must be separated from its keyword with a space. Parameters must be separated from each other by commas.
- You may use spaces to indent statements (usually done to improve readability), *but you must not use tabs*.

Note - the "#" character is the "comment" character. It can be used to designate a statement that should not be processed. This is usually done to include lines of descriptive information, or to "comment-out" a statement that is to be excluded from the file. <u>The "#" character must appear in the first position of the line</u>.

The following describes the syntax for each allowed *.MNU statement:

Statement:	**`columnbegin`** *Title, Position*
Description:	Marks the beginning of a menu description.
Parameters:	*Title* — The name of the menu as it will appear on the *Image-Pro* command bar. The "&" character precedes the accelerator key (the character that can be used with the Alt key to open the menu). *Position* — A number denoting the position of the menu on the command bar. The first position on the bar is position 0, (not 1). By convention, the *File* menu occupies position 0, and the *Help* menu occupies the last position.

Example:	**`columnbegin &File`**
	`progitem &Reload,Reload document from disk.,0x6B0F,112`
	`progitem &Close...,Close current document.,0x6B0F,103`
	`progitem &Save...\tCtrl+S,Save current document to disk.,0x6B0F,104`
	`endcolumn`
	
	The **`columnbegin`** statement in this example marks the beginning of a *File* menu description.

Statement:	**`endcolumn`**
Description:	Marks the end of a menu description.

Example:	`columnbegin &File`
	`progitem &Reload,Reload document from disk.,0x6B0F,112`
	`progitem &Close...,Close current document.,0x6B0F,103`
	`progitem &Save...\tCtrl+S,Save current document to disk.,0x6B0F,104`
	`endcolumn`

Statement:	`progitem` *Title, Descrip, BitFlags, ID*	
Description:	Defines a menu item (i.e., a command) that resides in *Image-Pro's* main program. It is also used to assign a user-developed macro to a menu.	
Parameters:	*Title*	The name of the command as it will appear in the menu. The "&" character must precede the accelerator key (the character that can be used to select this command once the menu is open). The "\t" character string can be used to right-align a string of text with the right margin of a menu. *Note - if the* `progitem` *statement is moved outside of a* `columnbegin/endcolumn` *structure, the command itself will be listed on the command bar. Do not include any tab-aligned text in such a statement (i.e., "\t").*
	Descrip	The string of text to be displayed in the status bar when this command is highlighted.
	BitFlags	A number that determines the image class to which the command can be applied, and, when selected, the dialog-box mode that will result. **For built-in *Image-Pro* commands, this value must not be changed!** For user-defined macros, refer to the *Bit Flag Options* at the end of this section.
	ID	A number that identifies the command procedure or macro. **For built-in *Image-Pro* commands, this value must not be changed!** For user-defined macros, this parameter must specify a value between 5000 and 5999, which indicates the macro's position in the current script file. Where: 5000 specifies the first macro in the script file, 5001 specifies the second macro, 5002 specifies the third macro and so forth.

Example:	`progitem &Save...\tCtrl+S,Save current document to disk.,0x6B0F,104`
	
	The example above defines the *Save* menu item. "&S" designates "S" as the accelerator key. *Save current document* will be displayed in the status bar when this command is highlighted.
	
	`REPORT,Launch Report Generator, 0x0000, 5003`
	The example above assigns the second macro in the script file to a menu item called REPORT. *Launch Report Generator* will be displayed in the status bar when this command is highlighted.	

Statement:	`item` *Title, Descrip, DLL, BitFlags, ID*	
Description:	Defines a menu item (i.e., a command) that resides in a DLL.	
Parameters:	*Title*	The name of the command as it will appear in the menu. The “&” character must precede the accelerator key (the character key that can be used to select this command when its menu is open). The "\t" character string can be used to right-align a string of text with the right margin of the menu. *Note - if the* `item` *statement is moved outside of a* `columnbegin/endcolumn` *structure, the command itself will be listed on the command bar. Do not include any tab-aligned text in such a statement (i.e., "\t").*
	Descrip	The string of text to be displayed in the status bar when this command is highlighted.
	DLL	The name of the DLL in which the command procedure resides. **This parameter must not be changed!**
	BitFlags	A number that determines the image class to which the command can be applied, and, when selected, the dialog-box mode that will result. **This value must not be changed!**
	ID	A number that identifies the command procedure. **This value must not be changed!**
Example:	`item &Scan...,Scan to new document.,ipscan32,0x1020, 101` This example defines the *Scan* menu item. *Scan* will be displayed in the status bar when this command is highlighted.	

continued on next page

Statement:	**`group`** *Title*
Description:	Defines a menu item that displays a pop-out menu when selected. This statement marks the beginning of a sub-menu definition.
Parameters:	*Title* — The name of the menu item (sub-menu) as it will appear in the menu.

Example:	**`group Sequence &Tools`**
	`item &Toolbar...,Show sequence Toolbar.,ipmotn32, 0x0480, 0`
	`item &Merge Files...,Merge images into current workspace.,ipmotn32,0x6B0F,100`
	`endgroup`
	The **`group`** statement in this example creates a menu item called *Rotat*e, and marks the beginning of the definition for the pop-out sub-menu, which is presented when the Rotate item is selected. "&o" designates "O" as the accelerator key.

Statement:	**`endgroup`**
Description:	Marks the end of a sub-menu description.

Example:	`group Sequence &Tools`
	`item &Toolbar...,Show sequence Toolbar.,ipmotn32, 0x0480, 0`
	`item &Merge Files...,Merge images into current workspace.,ipmotn32,0x6B0F,100`
	`endgroup`

Statement:	**`separator`**
Description:	Draws a line between menu items.

Example:	`item Set&up Acquire,Choose acquisition driver.,mccapt32,0x0080,105`
	`separator`
	`item &Scan...,Scan to new document.,ipscan32,0x1020,101`
	The **`separator`** statement in this example creates a line between the *Save As* and *Print* commands listed in the menu.

Statement:	**`progbutton`** *Bitmap, Descrip, BitFlags, ID*
Description:	Creates a button on the Toolbar for a command that resides in *Image-Pro's* main program. It is also used to assign a user-developed macro to a button on the Toolbar.
Parameters:	*Bitmap* — The name of one of the pre-defined icons (button bitmaps) supplied with *Image-Pro*. The icons and their names are listed at the end of this section.

continued on next page

`progbutton` *(continued)*

	Descrip	The string of text to be displayed in the status bar when the cursor touches this button.
	BitFlags	A number that determines the image class to which this button can be applied, and, when selected, the dialog-box mode that will result. **For built-in *Image-Pro* commands, this value must not be changed!** For user-defined macros, refer to the *Bit Flag Options* at the end of this section.
	ID	A number that identifies the command procedure or macro. **For built-in *Image-Pro* commands, this value must not be changed!** For user-defined macros, this parameter must specify a value between 5000 and 5999, which indicates the macro's position in the current script file. Where: 5000 specifies the first macro in the script file, 5001 specifies the second macro, 5002 specifies the third macro and so forth.

Example:

```
progbutton OPEN,Open document from disk.,0x0000, 102
```

The above example displays the *Open* icon on the Toolbar. When the cursor touches this icon, the description Open document from disk will be displayed in the status bar. When selected, this button will execute procedure #102, which is the *Open* command.

```
progbutton SAVE,Save current document to disk.,0x6B0F,
104
```

The above example displays the **SAVE** icon on the Toolbar. When the cursor touches this icon, the description *Save Document* will be displayed in the status bar. When selected, this button will execute procedure #104, the *Save* command.

Statement:	**`button`** *Bitmap, Descrip, DLL, BitFlags, ID*	
Description:	Creates a button on the Toolbar for a command that resides in an *Image-Pro* DLL.	
Parameters:	*Bitmap*	The name of one of the pre-defined icons (button bitmaps) supplied with *Image-Pro*. The icon names are listed at the end of this section.
	Descrip	The string of text to be displayed in the status bar when the cursor touches this button.

continued on next page

`button` *(continued)*

	DLL	The name of the DLL in which the command procedure resides. **This parameter must not be changed!**
	BitFlags	A number that determines the image class to which this button can be applied, and, when selected, the dialog-box mode that will result. **This value must not be changed!**
	ID	A number that identifies the command procedure. **This value must not be changed!**
Example:	`button CAMERA,Video/digital capture., mccapt32, 0x0080,100`	
	This example displays the **CAMERA** icon on the Toolbar. When the cursor touches this icon, the description *Video Capture* will be displayed in the status bar. When selected, this button will load and execute procedure 100 from CAPT.DLL, which is the *Acquire* command.	

Statement:	**%** *<normal menu item>*
Description:	The % prefix can be applied to any menu item statement, including any using the **comitem**, **item**, **macroitem**, and **progitem** keywords. The item will not appear on the *Image-Pro* menu structure.
Comments:	The % prefix is only useful with items that have the STARTUP flag defined (see *the Bit Flag Options* topic later in this chapter). In this case, the item will be hidden from the menu, but will be executed exactly once, when the application starts up. This is especially useful for any plug-ins that need to be aware of any images that are loaded (i.e. in case those images contain plug-in specific data). It is also used with **comitem** to allow IPCOM plug-ins to register new *Auto-Pro* functions.
Example:	`%item XYZ Startup,startup,xyz32.dll,0x480,100`
	This example defines a Startup menu item for the fictional XYZ32 module. The XYZ32 module will be invoked at *Image-Pro* startup with an ID of -1, and remain loaded for the duration of that *Image-Pro* session. Note that the ID parameter of the **item** line is ignored when used with the STARTUP flag.

Statement:	**combutton** ***Bitmap, Descrip, DLL, BitFlags, Server.Object[.Method]***	
Description	Creates a tool bar button for a command that resides in a COM server.	
Parameters	*Bitmap*	The name of the bitmap to be shown on the tool bar.
	Descrip	The tooltip to be displayed when the cursor is over this button.
	DLL	The name of the DLL in which the IPCOM architecture resides. *Note: this parameter must always be* ipcom32.dll *unless a different COM support module is provided.*
	BitFlags	A number that determines the image class to which this button can be applied, and, when selected, the dialog-box mode that will result. See more under *Bit Flag Options* at the end of this chapter.
	Server	The name of the COM server that is registered as providing the specified object.
	Object	The name of the COM object that is providing the plug-in method.
	[.Method]	An optional specification of the method to use when invoking the particular function. This parameter can be used to support multiple commands in a single COM object.
Example	`combutton HISTO,Histogram.,ipcom32.dll,32,VBPlugin.Task1` This example displays the **HISTO** icon on the tool bar. When the cursor is over this icon, the description Histogram will be displayed near the cursor. When selected, this button will load and execute the Task1 COM object in the VBPlugin COM server.	
See Also:	Comitem	

Statement:	**comitem *Title, Descrip, DLL, BitFlags, Server.Object[.Method]***	
Description:	This statement defines a menu item (i.e., a command) that is supported by an object that resides in a COM server.	
Parameters:	*Title*	The name of the command as it will appear in the menu. The "&" character indicates an accelerator key . The **\t** character string can be used to right-align a text string with the right margin of a column. *Note - if the comitem statement is moved outside of a columnbegin/endcolumn structure, the command itself will be listed on the main menu. Do not include any tab-aligned text in such a statement (i.e.,* \t *)*
	Descrip	The string of text to be displayed in *Image-Pro's* status bar when this command is highlighted.
	DLL	The name of the DLL in which the IPCOM architecture resides. *Note: this parameter must always be* ipcom32.dll *unless a different COM support module is provided.*
	BitFlags	A number that determines the image class to which this command can be applied, and, when selected, the dialog-box mode that will result. See more under *Bit Flag Options* at the end of this chapter.
	Server	The name of the COM server that is registered as providing the specified object.
	Object	The name of the COM object that is providing the plug-in method.
	[.Method]	An optional specification of the method to use when invoking the particular function. This parameter can be used to support multiple commands in a single COM object.
Comments:	The methods of the default interface (like **Histo** in the example below), are automatically available in IPBasic as *Object.Method* thus allowing control of them from a macro.	

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Example:

```
comitem &VB Plugin,VB
Plugin.,ipcom32.dll,32,VBPlugin.Task1
```

This example defines a command **VB plugin** with an accelerator of **V**, which when selected will invoke the VB Plug-in COM server and Task1 object. The selection of this menu item by the user will result in the IPCOM architecture calling the **Initialize** and **Invoke** methods of the Task1 object.

```
%comitem &Java Plugin,Java Plugin.,
ipcom32.dll, 0x480, JavaPlugin.Class1
```

```
comitem &Histogram, Histogram., ipcom32.dll,
0x80, JavaPlugin.Class1.Histo
```

This example defines a hidden item that will invoke and initialize the COM object at application startup, as well as the command **Histogram** with an accelerator of **H**, which when selected will invoke the JavaPlugin COM server and Class1 object. The selection of this menu item by the user will result in the IPCOM architecture calling the **Histo** method of the Class1 object.

See Also: % prefix, combutton, helpitem, item, macroitem, progitem

Statement: **endhints**

Description: The endhints statement marks the end of section defining hints for the MNU file’s menu and toolbar configuration. It must follow a hintsbegin statement.

Comments: See the section on Hints Statements for a discussion of menu hints..

Example:

```
hintsbegin
      type = 2
      level = 1
endhints
```

This example defines this menu configuration as a biological application using a Basic (simplified) interface.

See Also: Hintsbegin, level, type

Statement:	**helpitem *Title, Descrip, Helpfile, BitFlags, ID***
Description:	This statement defines a help menu item (i.e., a command) accessing a Windows help file that resides in a separate file.
Parameters:	*Title* — The name of the command as it will appear in the help menu. The **&** character indicates an accelerator key. *Note: the helpitem statement must not be moved outside of the Help columnbegin/endcolumn structure, and should not include any tab-aligned text in the statement (i.e.,* `\t`*).* *Descrip* — The string of text to be displayed in *Image-Pro's* status bar when this help item is highlighted. *Helpfile* — The name of a Windows online help file. *BitFlags* — Ignored: set to 0. *ID* — 0 = help is a Winhelp file (.hlp) 1 = help is an Acrobat file (.pdf) 2 = displays the Winhelp table of contents (.cnt)

Example:	`helpitem &Macro Language,Auto-Pro help.,`
	`       autopro.hlp,0,0`
	
	This example defines the **Macro Language** menu item, which uses the `AutoPro.hlp` Windows online *Auto-Pro* help file.

Statement:	**hintsbegin**
Description:	The hintsbegin statement marks the beginning of section defining hints for the MNU file's menu and toolbar configuration. It can be followed by a type statement and/or a level statement, and must be followed by an endhints statement.
Comments:	See the section on Hints Statements for a discussion of menu hints..

Example:	`hintsbegin`
	`       type = 2`
	`       level = 1`
	`endhints`
	
	This example defines this menu configuration as a Biological application using a Basic (simplified) interface.

See Also:	endhints, level, type

Statement:	**htmlhelpitem** ***Title, Descrip, Helpfile, BitFlags, ID***	
Description:	This statement defines a help menu item (i.e., a command) accessing a Windows help file that resides in a separate file.	
Parameters:	*Title*	The name of the command as it will appear in the help menu. The **&** character indicates an accelerator key. *Note: the htmlhelpitem statement must not be moved outside of the Help columnbegin/endcolumn structure, and should not include any tab-aligned text in the statement (i.e.,* `\t`*).*
	Descrip	The string of text to be displayed in *Image-Pro's* status bar when this help item is highlighted.
	Helpfile	The name of a Windows HTML help file (typically a .CHM file). The file name can be qualified with the desired page of the help file – see the Comments section for details
	BitFlags	Ignored: set to 0.
	ID	0 = displays the help index 1 = displays the help table of contents

Example:	`htmlhelpitem &Macro Language,Auto-Pro help., AutoPro5.chm,0,0`
	
	This example defines the **Macro Language** menu item, which uses the `AutoPro5.chm` *Auto-Pro* HTML help file.
	
	`htmlhelpitem &Solutions Zone, Display Solutions Zone, help\Support5.chm::Solutions_Zone_.htm, 0, 0`
	
	This example defines the **Solutions Zone** menu item, which goes directly to the specified page of the technical support help file.	

Statement:	**level**
Description:	The level statement indicates the level of complexity desired from the application features.
Comments:	See the section on Hints Statements for a discussion of menu hints. The levels defined at this time are 0 (zero) for advanced and 1 for basic. See the hintsbegin statement for an example.
See Also:	endhints, hintsbegin, type

Statement	**macrobutton *Bitmap, Descrip, Script, BitFlags, Macro***
Description:	Creates a button on the tool bar for a macro.
Parameters:	*Bitmap* — The name of the bitmap to be shown on the tool bar *Descrip* — The tooltip to be displayed when the cursor is over this button. *Script* — The name of the script file in which the macro resides. *BitFlags* — A number that determines the image class to which this button can be applied, and, when selected, the dialog-box mode that will result. See more under *Bit Flag Options* at the end of this chapter. *Macro* — The name of the macro to execute.
Example:	`macrobutton USER1, Macro: Histogram, C:\IPWIN7\DEFAULT.IPM , 0, Histogram` The example above displays the **USER1** icon on the tool bar. When the cursor is over this icon, the description `Macro: Histogram` will be displayed near the cursor. When selected, this button will execute the **Histogram** macro from the script file `c:\IPWIN7\default.ipm`
See Also:	button, combutton, macroitem, progbutton

Statement:	**type**
Description:	The type statement indicates the type of the application configuration.
Comments:	See the section on Hints Statements for a discussion of menu hints. The types defined at this time are 0 (zero) for general-purpose applications, 1 for industrial applications, and 2 for biological applications. See the hintsbegin statement for an example.
See Also:	endhints, hintsbegin, type

Statement:	**macroitem *Title, Descrip, Script, BitFlags, Macro***	
Description:	Creates an item in the menu for a macro.	
Parameters:	*Title*	The name of the command as it will appear in the menu. The & character indicates an accelerator key. The **`\t`** character string can be used to right-align a string of text with the right margin of a column.
	Descrip	The text string to be displayed in the status bar when this command is highlighted.
	Script	The name of the script file in which the macro resides.
	BitFlags	A number that determines the image class to which this button can be applied, and, when selected, the dialog-box mode that will result. See more under *Bit Flag Options* at the end of this chapter.
	Macro	The name of the macro to execute.
Example:	`macroitem &Histogram, Macro:Histogram` The example above defines the **Histogram** menu item. **&H** designates **H** as the accelerator key. `Macro:Histogram` will appear in the status bar when this command is selected. When selected, this command will execute the **Histogram** macro from the script file `c:\IPWIN7\default.ipm`	
See Also:	comitem, macrobutton, progitem, item	

Bit Flag Options

The *Bit Flag* parameter is used by the `item, comitem, progitem, macroitem button, combutton, macrobutton,` and `progbutton` statements. It determines the class of image that must be active for the menu item to be enabled, and the mode in which a plug-in's dialog box (if there is one) will be opened. If the bit flag for a particular class is disabled (i.e., not "on"), the command will be disabled when an image of that class is active.

The table below describes the basic *Bit Flag* parameter values and their meanings. The flags can be combined by simply adding their values (e.g., a value of 6 would denote *Gray Scale* (4) and *Palette* (2) images 4 + 2 = 6). The *Bit Flag* parameter can be specified in binary, decimal, or hexadecimal. It must use the appropriate prefix for the method selected. If you were to set all possible image class flags, the representation would be as follows:

```
Binary format (0b prefix):    0b0110101100001111
Hexadecimal format(0x prefix): 0x06B0F
Decimal format (no prefix):   27407
```

VALUE	HEX	DESCRIPTION
2	0x0002	GRAY SCALE – specifies that the command can be invoked when a *Gray Scale* image is active. This value "turns on" the GRAY flag in bit position 1.
4	0x0004	PALETTE – specifies that the command can be invoked when a *Palette* image is active. This value "turns on" the PALETTE flag in bit position 2.
8	0x0008	RGB – specifies that the command can be invoked when a *True Color* (RGB 24) image is active. This value "turns on" the RGB flag in bit position 3.
16	0x0010	Reserved. Do not use.
32	0x0020	Reserved. Do not use.
64	0x0040	Reserved. Do not use.

VALUE	HEX	DESCRIPTION
128	0x0080	MODELESS – specifies that a DLL procedure invokes a modeless dialog. This value "turns on" the MODELESS flag in bit 7. If you set the MODELESS flag, hVriInst in the Interface Function will be Null. This flag is used only by the button and item statements.
256	0x0100	GRAY12 – specifies that the command can be invoked when a Gray Scale 12 image is active. This value "turns on" the GRAY12 flag in bit position 8.
512	0x0200	FLOAT – specifies that the command can be invoked when a Floating Point image is active. This value "turns on" the FLOAT flag in bit position 9.
1024	0x0400	STARTUP – specifies that the DLL is to be invoked automatically when the application is loaded. This value "turns on" the STARTUP flag in bit 10. At start-up, the application calls the InterfaceFunction of the plug-in with Id = -1 Note: This flag is used only by the button, combutton, comitem, and item statements.
2048	0x0800	GRAY16 – specifies that the command can be invoked when a Gray Scale 16 image is active. This value "turns on" the GRAY16 flag in bit position 11.
4096	0x1000	Reserved. Do not use.
8192	0x2000	RGB36 – specifies that the command can be invoked when a 36-bit True Color image is active. This value "turns on" the RGB36 flag in bit position 13.
16,384	0x4000	RGB48 – specifies that the command can be invoked when a 48-bit True Color image is active. This value "turns on" the RGB48 flag in bit position 14.
32,768	0x8000	Reserved. Do not use.
65,536	0x00010000	Multi-frame – specifies that the command can only be invoked when the active workspace contains multiple image frames.

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VALUE	HEX	DESCRIPTION
(Use Hex values!)	0x00020000	Multi-workspace – specifies that the command can only be invoked when there are multiple image workspaces open.
	0x00040000	Multi-frame or workspace – specifies that the command can only be invoked when there are multiple frames available, either from multiple frames in the active workspace, or from multiple image workspaces.
	0x00080000	Disallow on FFT – specifies that the command **cannot** be invoked when the active workspace is an FFT workspace.
	0x00100000	Only when IQbase installed – specifies that the command can only be invoked if the Media Cybernetics integration with IQbase is installed to the application.
	0x00200000	Hide the menu entry if the plug-in is not installed.
	0x00400000	Reserved. Do not use.
	0x00800000	Reserved. Do not use.
	0x01000000	Reserved. Do not use.
	0x02000000	Reserved. Do not use.
	0x04000000	Reserved. Do not use.
	0x08000000	Reserved. Do not use.
	0x10000000	Reserved. Do not use.
	0x20000000	Reserved. Do not use.
	0x40000000	Reserved. Do not use.
	0x80000000	Reserved. Do not use.

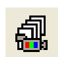
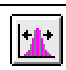
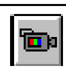
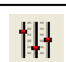
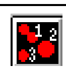
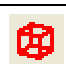
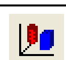
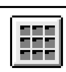
If *none* of the image class flags (GRAY, PALETTE, RGB, GRAY12, GRAY16, FLOAT, RGB 36 or RGB 48) are set, your macro will never be dimmed, even no image is active. Therefore, to make a macro available to all image classes, set its Bit Flags parameter to 0.

If any of the image class flags (GRAY, PALETTE, RGB, GRAY12, GRAY16, FLOAT, RGB 36 or RGB 48) are set (i.e., turned “on”), your macro will be available only if the active image is of one of the enabled types. Otherwise it will be dimmed. For example, if a macro were valid for Gray Scale and True Color images, its Bit Flags would be set to 10 (i.e., 2 + 8).

Pre-defined Button Bitmaps

The following table describes *Image-Pro's* pre-defined buttons and their bitmap names (required in the Bitmap parameter of the button and progbutton statements). It also shows the command to which each button is assigned in the *.MNU file delivered with *Image-Pro* (unused icons are denoted with a dash).

These button assignments can be changed by editing the *.MNU file. See **Customizing Command Buttons** for procedures.

ICON	BITMAP NAME	DEFAULT COMMAND ASSIGNMENT
	ACQUIRE	The *Acquire Image Set* (*AFA*) command
	ANNOTATE	The *Show Annotation Toolbar* command
	BACKSUB	The *Background Operations* command
	BESTLUT	The *Best Fit Equalization* command
	CAMERA	The *Video/Digital Capture* command
	CLOSEDOC	The *Close Open Document* command
	CLOSETOOL	The *Close All Open Tool Dialogs* command
	CONTRAST	The *Contrast Enhancement* command
	COUNT	The *Count and Measure Objects* command
	3D VIEWER	The *3D Viewer* command
	3D VISUALIZE	The *3D Visualize* (*3D Constructor*) command
	DARK MODE	The *Dark Mode* command
	DATABASE	The *Image Database* command

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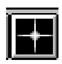
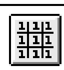
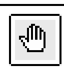
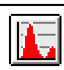
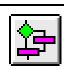
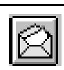
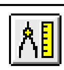
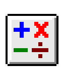
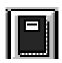
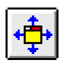
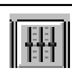
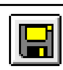
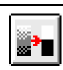
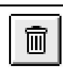
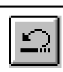
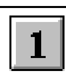
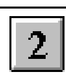
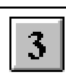
ICON	BITMAP NAME	ASSIGNED COMMAND
	DATA	—
	DECONVOLVE	The *Deconvolve Image Set* (*SharpStack*) command
	DRAW	The *Annotate* command
	FFT	The *FFT* command
	FILTER	The *Spatial Filtering* command
	HAND	The *Pan Image* tool
	HISTO	The *Histogram* command
	INFO	The *Info* command
	IRREG	The *Freeform AOI* command
	MACRO	The *Macro* command
	MACREC	The *Record Macro* command
	MACEDIT	The *Edit Macro* command
	MAIL	The *Send Mail* command
	MEASURE	The *Measurements* command
	OPEN	The *Open* command
	OPER	The *Arithmetic and Logical Operations* command

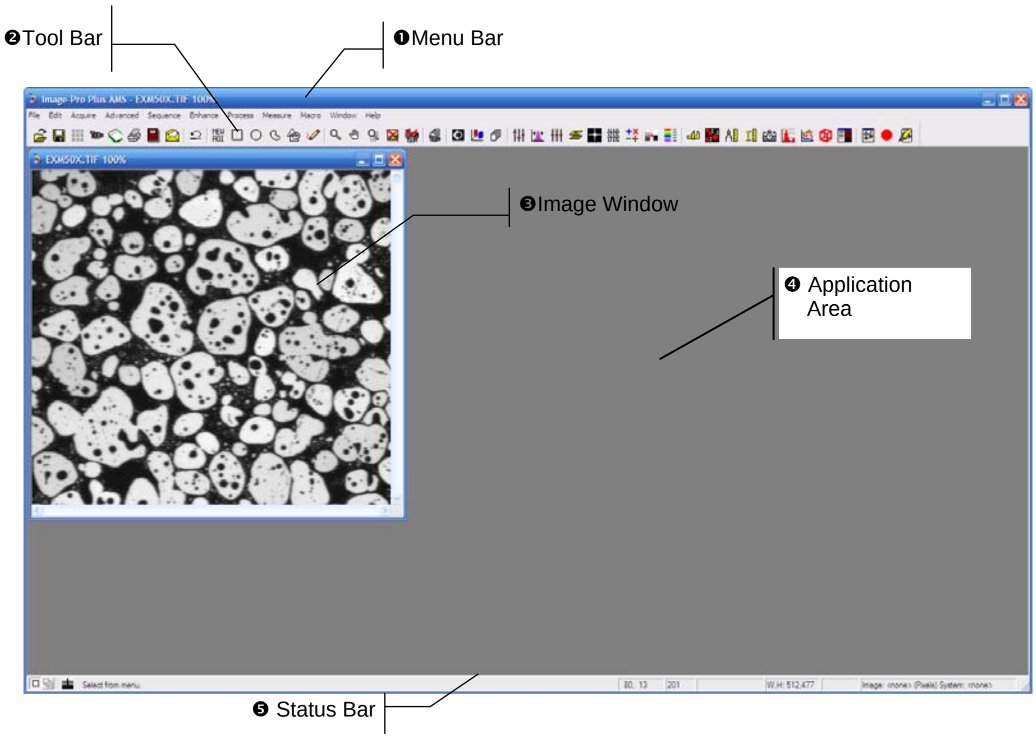
continued on next page

ICON	BITMAP NAME	ASSIGNED COMMAND
	PRINT	The *Print* command
	PROFILE	The *Line Profile* command
	PSEUDO	The *Colorize Gray Scale Image* command
	REPORT	The *Report Generator* command
	RESIZE	The *Resize* command
	RESET	The *Reset Contrast Table* command
	SAVE	The *Save* command
	SCAL	The *Spatial Calibration* command
	SCAN	The *Scan* command
	SEGMENT	The *Perform Segmentation* command
	SET	The Set Manager command
	SYNC	Sync *Scroll, Pan, and Zoom* commands
	SNAP	The *Snap* command
	TRASH	—
	UNDO	The *Undo* command
	USER1	First user-defined macro
	USER2	Second user-defined macro
	USER3	Third user-defined macro

Getting Started

The *Image-Pro Plus* Application Window

When *Image-Pro Plus* is loaded, its application window becomes active. The application window consists of the Menu Bar, the Tool Bar, the Image Window(s), the Application Area, and the Status Bar. The following diagram introduces you to these and other features of the *Image-Pro Plus* application window.



❶Menu Bar: The menu bar contains the *Image-Pro Plus* commands selected from within the menus listed on this bar. As with most applications, you must identify the object of your command before selecting the command itself (i.e., make sure to select the image data the command is to operate upon *before* selecting the command).

❷ **Tool Bar:** The tool bar is the button bar along the upper edge of the screen. This portion of the application window contains the basic intensity controls and imaging tools for daily *Image-Pro Plus* operations. The tool bar includes the following commonly used tools:

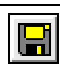
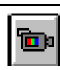
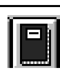
- *BCG* controls
- *AOI* tools
- *Zoom & Pan* tools.

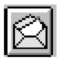
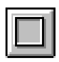
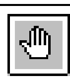
As the cursor is moved over the buttons, yellow tooltips appear providing a brief description of the button. If no tooltip appears, the button tool/function does not apply for that image.

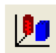
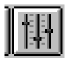
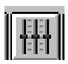
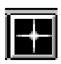
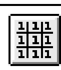
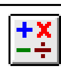
This Tutorial addresses **34** buttons in the tool bar. A description of these buttons and their uses appears below:

The following tools are located on the toolbar immediately below the menu bar. If no toolbar is being displayed below the menu bar, you may show it by invoking the *Hide*/*Show Tools* command on the *Window* menu or pressing <F5>. If the menu bar and title bar are not being displayed, you may invoke them by pressing <F4>.

When you position the mouse cursor over one of the icons, a brief description of the command appears.

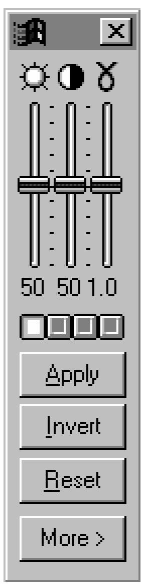
ICON	BITMAP NAME	COMMAND
	OPEN	The *File : Open* command
	SAVE	The *File : Save* command
	DATABASE	The *Image Database* command
	CAMERA	The *Acquire* command
	SCAN	The *File : Scan* command
	PRINT	The *File : Print* command
	REPORT	The *Report Generator* command

ICON	BITMAP NAME	COMMAND
	MAIL	The *Electronic Mail* command
	UNDO	The *Edit: Undo* command
	NEW AOI	The create *New Area of Interest* (AOI) Command
	RECTANGULAR AOI TOOL	The create *Rectangular Area of Interest* (AOI) Command
	ELLIPTICAL AOI TOOL	The create *Round (or Oval) Area of Interest* (AOI) Command
	FREEFORM AOI TOOL	The create *Irregular Area of Interest* (AOI) Command
	MULTIPLE AOI TOOL	The create *Multiple Area of Interest* (AOI) Command
	DRAW	The *Edit: Annotate* command
	ZOOM	The *Zoom* (*Enlarge/Reduce*) command
	PAN	The *Pan* (*Move Image*) command
	SYNC	The *Synchronize Zoom/Pan/Scroll* command
	CLOSEALL	The *Close All Open Documents* command
	CLOSETOOLS	The *Close All Open Tool Dialogs* command
	3DACQUIRE	The *Acquire 3D Image Stacks (AFA)* command
	DECONVOLVE	The *Deconvolve 3D Image Stacks (SharpStack)* command

ICON	BITMAP NAME	COMMAND
	3DVISUALIZE	The *Visualize 3D Image Stacks (3D Constructor)* command
	CONTRAST	The *Enhance: Contrast Enhancement* command
	BESTLUT	The *Enhance: Best Fit Equalization* command
	RESET	The *Enhance: Reset Contrast Table* command
	BACKSUB	The *Process: Background Correction* command
	FFT	The *Process: FFT* command
	FILTER	The *Process: Filtering* command
	OPER	The *Process: Operations* command
	SEGMENT	The *Process: Segmentation* command
	PSEUDO	The *Process: Pseudo-Color* command
	SPATIAL	The *Measure : Spatial Calibration* command
	COUNT	The *Measure: Count/Size* command
	MEASURE	The *Measure: Measurements* command
	SNAP	The *Measure:Snap* command
	HISTO	The *Measure: Histogram* command
	PROFILE	The *Measure: Line Profile* command

ICON	BITMAP NAME	COMMAND
	3D VIEWER	Open the *3D Viewer* command
	DARK MODE	Change to dark mode
	MACRO	The *Macro* command
	MACREC	The *Record Macro* command
	MACEDIT	The *Edit Macro* command

The Contrast Enhancement Tool: The *Contrast Enhancement* tool shows or hides the *Contrast Enhancement* control panel.



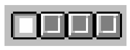
This panel lets you easily adjust the Brightness, Contrast, and Gamma (BCG) attributes of the active image. There is an independent slider control panel for each characteristic. The slider button changes color (from red to blue) when the specific attribute is selected.

Use the *Brightness* control to modify the overall amount of light in your image.

Use the *Contrast* control to change the degree of difference between the brightest and darkest components in your image.

Use the *Gamma* control to enhance the contrast in the very dark or very light areas in your image without significantly affecting the contrast in the midtone regions.

Moving the **Slider** (▤) button up the scale increases the attribute. Moving it down the scale reduces the attribute. For single incremental changes, press the up (⇧) or down (⇩) arrow keys on the keypad.

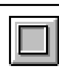


These four buttons are the **Color Channel** buttons. Select the Luminance, Red, Blue, or Green channel button corresponding to the color in your image. If your image is not in *True Color*, the **Luminance** channel will be your only option here. The **Luminance** channel is the intensity produced by the combined RGB channels. Click on the color button, and move the slider controls until the new color values are reflected in your image.

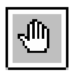
Use this button to permanently apply the current contrast enhancement adjustments to the active image.

Use this button to reset the BCG sliders to the default values.

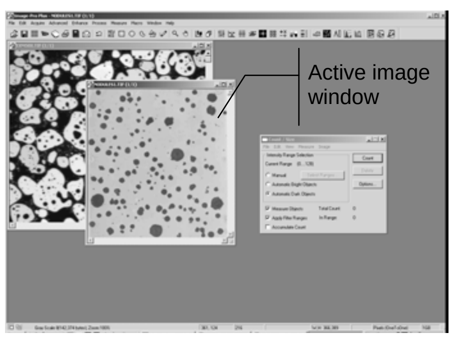
AOI Tools: The *Area of Interest* (AOI) tools define a portion of your image. Many of *Image-Pro Plus'* commands can be constrained by defining an AOI. When such commands are executed, their effects are applied to just the pixels within the AOI, leaving the pixels outside the AOI unaffected. There are several *AOI* tools which can determine the size or shape of the AOI as follows:

ICON	TOOL	FUNCTION
	**New AOI**	Use this tool to create a new AOI.
	**Rectangular AOI Tool**	Use this tool to create and/or activate a square or rectangular AOI in the image.
	**Elliptical AOI Tool**	Use this tool to create and/or activate a circular or elliptical AOI in the image.
	**Freeform AOI Tool**	Use this tool to create and/or activate any irregular AOI in the image. You can use the *AutoTrace* feature of the *Freeform AOI* tool to outline the edge of an object.
	**Multiple AOI Tool**	Use this tool to create and work with multiple AOIs.

Zoom & Pan Tools: Uses for the *Zoom* and *Pan* tools are described in the following table:

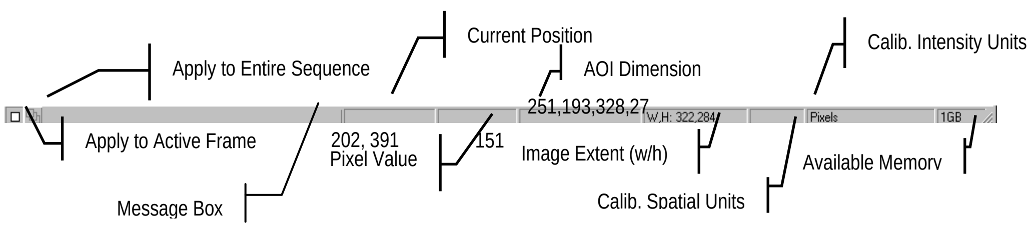
ICON	TOOL	FUNCTION
	**Zoom**	Use this tool to magnify or reduce the appearance of the image in the window. Pressing the **right** mouse button accesses the *Zoom* sub-menu containing other zoom options.
	**Pan**	The *Pan* tool is used to position an image that does not entirely fit within the image window. It is an alternative to using the arrows on the scroll bars for accessing image data that falls outside the image window.
	**Sync**	The *Sync Zoom/Pan/Scroll* tool is used to move several image workspaces at once.

❸**Image Windows:** Images are maintained in separate image windows within the application area. An image window contains a single image. You may work with several open images at the same time. When you do so, *Image-Pro Plus* will perform a selected operation upon the *active* window. The *active* window is the frontmost image in the application area, and its title bar is highlighted, as shown in the diagram on the left.



❹ **Application Area:** The application area is the workspace in the desktop where *Image-Pro Plus* performs its functions. This is where the image windows are displayed, where the dialog boxes appear, and where you find the graphs and tables produced by *Image-Pro Plus*. The *Image-Pro Plus* workspace is enclosed by the menubar and toolbar at the top edge, and by the status bar at the bottom. (For more information about the menu bar and toolbar components, please refer to the *Image-Pro Plus Reference Guide*.)

❺**Status Bar:** The status bar is the bar along the bottom edge of the screen. It displays information about the image open on the screen, such as calibration and spatial resolution. It also conveys messages about the current *Image-Pro Plus* operation and indicates the memory available on your system. Use the **Set Preferences** dialog to control what information appears in the status bar (see the *Edit* menu section in the *Image-Pro Plus Reference Guide*).



The *Status Bar* provides general-purpose information about the open image:

Message Box describes a selected command or menu and acts as a progress indicator for lengthy operations.

Current Position gives the location of the cursor in pixels.

Pixel Value displays the intensity value of the pixel underneath the cursor.

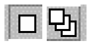
AOI Extent displays the location of the AOI in terms of four pixel locations.

AOI Dimension gives the width and height of the AOI in pixels.

Intensity Units indicates the intensity calibration units.

Spatial Units indicates the spatial calibratio units.

Available Memory indicates how much memory is available for *Image-Pro* operations.

 The *Apply to Active Frame* and *Apply to Sequence* buttons appear at the extreme right of the status bar. These buttons can be used to apply a specific operation to the frame currently in the application area, or to the active portion of a sequence of images.

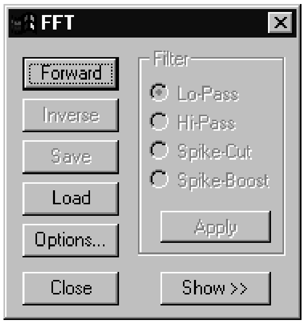
When an operation is in progress, the message bar will display a tracking bar, and the sequence buttons will be replaced with a *Cancel* button. To cancel the operation, click the *Cancel* button or press **<Esc>.** (Please refer to the description of the *Sequence Toolbar* later in this manual.)

Image-Pro Plus v. 7.0 allows you to enable or disable the status bar fields by right clicking on the status bar. You will see a pop-up menu of the indicator fields:

Click the cursor next to the name of the field you wish to enable. Enabled fields are checked.

To resize any of the fields in the status bar, place your cursor over one of the dividers between the fields. The cursor will change to a double-headed arrow. Click and drag the cursor left to make the field smaller, or to the right to enlarge the field.

Dialog Boxes: *Image-Pro Plus* provides many of its image processing features using dialog boxes which display data for, or apply commands to, any open image window.



The *FFT* and *Filter* windows on the left are examples of common dialog boxes. They reflect the data or choices available, and/or apply their effects to whatever image is active. Many boxes of this type have additional options available with tabs (as shown in the *Filter* tabbed dialog box).

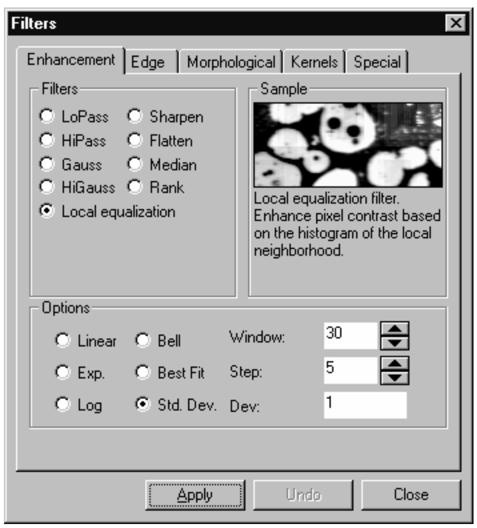
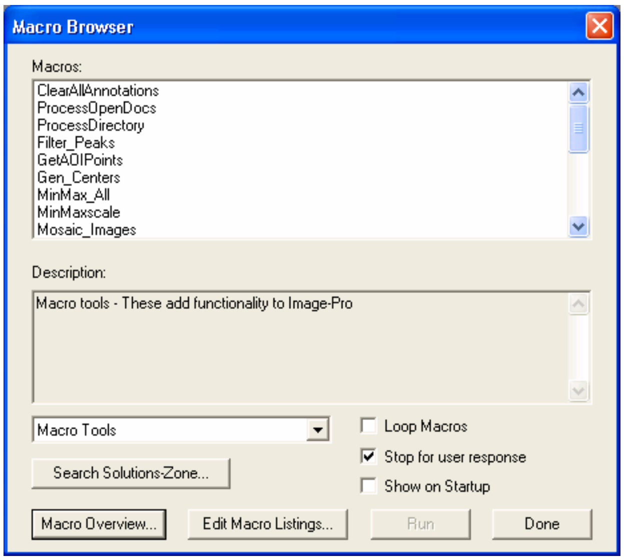


Image-Pro Plus Demonstration Macros

Image-Pro Plus version 7.0 contains a large number of macro scripts that include both demonstration and utility macros. The demonstration macros can be used to view highlighted features of *Image-Pro*, and the utility macros can be used to help automate your tasks.

When you select the *Macro* command, the **Macro Browser** dialog box is presented.



Some of the macros provided with *Image-Pro Plus 7.0* include:

`Separate_Fluorochromes`: This demo macro demonstrates separating a 24-bit color image into its Red, Green, and Blue channels. After enhancing the blue channel, the individual channels will be re-combined to a 24-bit color image.

`CountSmall_Cells`: This is a demo macro showing how to count the number of cells in a sample.

`MeasureStain`: Using an image of a tissue section, this macro demonstrates using the *Count/Size* tools to select ovjects of interest based on color.

`MeasureAngles`: Using an x-ray film image, this demo macro calculates the angle of the leg by first processing the image, defining areas of interest (AOIs), and then measuring angle using *Count/Size*.

`CountAndClassify`: This macro demonstrates one possible way to count the number of holes (pores) in an object (or objects), and then determines the percent area of the holes compared to the objects of interest.

`MeasureIronPhases`: This demo macro first locates and counts individual phases of a multiple phase material, then demonstrates how to segment individual phases and then measure them using the *Count/Size* tool.

`ThroughfocusSequence`: This demo macro opens a sequence of images using the *Sequencer* tool and applies a pseudocolor to the sequence. For additional details, the macro opens the histogram of the image sequence to view the histogram from frame to frame.

`MeasureAutoradiograph`: This macro demonstrates creating an intensity calibration curve and then deriving measurements from selected areas of an autoradiograph.

`SprocketCountDemo`: This demo macro creates a mask image, and then uses such filters as **Thinning** and **Branch/End-Point** to locate endpoints and uses *Count/Size* to count the number of endpoints.

`Barcode`: This demo macro shows how to locate edges and derive useful measurements based upon the location of the edges and the distances between them.

`Watermark`: This macro applies a watermark to your images.

In addition, some new macros have been created for *Image-Pro Plus 7.0*. They include:

`Trim Display Range`: Presents an interface for setting the display range of an image, clipping a set percentage of high and low pixels. For example, clipping the top and bottom 2% from the histogram to better display image intensities.

`White Balance`: Measures the RGB values in a known white or gray section of the image, then uses those values to white balance the image.

`Measure White Balance`: Places an AOI on the image, which should be moved over a known white or gray area. When the AOI is selected, the average Red, Green, and Blue values are read, and conversion factors to make them equal are calculated.

`Balance Active Image`: Balances the active image by scaling RGB values as computed.

`Save/Load White Balance Setting`: Saves or loads the current conversion factors to a text file for later use.

`Calibrated Plot`: Allows you to apply a pseudo-color with a known number of color steps to an image, and from that to generate a color output with a labeled/calibrated scale bar.

`Process in Place`: Converts the current image if checked, or otherwise creates a new image with the applied white balance factors.

`Average/Scale to Max`: Either averages the conversion factors to 1.0, or scales up the dimmer components to match the brightest. Averaging retains the image intensity, while scaling to max can better handle cases where one of the channels is saturated.

`Quick Contrast`: Scales the current image for visibility. If this is a sequence or set, the central image will be chosen and scaled, as end images often are not very interesting.

`Sequence Projection`: Creates a quick projection of the currently active sequence. This is done by averaging all frames in the sequence and then enhancing the contrast of the result.

`3D Slicer`: This is a very simple 3D volume viewer. It prompts you for a slice thickness, the ratio of the Z distance to the XY pixel distance, and displays an XY, an XZ, and a YZ view of the volume. Clicking in any one view changes the other two to intersecting cut planes.

`Sigma Filter`: This is a very specific Median filter. The image is analyzed to identify the local mean and standard deviation, and if individual pixels differ from the mean by more than the stated standard deviations they are replaced by a median filtered pixel. This is an excellent removal of salt/pepper noise in images, while limiting modifications to a few percent of the original pixels. The dialog allows choosing the limit of Standard Deviation, the base filter size for variance, mean, and median filters, and displays the percentage of pixels modified.

`Remove Periodic Noise`: Using FFT techniques, peaks in the frequency domain are identified, indicating periodic patterns in the image. A scaling factor is requested: a value of 1.0 does nothing, a value <1.0 suppresses the periodic pattern, while a value >1.0 enhances it. This can be used to enhance periodic patterns such as fingerprints, or to remove periodic noise.

`3D Restricted Dilation`: Given a mask volume (0-255, or otherwise ‘binary’ images) and a seed volume, dilate the seed into the mask. This may be used, for example, to seed on a nerve body and dilate along the nerve processes, avoiding external volumes that might otherwise be shown if just using a straight threshold.

`3D Colocalization`: Applies the 2D colocalization functions to a 3D volume and accumulate the results.

`Color Deconvolution`: Applies deconvolution to an RGB volume, with estimated emission wavelengths for the three components.

`Min/Max Circles`: Calculates and draws the minimum enclosed circle and maximum enclosing circle for counted objects.

`Sequence Histogram`: Calculate the accumulated histogram of an entire sequence, as opposed to the single frame histogram tool.

`Read/Write ASCII`: Writes an image to disk as a text file with comma, tab, or space delimiters, and read such images from disk. The images are saved as XY arrays of delimited numbers, one row of numbers per row in the image. Note that this is limited to grayscale images. Read images are of type Float, allowing any range of values to be read.

Other macros may be added in the future. Please visit our website www.mediacy.com for updates.

Learning the Basics

The Tutorial in this section is designed to introduce you to the basic image manipulation tools within *Image-Pro Plus*. In this first exercise you will:

- Load an image and create a working copy.
- Adjust the intensity with the *Brightness*, *Contrast*, and *Gamma* (*BCG*) controls.
- Enhance the working image with various filters.
- Cut and resize the working image for comparison with the original image.
- Print and save the final image.

This exercise will take about 25 minutes to complete.

Setup: If you have not yet started *Image-Pro Plus*, do so now by double-clicking on the *Image-Pro Plus* icon within the *Image-Pro Plus* folder. Once *Image-Pro Plus* is running, you may begin following the steps below.

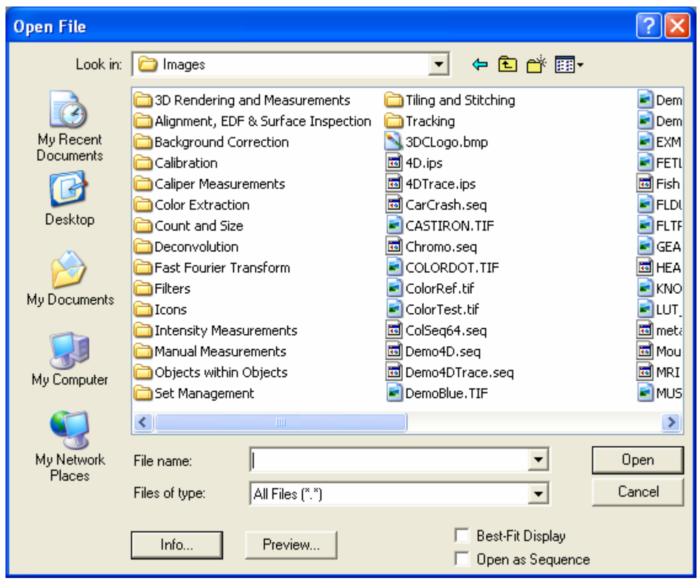
Important*: Please check that your Image-Pro settings for this exercise* ***match*** *those in the dialog boxes shown in the tutorial. Image-Pro Plus retains the settings from previous experiments, which may not be the same as the ones used in this exercise.*

■ Loading the Image

In this step, you will open the first image, which is a captured image from a surveillance camera in a bank..

1. **Select the *Open...* command from the *File* menu.**

The **Open File** dialog box appears, displaying the *Images* folder.



2. Open the *Bankvid.tif* file in the *Images/Filters/Sharpening* folder.

TIFF in the **Files of type** field indicates that this image is in Tagged Image File Format. *Image-Pro Plus* opens the **`Bankvid.tif`** image in its own image window.

This is an image from a bank surveillance camera, with an unclear image of a crime suspect. In this exercise, you will enhance this image to produce a clearer image of the suspect in the foreground.

Continue to the next set of steps in this exercise: *Copying and Pasting.*

■ Copying and Pasting

In this step you will make a working copy of the original image. Any further changes you make will only apply to this working copy of the image, leaving the original image unaltered.

1. Select the *Copy* command from the *Edit* menu.

The image is copied to the Clipboard.

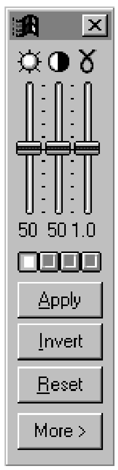
2. Select the *Paste New* command from the *Edit* menu.

The Clipboard data appears in its own window **`Untitled1`**. Use this new image as the working copy.

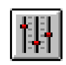
Continue to the next set of steps in this exercise: *Enhancing the Image*.

■ Enhancing the Image

In this step you will enhance the working image **`Untitled1`** using the *Brightness*, *Contrast*, and *Gamma* (*BCG*) controls on the *Contrast Enhancement* panel to improve the visual effect and extract unique features currently hidden from view. In this case, you will "clean up" the image to distinguish the suspect in the foreground.



Before beginning this exercise, display the *Contrast Enhancement* panel on the screen by clicking the **Contrast Enhancement** button on the tool bar at the top of the screen.

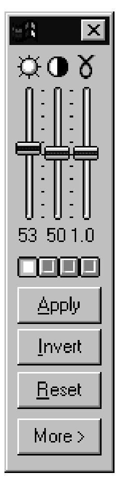


Contrast Enhancement button

Move this panel to any convenient position on the screen by dragging its title bar.

In the following steps, use the slider controls on this panel to adjust the visual characteristics of this image.

1. Increase the *Brightness* value.



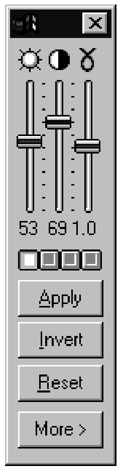
Because the overall image is somewhat dark, lighten it using the *Brightness* control.

Place the cursor on the **Slider** (▤) of the *Brightness* control:

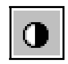
Brightness control

Drag the **Brightness** slider up, until the indicator beneath it reads **53**, or press the up arrow key (⇧) on the keypad to increase the value by single increments. This increases the brightness of the image, by lightening it.

2. **Increase the *Contrast* value.**



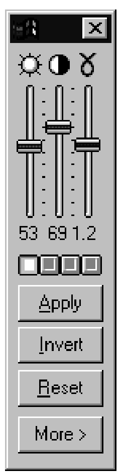
To better distinguish the suspect in the foreground from the background, heighten the contrast in the image. Drag the **Slider** ( ) of the *Contrast* control:



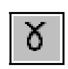
Contrast control

until the indicator beneath it reads **69**. This increases the intensity differences between the suspect and the background in the image.

3. **Increase the *Gamma* value.**

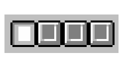


To bring out the variations in the dark foreground, increase the **Gamma** value. High gamma values increase contrast in dark areas of an image. Low gamma values increase contrast in the lighter areas of an image. Drag the **Slider** ( ) of the *Gamma* control:

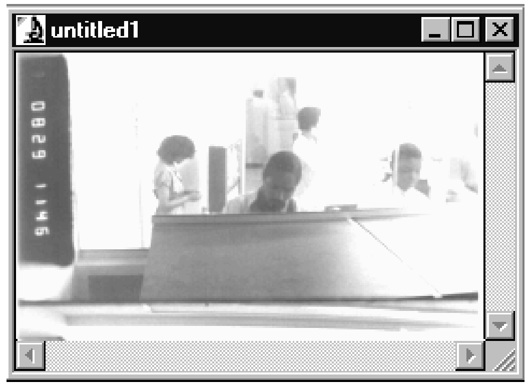


Gamma control

until the indicator beneath it reads **1.2**. All the image elements, the suspect in the foreground and the background, should now be distinguishable in the image.



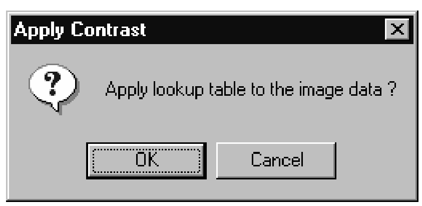
*Note: Since this image is not true color, the **Luminance** channel (selected left button) is the only option for the color channel, which is represented by this four-button panel. The **Luminance** channel is the intensity produced by the combined RGB channels.*



4. Apply the *Brightness*, *Contrast,* and *Gamma* Control changes.

The BCG intensity adjustments performed in the preceding steps were not permanently applied to the image. Instead, they were recorded into a Lookup Table (LUT) through which the original image is interpreted. To permanently modify the image, the LUT must be applied to the image.

Click **Apply** and the **Apply Contrast** dialog box appears.



5. Click *OK*.

Clicking **OK** uses the BCG settings to permanently modify your image bitmap.

Notice that after clicking **OK**, all three sliders return to their initial, unaltered positions.

Continue to the next set of steps in this exercise: *Working with Filters.*

■ Working with Filters

In this step you will further enhance the working image by using the **Sharpen** filter to clarify the suspect's face.

1. Select the *Filters…* option from the *Process* menu.

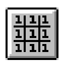
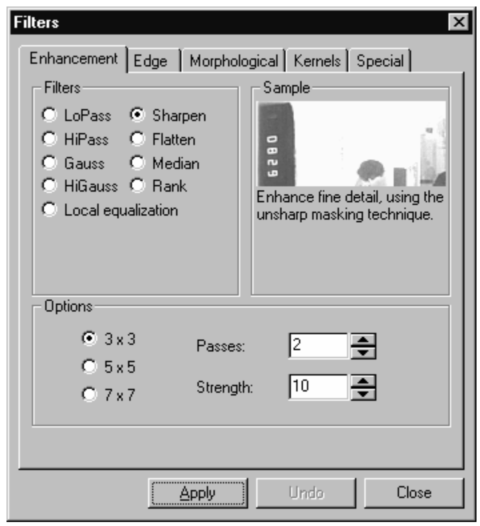


Image-Pro Plus opens the **Filters** tabbed dialog box.



2. Click on the *Sharpen* filter radio button from the *Enhancement* tab label.

3. Increase the number of passes to *2* in the *Passes* edit box.

This demonstrates how multiple applications of filters can be combined.

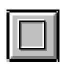
4. Click *Apply*.

Image-Pro Plus applies the selected filter to the working image.

■ Defining an AOI

In this step you will define an **Area of Interest (AOI)** around the suspect and cut this portion from the working image. The cut portion will be used for later comparison to the original image.

1. Select the *Rectangular AOI* tool.



Click the *Rectangular* tool located on the Tool Bar along the top of the screen. Notice that the **Rectangular** button illuminates, indicating that it is the activated tool.

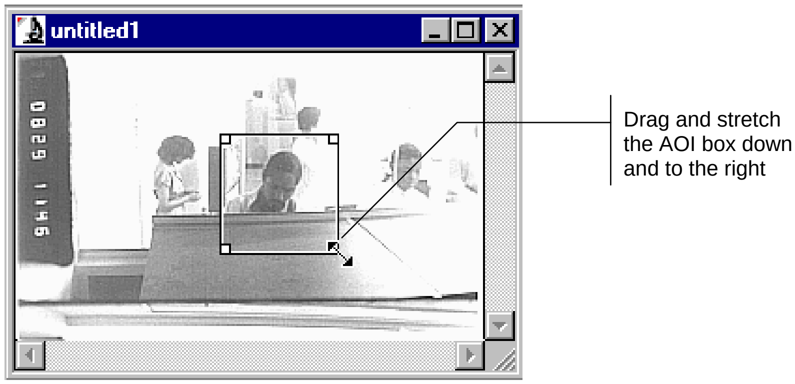
2. Move the cursor to the *Untitled1* image window.

The cursor appears as a right-angle.

Place this cursor above the left side of the suspect's head in the image, as shown in the following example.

3. Define the Area of Interest (AOI).

Then, press the mouse button and drag the cursor past the lower-right corner of the suspect's head.



When the cursor is below the right-hand corner of suspect's head, release the mouse button. The AOI rectangle you have just defined will remain in the image.

Definition... ***Area of Interest (AOI)***

An AOI is an area that is isolated from the rest of the image. It can be any shape you choose: a rectangle, an ellipse, or a freeform polygon. Most, but not all, of Image-Pro Plus commands can be constrained by an AOI. The command applies to only the pixels within the AOI, when one is defined.

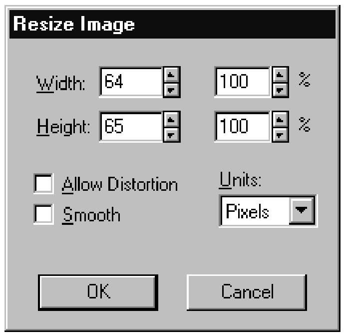
Examples of commands that are constrained by an AOI are: ***Save As****,* ***Print****,* ***Filter,*** *and* ***Rotate****. These commands will perform the requested operation using just the data within the AOI.*

A few operations affect the entire image regardless of whether an AOI is active or not. Examples of functions not constrained by an AOI are: ***Color Segmentation*** *and* ***Save.***

---

4. **Enlarge the AOI by selecting the *Resize…* command from the *Edit* menu.**

   The **Resize Image** dialog box opens.



5. **Increase the *Width* percentage value to *300%*.**

   Notice as the **Width** value changes, the **Height** value changes too. This occurs because the **Allow Distortion** checkbox is turned off, which forces the image to scale proportionally.

6. **Click *OK*.**

   An enlarged image of the AOI appears as **`Untitled2`**.

Move the **`Untitled2`** window over to the right side of the application area to view the original image **`Bankvid.tif`**.

Continue to the next set of steps in this exercise: *Zooming in on an Image*.

■ Zooming in on the Image

In this step you will magnify the original image (**`Bankvid.tif`**) of the suspect to compare against the enhanced image of the suspect (**`Untitled2`**).

1. Select the *Zoom* tool.

Click the *Zoom* tool located on the Tool Bar along the top edge of the screen. Notice that the **Zoom** button illuminates, indicating that it is the active tool, and the *Rectangular AOI* tool is no longer active.

***Note:** You can also position the cursor anywhere on the image and click the right mouse button. Select **Zoom In** from the pop-up menu.*

2. Zoom-in on the *Bankvid.tif* image.

Move the cursor to the **`Bankvid.tif`** image window. Note that the cursor appears as a magnifying glass once it is within the image.

Place the magnifying glass over the suspect, then click the left mouse button **once** to magnify the image **2X**. This action zooms-in on the region. The **`Bankvid.tif`** image window appears like this:

3. Place the original image (*Bankvid.tif*) and the modified image (*Untitled2*) side by side to compare the two images.

Note that the facial features of the suspect are easier to see in the enhanced and "cleaned up" image of **`Untitled2`**.

Continue to the next set of steps in this exercise: *Printing the Image.*

■ Printing the Image

In this step you will print the working image (**`Untitled2`**) in centered form.

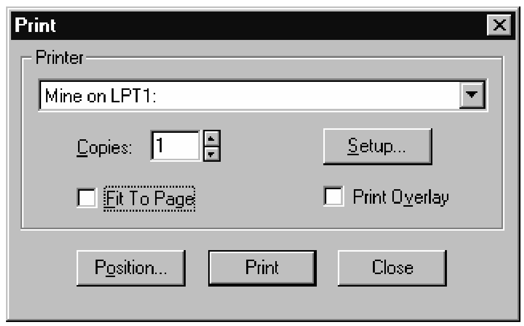
1. **Select the *Untitled2* image to print.**

   The **`Untitled2`** image is now active.

2. **Select the *Print* command from the *File* menu.**

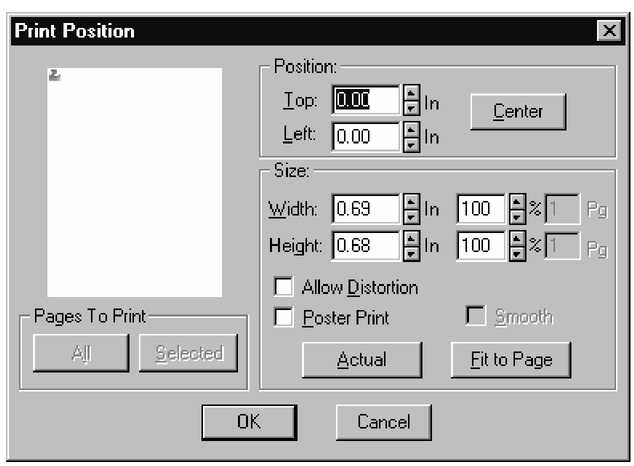
   The **Print** dialog box appears.

3. **Make sure the *Fit to Page* box is *<u>unchecked</u>*.**

4. **Select the *Position* button.**

   The **Print Position** dialog box appears.

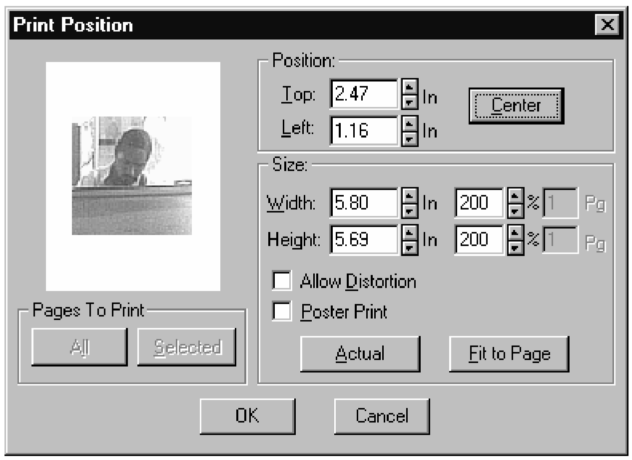
   

5. Increase the *Width* percentage value to 200%.

Notice as the **Width** value changes, the **Height** value changes too. The image thumbnail in the box on the left correspondingly increases in size.

6. Click the *Center* button in the *Position* group box.

The image thumbnail is now centered on the page.



7. Click *OK*.

The **Print** dialog box reappears.

8. Click *Print*.

The image is sent to the printer.

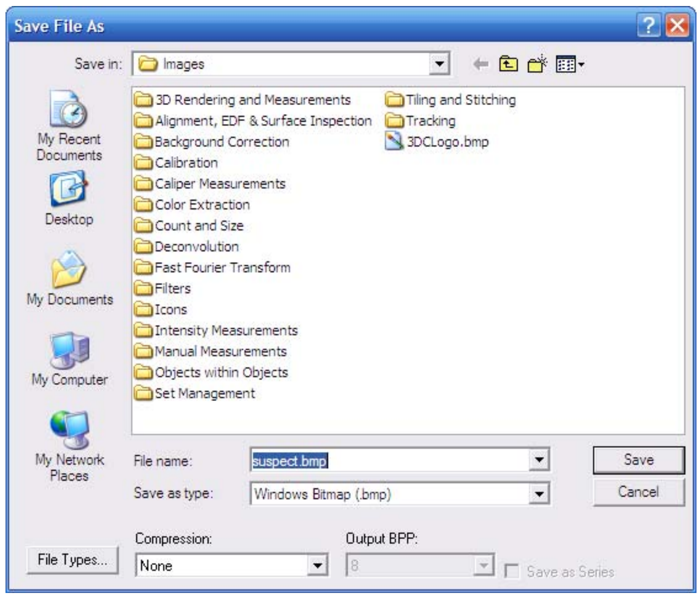
Continue to the next set of steps in this exercise: *Saving the Image.*

■ Saving the Image

In this step you will save the working image **`Untitled2`** to a new file, in a new file format.

1. **Select the *Save As...* command from the *File* menu.**

   The **Save File As** dialog box appears.



2. **Select the *Windows Bitmap (BMP)* option from the *Save as type* list box.**

3. **Type: `Suspect.bmp` in the *File name* box.**

4. **Click *Save*.**

   Image-Pro Plus saves the modified image to the **`Suspect.bmp`** file in Windows bitmap format.

Continue to the next set of steps in this exercise: *Closing the Image Windows and Exiting Image-Pro Plus.*

■ Closing the Image Windows and Exiting *Image-Pro Plus*

In this step you will close the **`Bankvid.tif`**, **`Untitled1`**, and **`Suspect.bmp`** image windows and exit out of *Image-Pro Plus*.

1. Close *Image-Pro Plus* by selecting the *Exit…* command from the *File* menu.

You can also double-click each image window's close box.

2. Do not save any changes.

Click on the **No All** button when the dialog box appears asking the question "Save changes to untitled1?"

This will close the *Image-Pro Plus* application.

Continue to the next exercise: *Filtering, Calibrating, and Measuring*.

Filtering, Calibrating, and Measuring

The Tutorial in this section is designed to introduce you to tools used for filtering and measuring. In this lesson you will:

- Use a sharpening filter to accentuate object edges in an image.
- Calibrate the spatial scale to measure in microns.
- Measure image objects using the manual and auto-trace measurement tools.
- Save the measurements to an ASCII file.

This exercise will take about 25 minutes to complete.

Setup: If you have not yet started *Image-Pro Plus*, do so now by double-clicking on the *Image-Pro Plus* icon within the *Image-Pro Plus* folder. Once *Image-Pro Plus* is running, you may begin following the steps below.

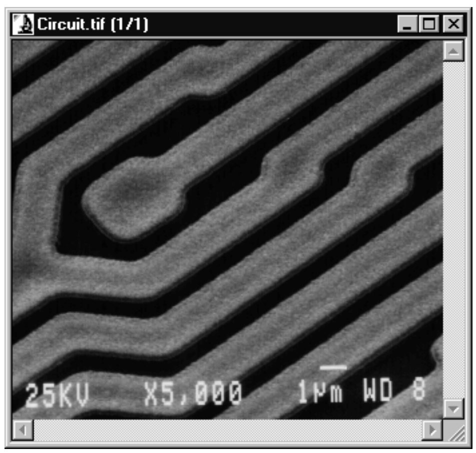
Important*: Please check that your Image-Pro settings for this exercise* ***match*** *those in the dialog boxes shown in the tutorial. Image-Pro 7.0 now retains the settings from previous experiments, which may not be the same as the ones used in this exercise.*

■ Loading an Image

.

1. Open the image *Circuit.tif* from the *Images/Manual Measurements* folder.

The **`Circuit.tif`** image window is opened in the *Image-Pro Plus* application window. This is an image of an etched printed circuit board (PCB).

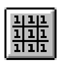


Continue to the next set of steps in this exercise: *Sharpening the Image.*

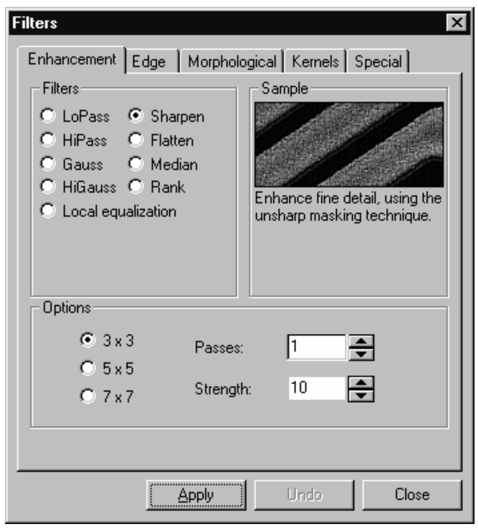
■ Sharpening the Image

In this exercise you will use the **Sharpen** filter to accentuate the edges in the **`Circuit.tif`** image. Because the circuit board traces in this image will be measured, you will want them to have clean and distinct edges. Sharpening will accentuate the places where a trace ends and the board begins.

1. Select the *Filters...* command from the *Process* menu.



The **Filter** dialog box appears.

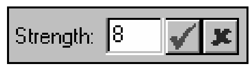


2. Click the *Sharpen* radio button under the *Enhancement* tab label.

3. Set the *Strength* value in the *Options* group box to the value of *8*.

This applies the sharpen filter at approximately 80% of its maximum strength, which will result in a significant, but not extreme, application of the filter.

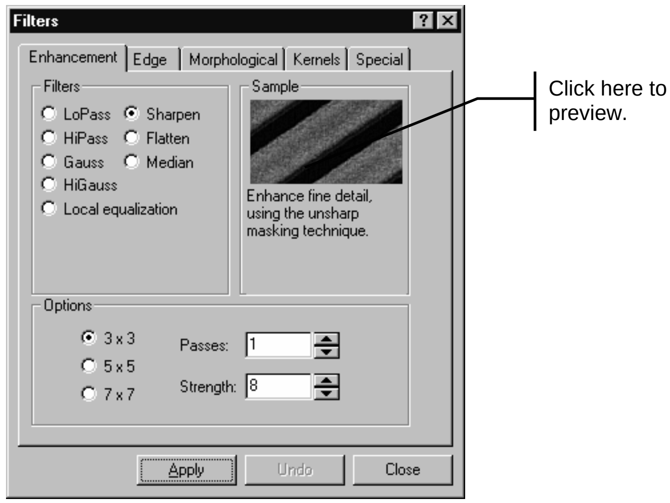
Note: *When you begin typing into the* **Strength** *field, two special data buttons appear:*



These buttons are used by Image-Pro Plus to enter or clear the data that is typed into a field. Clicking the **Checkmark** *(4) button enters the data into the field (pressing the <***Return***> key will do the same thing). A limits check is performed at this time, if appropriate. If the data is not within the allowed range, the* **Checkmark** *and* **X** *buttons will not be cleared. Clicking the* **X** *button clears the contents of the field and returns the value to the original unit measurements.*

If the **Passes** option is not set at the default value of 1, set it to **1** before continuing to the next step.

4. Click in the *Sample* window to preview the results.



Dragging in the **Sample** window will apply the filter to different portions of the image.

5. Click *Apply*.

The **`Circuit.tif`** image is sharpened. Edges and details within the image are now more pronounced.

6. Close the *Filter* dialog box.

Continue to the next set of steps in this exercise: *Calibrating the Spatial Scale*.

■ Calibrating the Spatial Scale

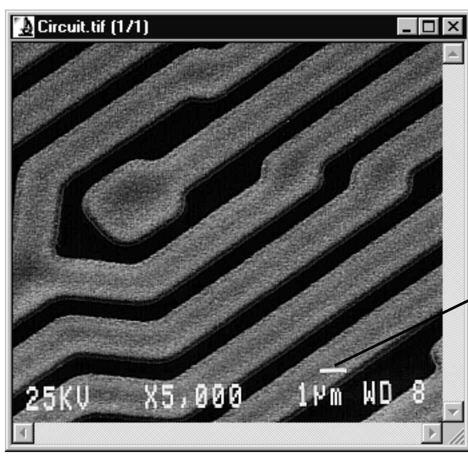
This set of steps establishes the unit-of-measure associated with this image. By default, *Image-Pro Plus* expresses all spatial measurements in terms of pixels. You will change the spatial scale to make measurements in terms of microns.

This particular image is marked with a Calibration bar identifying the length of 1 micron. This bar will be used to specify the length of 1 micrometer to *Image-Pro Plus.* You will use the *Zoom* tool to facilitate a precise measurement.

1. Select the *Zoom* tool from the tool bar.

The cursor appears as a magnifying glass.

2. Place the *Zoom* cursor (the magnifying glass) over the Calibration bar in the lower right corner.



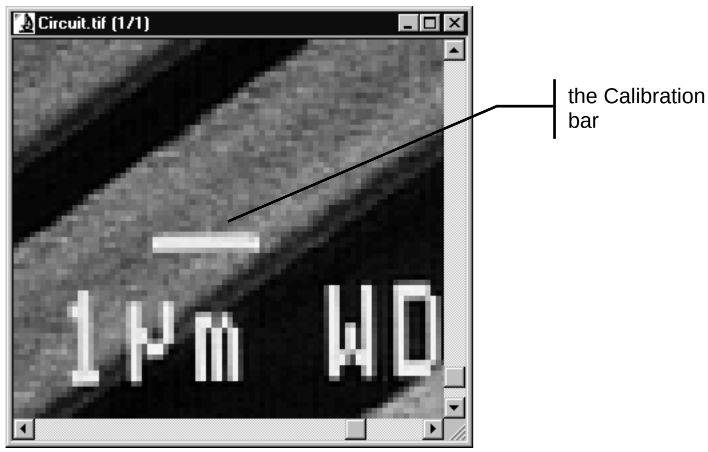
the Calibration bar

3. Click the left mouse button.

The **`Circuit.tif`** image is magnified to 200% of its original size. The status bar shows the amount of magnification.

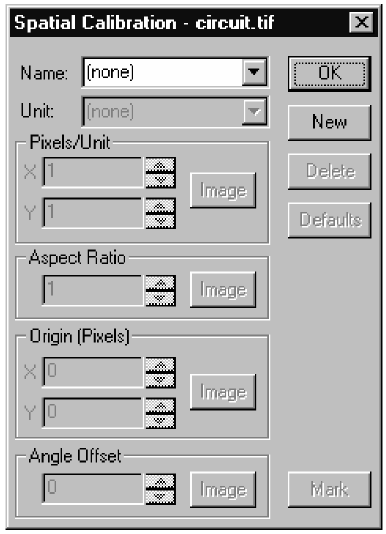
4. Place the *Zoom* cursor over the *Calibration* bar, and click the left mouse button, again for the second time.

The `Circuit.tif` image is magnified to 400% of its original size.



5. From the *Measure* menu, select the *Calibration* sub-menu, and then select the *Spatial...* option.

The **Spatial Calibration - circuit.tif** window appears.



6. Click the *New* button.

The calibration fields become active, and the **Spatial Cal 0** name appears in the **Name** field.

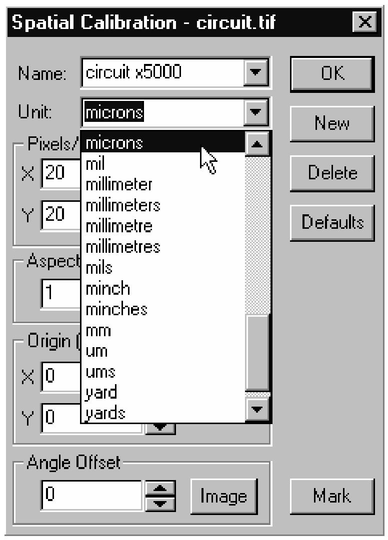
The **Name** field identifies the set of calibration values you are about to create. Calibration settings can be saved so they can be recalled when they are needed again.

7. Delete the contents of the *Name* field and type: **`circuit x5000`**

This descriptive name is assigned to the set of calibrations you are about to create.

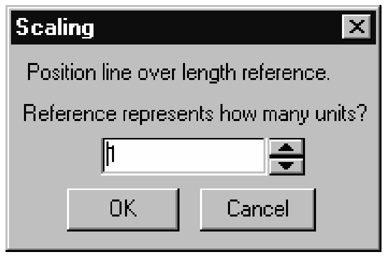
8. Delete the contents of the *Unit* field and type: `microns`

This instructs *Image-Pro Plus* to label your measurements as microns whenever a spatial measurement is reported (e.g., in a measurement data sheet or a histogram). You can also select the unit of measurement by scrolling through the **Unit** list box to view the available choices.

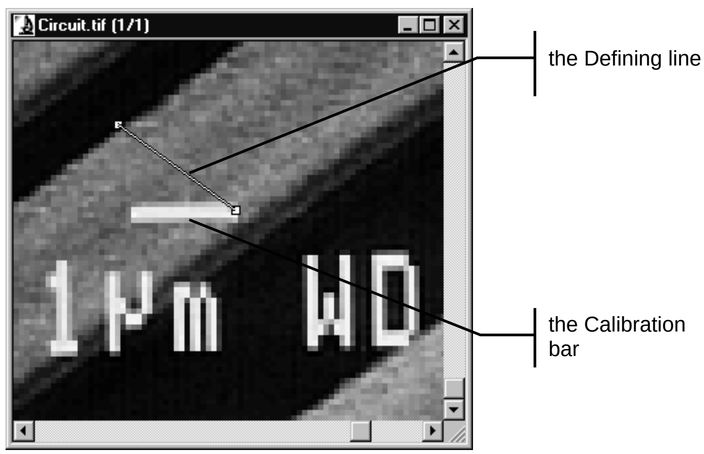


9. Click the *Image* button in the *Pixels/Unit* group box.

The **Scaling** dialog box appears, and a defining line is placed in the image. Make sure that the reference represents 1 unit (1 micron).



The **Scaling** dialog box appears, and a defining line is placed in the image.



The **Image** button allows you to define your spatial calibration unit from a known standard-of-measure in the image. In this case, the white mark (the bar) in **`Circuit.tif`** is 1 micron in length. In the next few steps, you will calibrate the defining line to the length of the bar.

10. Click the *Zoom* tool to turn *Zoom* off.

11. Place the cursor over the rightmost endpoint of the defining line.

The cursor changes to the crosshairs.

12. Match the length of the defining line to the length of the Calibration bar.

Click and hold the left mouse button to drag the endpoints of the defining line. Reposition the endpoints until the line is straight and exactly the same length of the Calibration bar, as shown in the following example:

13. Click *OK* in the *Scaling* dialog box.

The **Spatial Calibration - circuit.tif** window returns. The **Pixels/Unit** fields should read approximately 20, indicating that 20 pixels will represent 1 micron. *Image-Pro Plus* will report all spatial measurements in these terms (i.e., a measurement of 10 pixels will be expressed as .5 microns, a measurement of 40 pixels will be expressed as 2.0 microns, and so forth).

14. Click *OK* in the *Spatial Calibration - circuit.tif* dialog box.

The **Spatial Calibration** window closes.

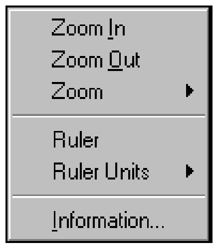
Continue to the next set of steps in this exercise: *Measuring Length.*

■ Measuring Length

In this exercise you will use the **Length** tool to measure the width of a gap in the circuit trace.

1. **Press the right mouse button when the cursor is on the *Circuit.tif* image.**

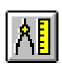
   The *Zoom* pop-out menu appears.



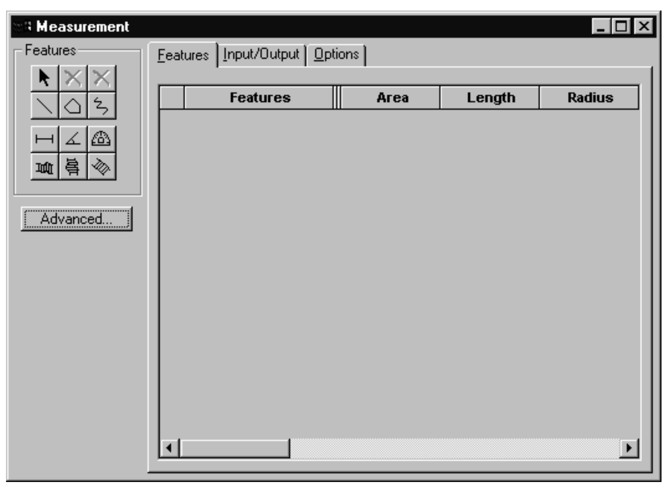
2. **Select the *Zoom* sub-menu, and then select the Zoom 100% option.**

   The **`Circuit.tif`** image returns to its original size.

3. **Select the *Measurements…* command from the *Measure* menu.**



   The **Measurements** tabbed dialog appears.



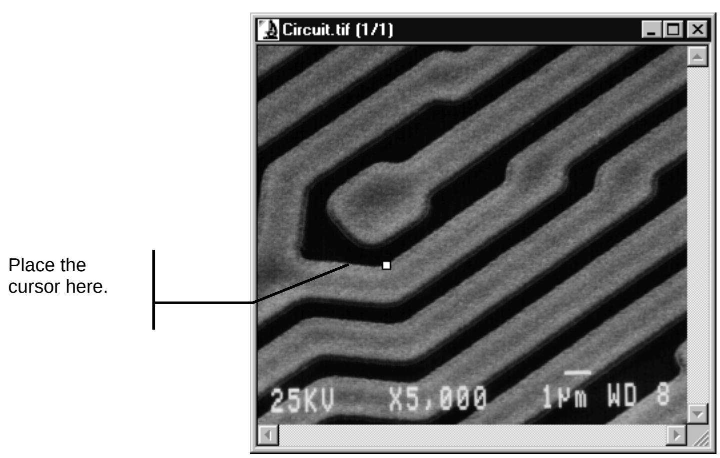
4. **Click the *Advanced* button** **.**

5. **Select the *Best Fit Line* feature. Make sure the *Auto-Add* checkbox is selected.** 

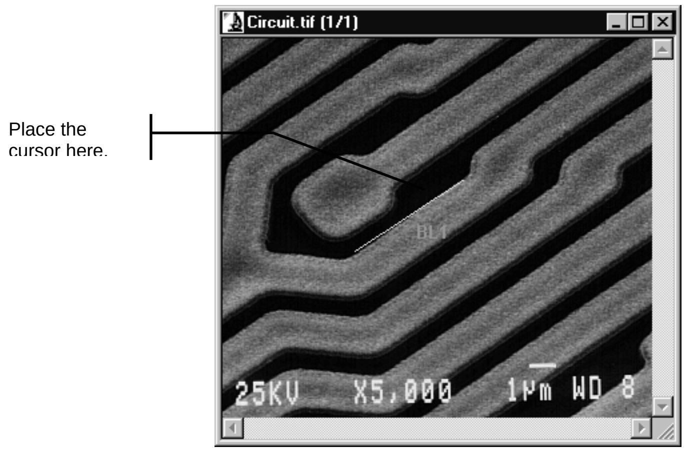
6. **The Create Best Fit Line message box appears**.
The **Best-Fit Line** feature is used to obtain a straight-line measurement.

In this case, you are measuring the length of the trace between the bends.

7. **Place the cursor over the trace as shown below, then click the left mouse button.**
The first point of the measurement is anchored.

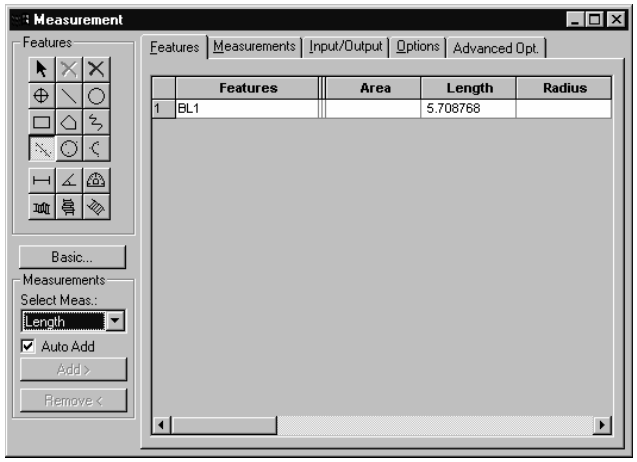


8. **Move the cursor to start of the bend in the trace (see illustration below), and double-click the left mouse button.**



A "measuring" line appears connecting the two anchor points on the image.

9. The **Measurements** tab reports the length of the line as approximately 5.7 microns.

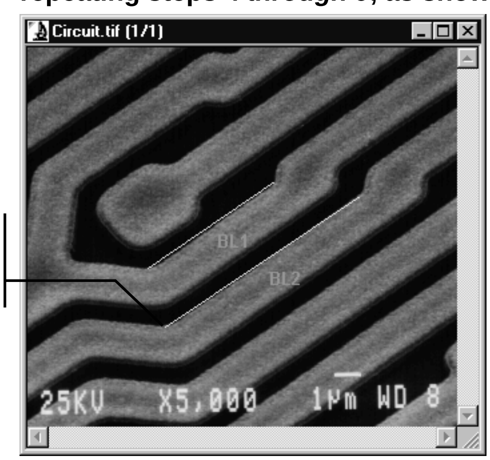


Note: *You can adjust this line in the image by dragging either of its ends to a new position. Use the selection tool (arrow) and press the <Shift> key to move the ends of the line. The measurement in the* ***Adjust Line*** *message box will automatically update as the line's length changes.*

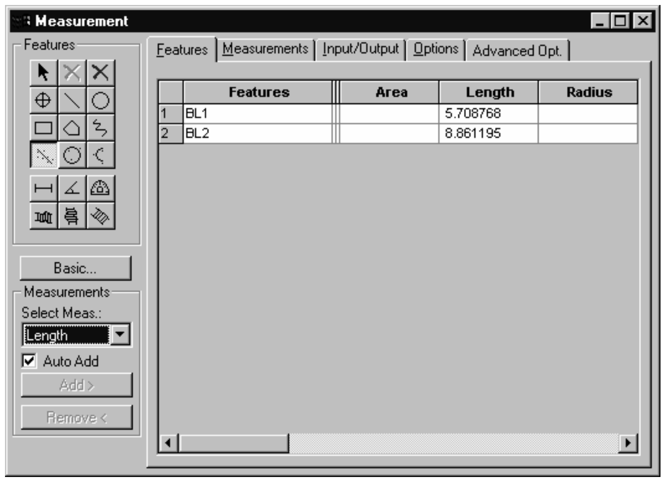
10. *Image-Pro* labels your measurement line as **BL1**.

11. **Perform the measurement of the second trace by repeating steps 4 *through 9*, as shown below.**

The second trace



12. The *Measurements* tab is automatically updated with data from both traces.



The new measurement is approximately 8.8 microns.

Note that the measurements are labeled **BL1** and **BL2**. The **BL** signifies that it is a best-fit line measurement, and the number indicates the measurement. For example, for **BL1**, the **1** signifies that it is the first measurement in this set of data.

■ Using the Trace Measurement Tool

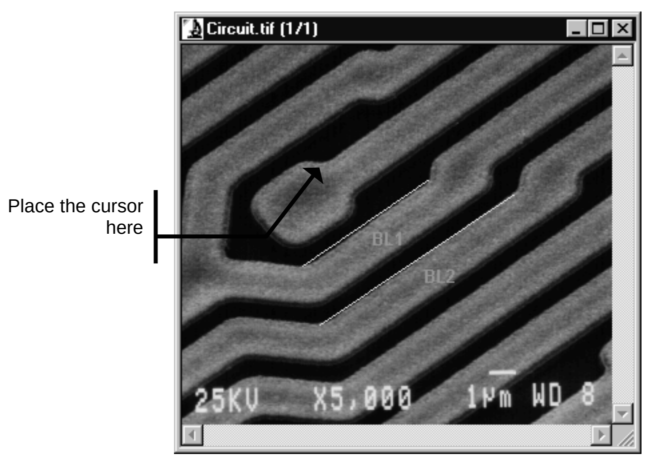
You will also use the **Trace** measurement tool to manually outline and measure the perimeter of the terminals, and automatically measure another trace on the PCB.

1. Click the *Trace* button in the *Measurements* toolbar.

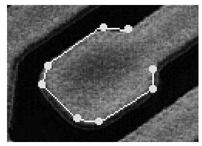
The **Creating Trace** message box appears.

The *Trace* measurement tool is used to measure lines that are not straight**.** In the following steps, first measure the terminal using this tool's manual mode, then measure a trace in automatic mode.

2. Place the cursor at the base of the circuit terminal as shown below, and click the *left* mouse button to define the beginning of the measurement line.

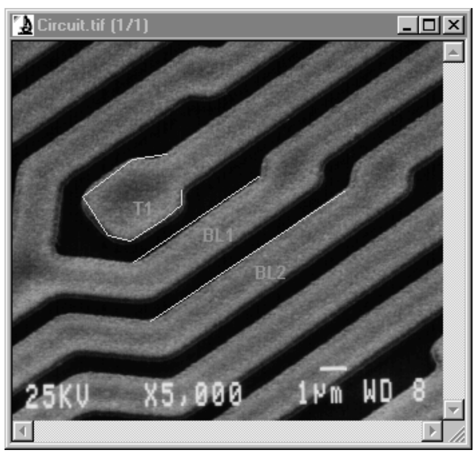


Move the cursor to each of the points illustrated below, and click the left mouse button at each position. This will produce a *polyline* (a line made up of many consecutive segments).

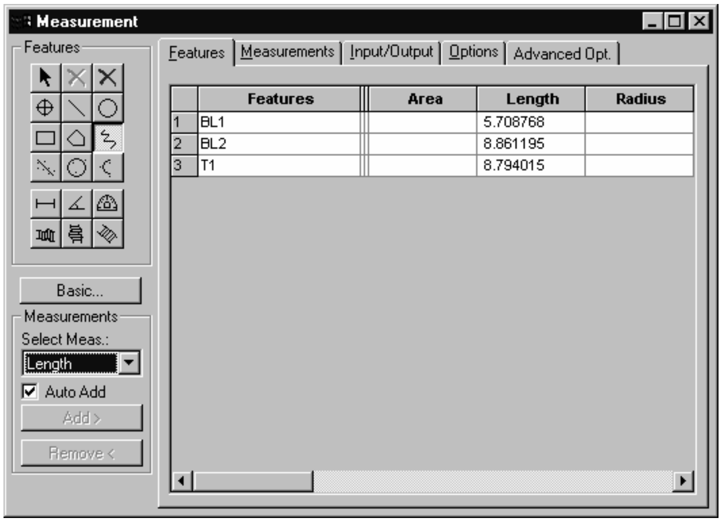


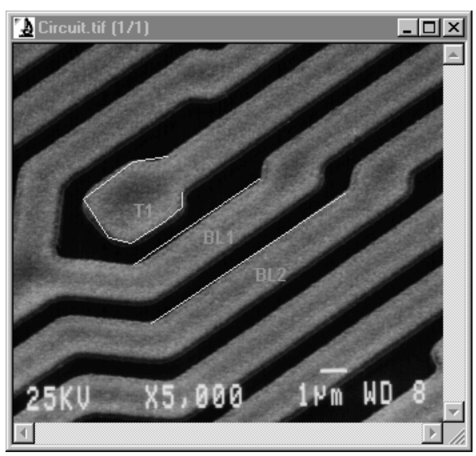
3. **After you have defined each of the points above, click the *right* mouse button to end the polyline measurement.**

The terminal is highlighted.



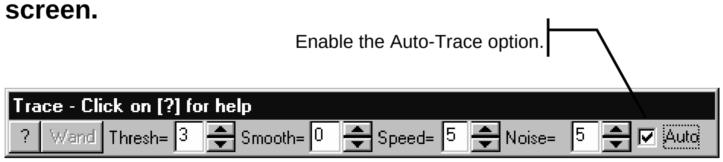
4. The measurement result will appear in the **Measurements** window datasheet, and the measurement will be outlined and labeled in the image.





Note that the measurement is labeled **T1**. The **T** signifies that it is a **Trace** measurement, and the **1** signifies that it is the first trace measurement in this set of data.

5. **To perform an automatic trace, locate and enable the *Auto-Trace* checkbox in the *Outline* dialog box on the screen.**

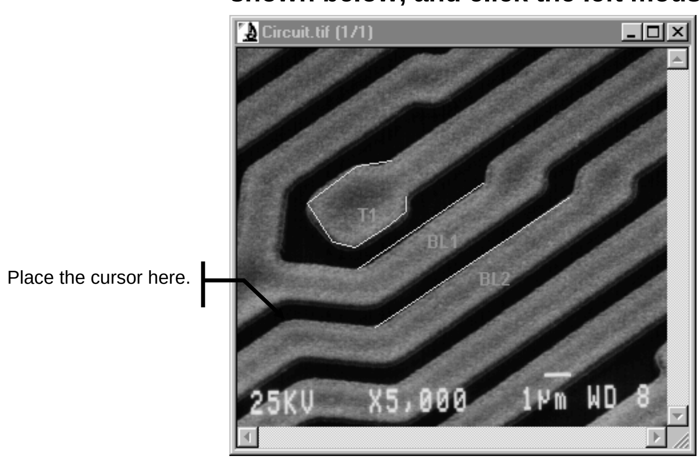


The **Outline** dialog box appears when the *Trace* measurement tool is activated. It controls the *Image-Pro Plus* automatic trace measurement feature. When the **Auto-Trace** checkbox is enabled, *Image-Pro Plus* automatically measures an edge that you specify.

6. **Set the *Smoothing* option to *5*.**

   This option tells *Image-Pro Plus* to produce a smooth trace, and not follow every nook and cranny produced by minor pixel variations along the edge.

7. **Place the cursor at the beginning of the edge as shown below, and click the left mouse button once.**



This action identifies the first point of the measurement line.

8. Place the cursor a few pixel positions further along the edge (see below) and click the left mouse button again.

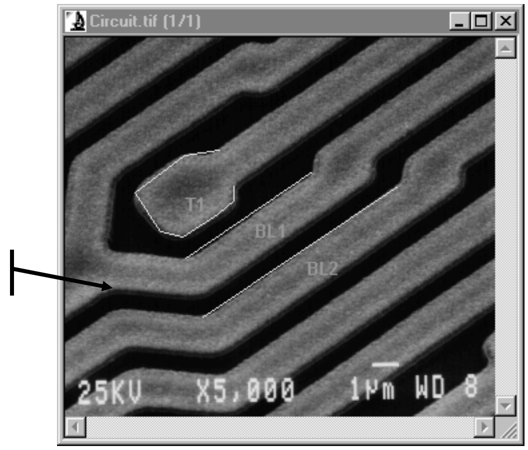


Image-Pro Plus begins measuring the edge of this trace and continues until it reaches the edge of the image. (You can stop the Auto-Trace operation by pressing the right mouse button at the same time.)

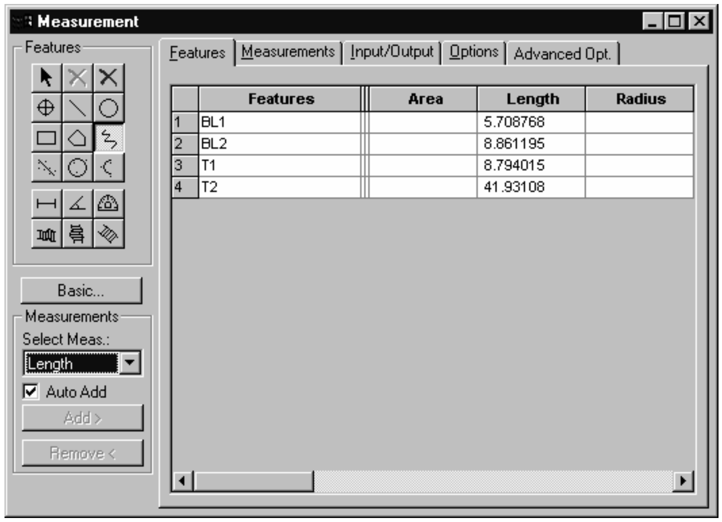
As illustrated by this exercise, *Image-Pro Plus'* automatic trace measurement facility requires you to define the first two points along the edge you want to measure. The first point specifies the beginning of the line (see Step **7** above) and the second point specifies the direction in which the line is to be drawn.

9. Once the line reaches the edge, click the right mouse button.

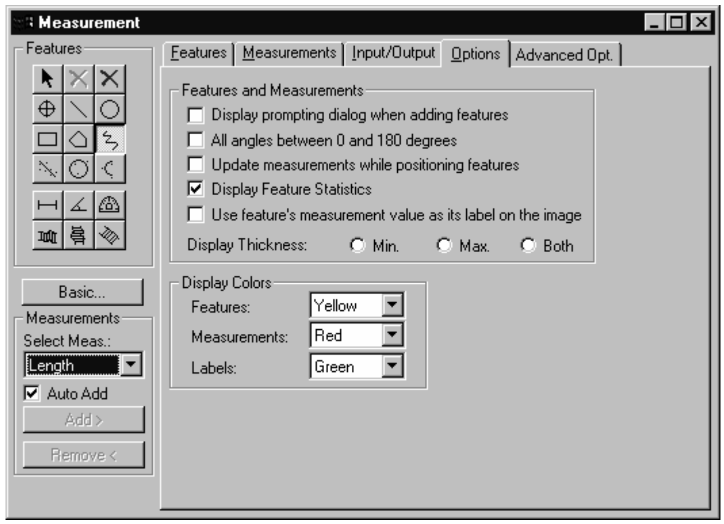
The edge is highlighted.

10. Click on *OK* in the *Trace Measurement* window.

The measurement result (approximately 21 microns) is placed in the **Measurements** window datasheet, and the measurement is outlined and labeled in the image as **T2**.

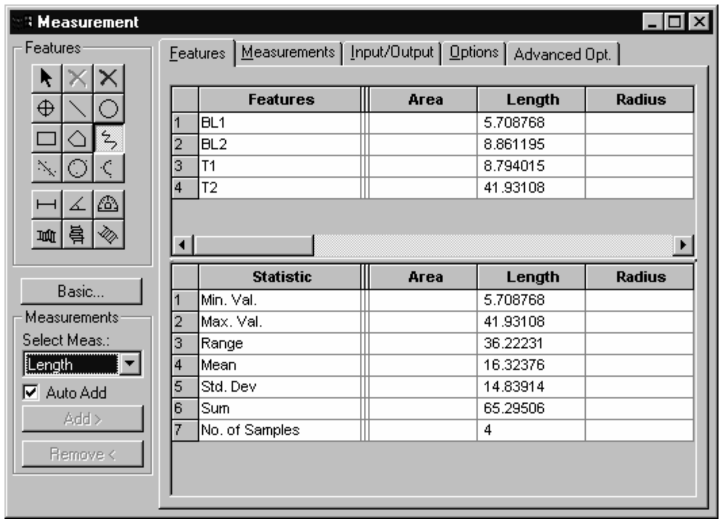


11. On the *Options* tab, select *Display Feature Statistics* to view the statistics associated with the current set of measurements.



***Note**: **Area** and **Angle** values are zeros , as those measurements have not been performed.*

12. **Select the Features tab.** You will see the measurement statistics displayed on the lower portion of the page.

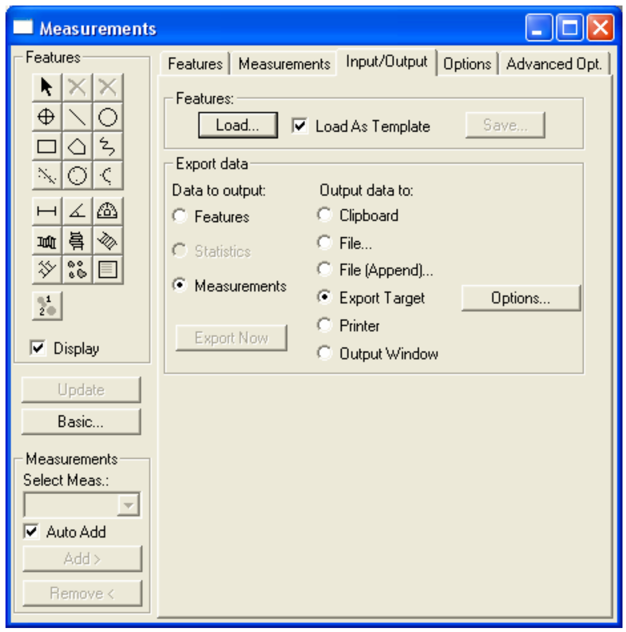


Continue to the next set of steps in this exercise: *Saving the Measurements.*

■ Saving the Measurements

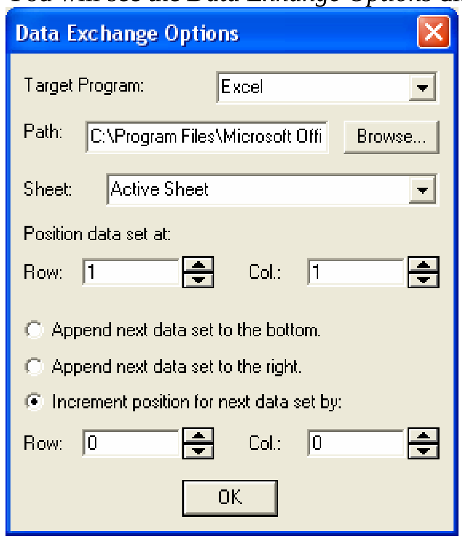
In this step you will export the measurement data to an Excel spreadsheet.

1. Select the *Input/Output* tab on the Measurements tabbed dialog.



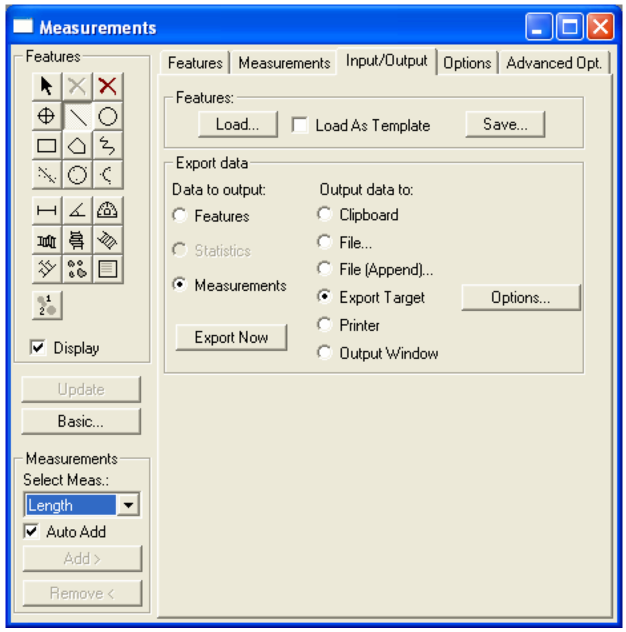
2. Select the *Measurements* from the *Data to Output* list, and *Export Target i*n the *Output data to* list.

3. **Click the *Options* button.**
You will see the *Data Exhange Options* dialog:



Indicate that you are sending the data to an *Excel* spreadsheet, and click **OK** to return to the ***Input/Output tab*** dialog.

4. Click *Export Now*.



The Measurement data will be sent to an *Excel* spreadsheet.

Continue to the last set of steps in this exercise: *Closing the Images and Exiting Image-Pro Plus.*

■ Closing all the Windows and Exiting *Image-Pro Plus*

Close all the windows and exit *Image-Pro* by selecting the *Exit* option under the *File* menu. Do **not** save any files.

Continue to the next exercise: *Optical Density Analysis.*

Optical Density Analysis

The Tutorial in this section introduces you to *Image-Pro Plus' Intensity* calibration and *Analysis* tools.

In this exercise you will:

- Load an image of a gel sample and calibrate the intensity scale in terms of optical density.
- Obtain density measurements from a band of pixels within the gel image.
- Establish a baseline measurement of incident light.
- Plot the density measurements of a band of DNA pixels against the incident light baseline.
- Capture the screen image of the plot.
- Store into an image file to be imported into another application.

This exercise will take about 15 minutes to complete.

Setup: If you have not yet started *Image-Pro Plus*, do so now. Once the *Image-Pro Plus* application window is active, you may begin following the steps below.

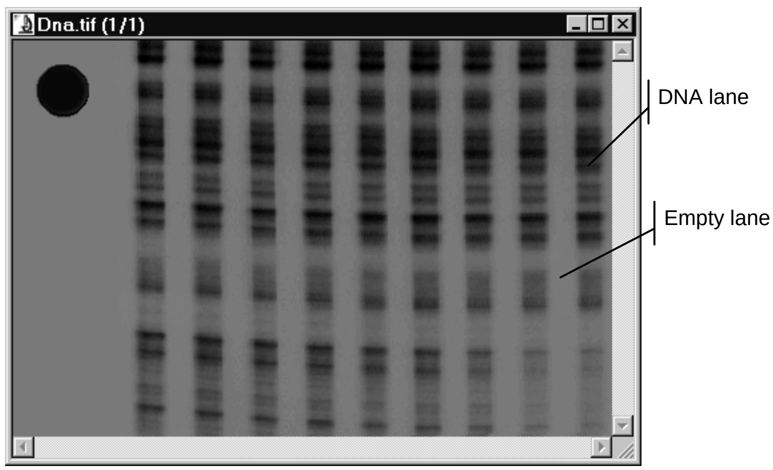
Important*: Please check that your Image-Pro settings for this exercise* ***match*** *those in the dialog boxes shown in the tutorial. Image-Pro 7.0 retains the settings from previous experiments, which may not be the same as the ones used in this exercise.*

■ Loading the Image

In this exercise, you will modify *Image-Pro Plus'* intensity scale so that the intensity values of a DNA gel sample image are expressed as density values (calibrated to a standard density curve). This image was captured in a light box with a CDD camera, with the UV light source positioned behind the sample.

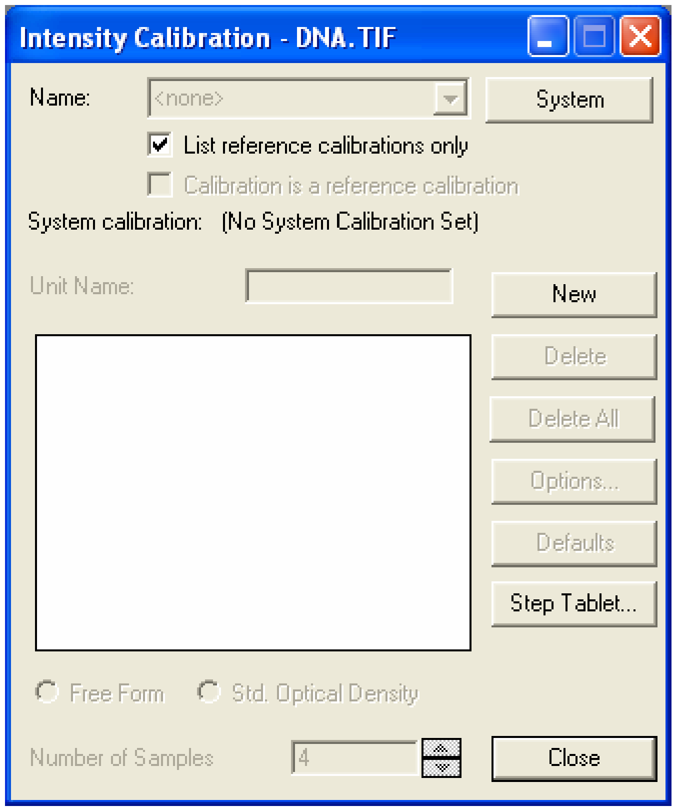
1. **Load the *DNA.tif* file from the *Images/Manual Measurements* folder of the *Image-Pro Plus* folder.**

The `DNA.tif` image window is opened. The dark stripes (lanes) indicate the presence of DNA.



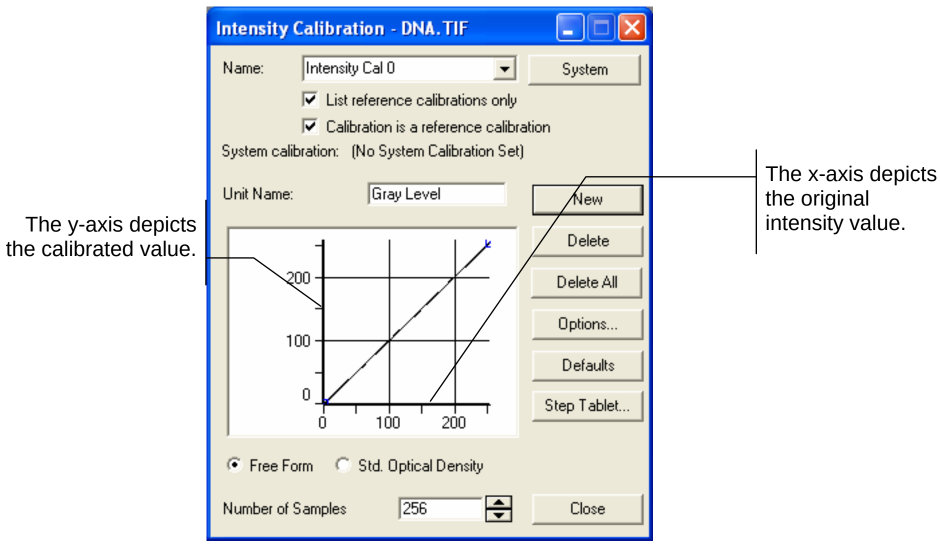
2. From the *Measure* menu, select the *Calibration* sub-menu, and then the *Intensity* command.

The **Intensity Calibration - dna.tif** dialog box appears.



3. Click the *New* button to create a new calibration.

The intensity curve appears and the **Intensity Cal 0** name appears in the **Name** field.



The **Name** field identifies the set of calibration values you are about to create. Calibration settings can be saved within *Image-Pro Plus* so they can be recalled when they are needed again.

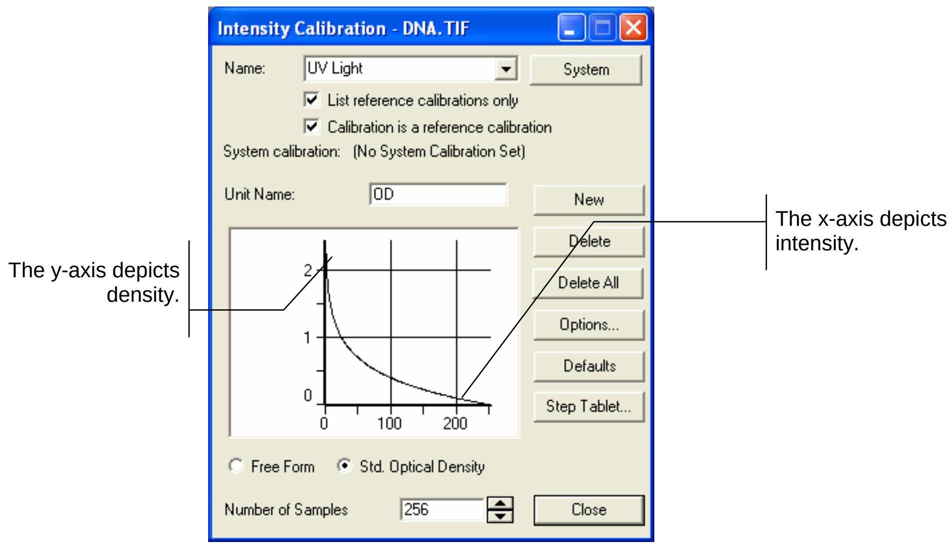
The intensity curve illustrates the effect of a calibration change upon the intensity scale. The **x**-axis describes the original intensity value (i.e., the value read from the image), and the **y**-axis reflects the way in which the value will be interpreted per the calibration changes.

4. Enter *UV Light* as the *Name* of the new calibration.

5. Enter *OD* as the *Unit Name* for optical density.

6. Click the *Standard Optical Density* radio button.

The intensity curve changes to reflect the density/intensity relationship.



The optical density scale reflects an exponential decay of light within a transmitting material. As you can see, the optical density curve is inversely related to the intensity scale. This means:

- Small intensity values reflect large density values.
- Large intensity values reflect small density values — i.e., the darker the pixel, the denser the material.

Continue to the next set of steps in this exercise: *Specifying the Black and Incident Levels.*

■ Specifying the Black and Incident Levels

In this exercise you will establish the levels that represent **black** (indicating that no light is present) and **incident** or white (indicating that no material is present).

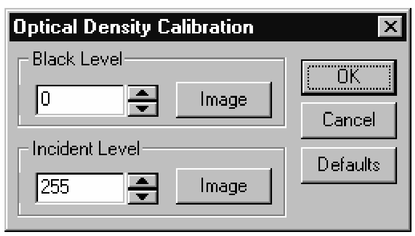
The black circular object (black spot) in the upper-left corner of the image is the calibration standard, which will be used to establish the black level. This object represents the intensity recorded by the camera when no light was passed to it (it was created by affixing a solid object to the sample when the image was captured in the light box).

The empty area along the **left** side will be used to establish the incident level or white level. This area represents the intensity recorded when no material was present, and all possible light was passed to the camera.

You can now enter values for the levels or click on the corresponding **Image** buttons to interactively set the levels.

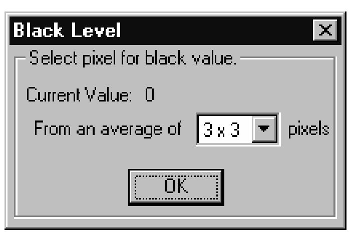
1. **Click the *Options* button in the *Intensity Calibration* dialog box.**

   The **Optical Density Calibration** dialog box appears.



2. **Click the *Image* button in the *Black Level* group box.**

   The **Black Level** message box appears, and the cursor appears as crosshairs over the image.

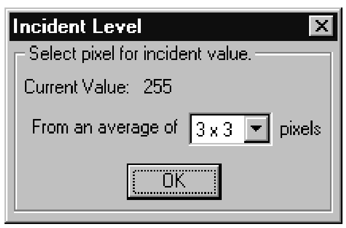


3. **Move the crosshairs to the center of the black spot in the upper-left corner. Click the left mouse button when the center of the cursor is positioned over the center of the dark circle, and the *Black Level* message box reads *8*.**

   The **Optical Density Calibration** dialog box reappears, and the **Black Level** field reads **8**.

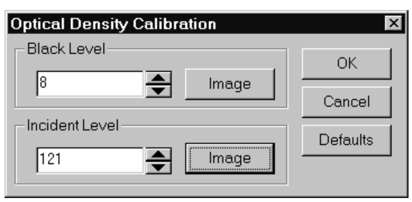
4. **Click the *Image* button in the *Incident Level* group box.**

   The **Incident Level** message box appears. The cursor appears as crosshairs.



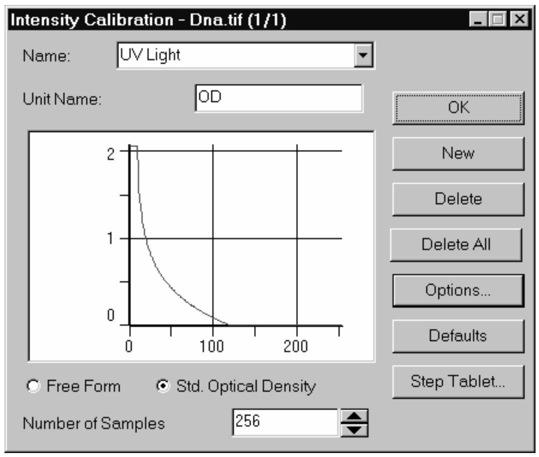
5. **Move the crosshairs to the empty area along the left side of the image. Click the left mouse button when the *Incident Level* message box reads *121*.**

   The **Optical Density Calibration** dialog box reappears, and the **Incident Level** field reads 121.



6. Click *OK*.

The **Intensity Calibration - dna.tif** dialog box appears. The end points of the density curve reflect the black and incident levels you have just entered.



7. Click *OK.*

The **Intensity Calibration - dna.tif** dialog box closes.

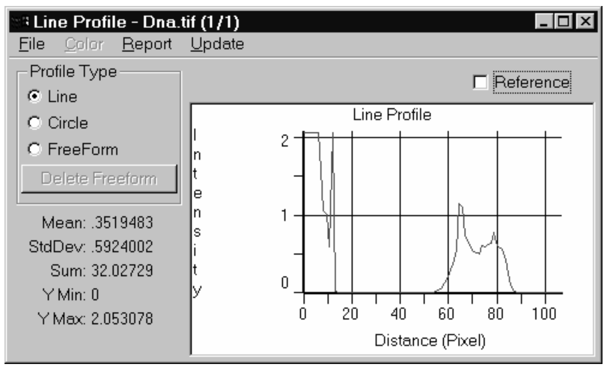
Continue to the next set of steps in this exercise: *Plotting the Density of a Lane.*

■ Plotting the Density of a Lane

In this exercise you will collect the density measurements for one of the DNA lanes in the gel, and plot its density using the *Line Profile* command. A Line Profile plots the values of each pixel in a line in the image. In this exercise, you will define a column of pixels (instead of a single line), and plot the "average" density value along the length of the column.

1. Select the *Line Profile* command from the *Measure* menu.

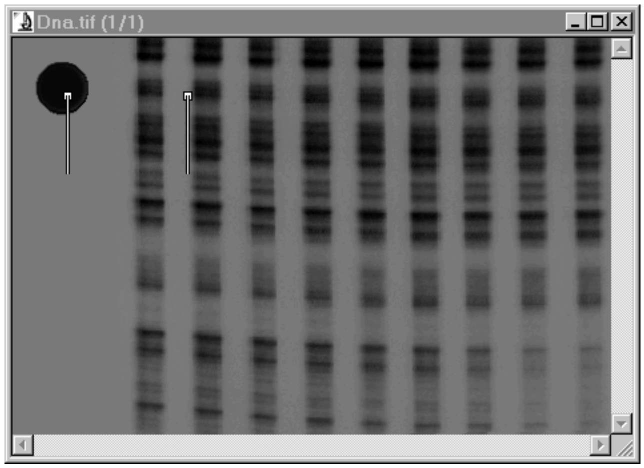
The **Line Profile - dna.tif** dialog box appears, and a diagonal defining line is placed in the upper-left corner of the **`DNA.tif`** image window.



Note: *Some of the example images used in this exercise show the profile's statistic values along with the graph. On your system, these statistics might not appear. Do not be concerned; this difference will not affect the objectives of this exercise.*

2. **From the *Line Profile-dna.tif* dialog box, select the *Report* menu, and then select the *Thick Vert* command.**

A pair of defining lines appear in the upper-left corner of the image.



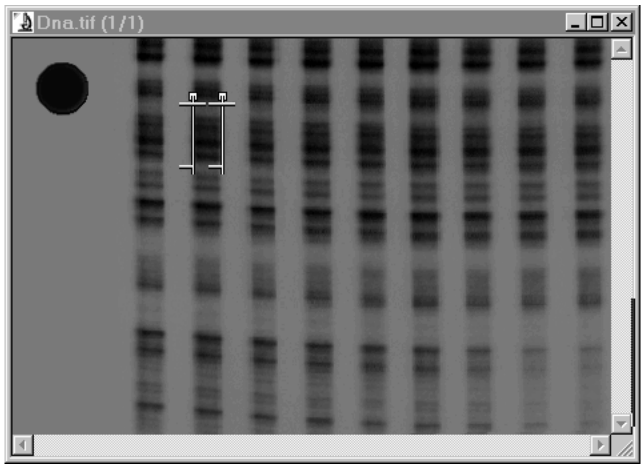
3. **From the same *Report* menu, make sure the *Intensity Cal* and *Range/Area* options are selected.**

4. **Deselect the *Full Scale* option in the *Report* sub-menu.**

The Y-axis of the graph is scaled to the minimum and maximum values of the profile line.

5. Match the width of the pair of lines to the second DNA lane in the image.

Position the pair of lines over the second DNA lane and drag the rightmost defining line to the right edge of the second DNA lane.



Using the same procedure, drag the leftmost defining line to the left edge o the second DNA lane.

6. Stretch the pair of lines the length of the lane.

Position your cursor between the pair of lines at the top end of the column. When the vertical double-arrow appears, drag the endpoints to the top edge of the DNA lane.

Using the same procedure, drag the bottom endpoints to the bottom of the DNA lane.

When complete, your defining pair should look as follows:

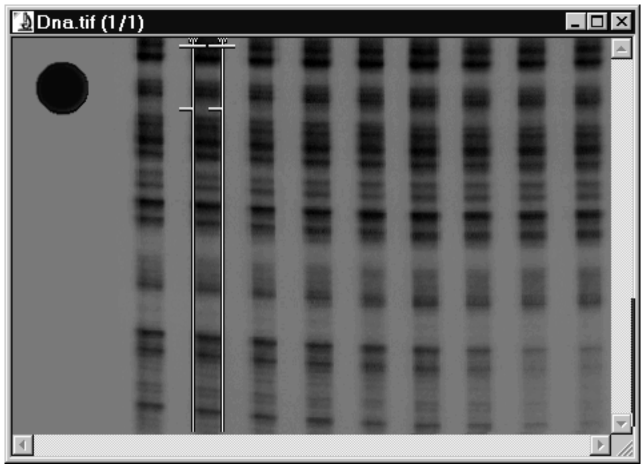


Image-Pro Plus measures, and plots, the average density down the length of the lane. You can see that the graph reflects the bands of material in the lane. The darkest (densest) bands produce the highest peaks.

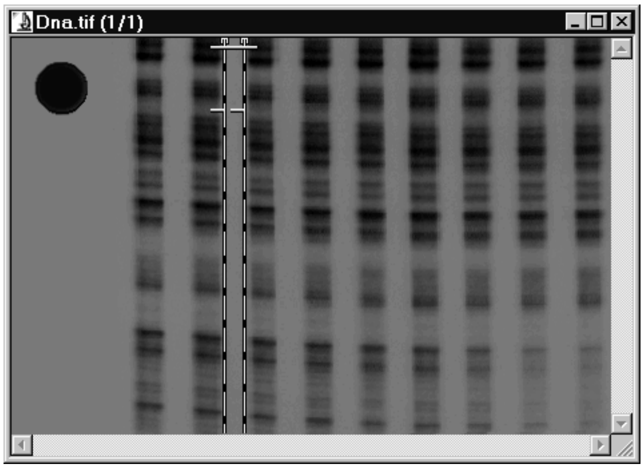
Continue to the next set of steps in this exercise: *Plotting the Density Against a Baseline.*

■ Plotting the Density Against a Baseline

In this exercise you will define an empty column as a baseline, and plot the differences between it and the sample lane.

1. Move the defining pair to an empty lane.

Position your cursor anywhere between the defining lines. When the 4-way cursor appears, drag the lines to the empty lane to the left of the current DNA lane.

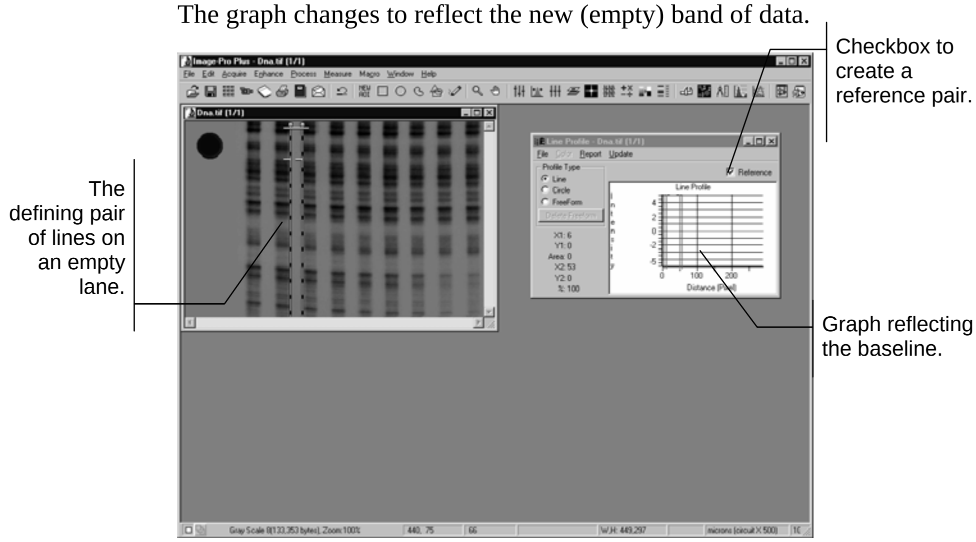


Note: *If necessary, adjust the lanes individually to ensure both are entirely within the empty lane. You do not want them touching the DNA lanes on either side.*

2. Click the *Reference* checkbox in the Line Profile - dna.tif window.

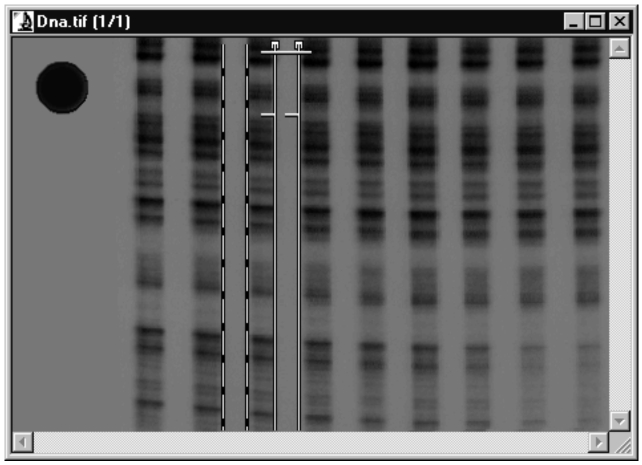
The defining-line pair appears as dashed lines, and *Image-Pro Plus* plots their entire length as **0**. This creates a baseline against which other profiles can be measured.

The graph changes to reflect the new (empty) band of data.



3. Place a defining pair over the second DNA lane.

Place your cursor between the reference lines. When the 4-way cursor appears, click the left mouse button. A new defining pair will be cloned from the reference pair and placed directly over the initial pair. Drag the new pair over the second lane as shown.



Now, two pairs of lines appear in the image. The dashed pair in the empty lane represents the baseline, and the solid pair in the DNA line represents the measured pair. The graph shows the variance of the measured pair from the baseline pair.

In the next set of steps, you will copy the graph to an image file.

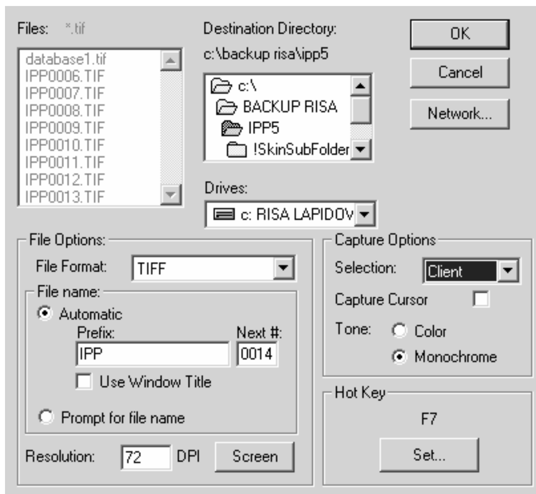
Continue to the next set of steps in this exercise: *Storing the Plot to an Image File.*

■ Storing the Plot to an Image File

In this exercise you will save the line plot to a TIFF file using *Image-Pro Plus'* Screen Capture utility. Once your plot has been saved in TIFF form, it can be pasted or imported into a word processor or desktop publishing program.

1. Select the *Screen Capture* command from the *Image-Pro Plus' File* menu.

The **Screen Capture Options** dialog box appears.



2. Select the *Client* option in the *Selection* list box under the *Capture Options* group

This option lets you copy the *content* of the active window to an image file (just those pixels *inside* the window; not the border and title bar). The other choices in this list box let you capture the entire screen, an entire window, or just a particular area of the screen that you specify.

3. Select the *c:\ipwin5\images* folder.

This directs *Image-Pro Plus* to store the captured screen to this directory. By default, *Image-Pro Plus* stores captured screens to files prefixed with **GRAB**. You will not change this naming convention in this exercise, so *Image-Pro Plus* will store the screen to a file called **GRAB0000.tif** in the *c:\ipwin5\images* directory.

4. Click *OK*.

The **Screen Capture Options** dialog box closes**.**

5. Press *<F7>.*

The **Select Window/Client** dialog appears.

6. Click and drag the *Window Selector* tool over the *Line Profile—dna.tif* window*.*

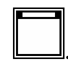
Use the mouse to click and drag the *Window Selector* tool . Release the left mouse button after selecting the **Line Profile—dna.tif** window.

Image-Pro Plus reads the contents of the **Line Profile—dna.tif** window and creates an image from the pixels that it contains. A camera icon appears, and a progress line runs down the length of the window as *Image-Pro Plus* captures the window. The image is stored to **GRAB0000.tif** in the *ipwin4\images* folder.

Note: *You have just stored an image of the profile graph. The data used to generate the graph are not a part of this file. To store the data associated with this graph, use the **Save…** command on the **File** menu in the **Line Profile—dna.tif** window.*

More about... Screen Capture

You could now use this captured image just as you would any image file. You could read the file into Image-Pro Plus to edit it, enhance it, or combine it with another image. Moreover, you could read (or paste) it into a word processing or desktop publishing document.

*In this exercise, Image-Pro Plus stored the image to a file called GRAB0000.tif. Subsequent screen images stored to this directory would be assigned the same prefix **GRAB**, but a new consecutive number (e.g., GRAB**0001**.tif, GRAB**0002**.tif, etc.) would be assigned.*

You also have the option to change the GRAB prefix to something else, for example IPP.

*You may store a captured screen data in any file format supported by Image-Pro Plus. If your application does not accept TIFF, select another format with the **Screen Capture** command.*

By default, ***<F7>*** *is assigned as the hot key to invoke the Screen Capture facility. You may change this assignment with the* ***Screen Capture*** *command.*

Continue to the next set of steps in this exercise: *Closing all the Windows and Exiting Image-Pro Plus.*

■ Closing all the Windows and Exiting *Image-Pro Plus*

Close all the windows by selecting the **CloseAll button**  from the *Image-Pro Plus* toolbar. You may continue working with *Image-Pro Plus* or exit *Image-Pro Plus* by selecting the *Exit* option under the *File* menu. Do not save any changes.

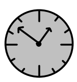
Continue to the next exercise: *Filtering with Fast Fourier Transforms.*

Filtering with Fast Fourier Transforms

The Tutorial in this section introduces you to the *Fast Fourier Transform (FFT)* command. The *FFT* command transforms an image into a frequency spectrum. Image **noise** appears as a repetitive pattern which is difficult to remove using standard spatial-filtering tools. The *FFT* tool is especially designed to isolate and filter this noise, then transform and edit the noise pattern out of the image in easy steps.

In this exercise you will:

- Load an image containing pattern noise and transform that image into its energy spectrum using a *Fast Fourier Transform*.
- Edit the spectrum to remove the pattern frequency.
- Regenerate the edited image through an *Inverse Transform*.
- Send the file as an e-mail attachment.



This exercise will take about 25 minutes to complete.

Setup: If you have not yet started *Image-Pro Plus*, do so now by double-clicking on the *Image-Pro Plus* icon in your *Image-Pro Plus* folder. Once the *Image-Pro Plus* application is active, you may begin following the steps below.

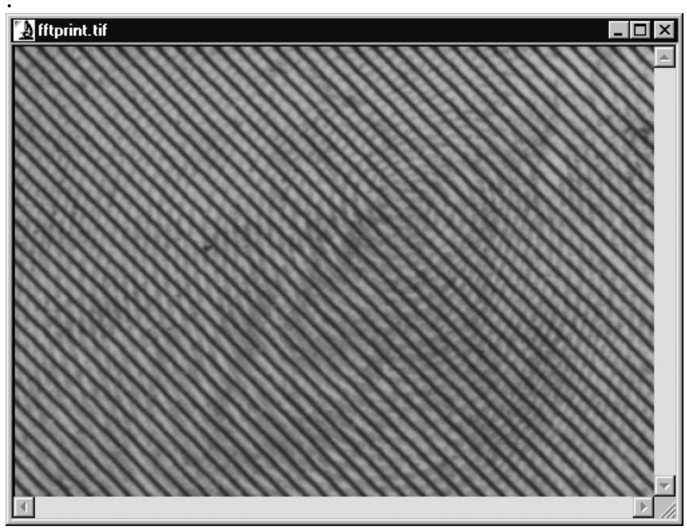
■ Transforming the Image

In this exercise, you will use an image embedded with a strong diagonal interference signal and transform that image into its frequency spectrum using the *Image-Pro Plus FFT* command.

1. Load the *Fftprint.tif* file from the *Images/Fast Founier Transform* folder.

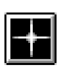
The **`Fftprint.tif`** image window appears. Notice the strong diagonal noise patterns.

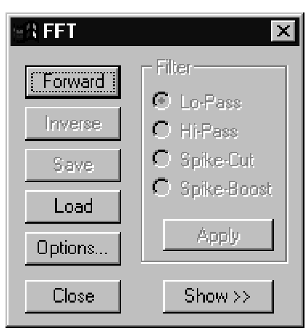
.



2. Select the *FFT…* command from the *Process* menu.

The **FFT** window appears.

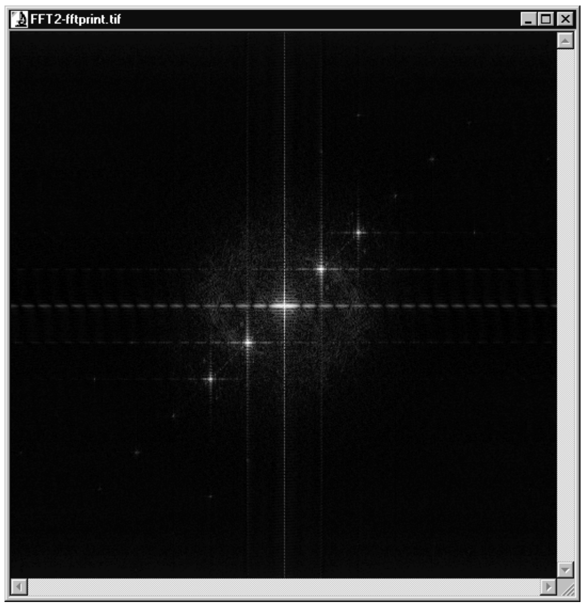




3. Transform the image to its frequency spectrum.

Click the **Forward** button in the **FFT** window to transform the image. This process may take a few moments. You can monitor its progress by observing the message bar at the bottom of the screen.

When the transformation process is complete, the **FFT1-`fftprint.tif`** image window appears, containing the spectrum.



The spectrum appears as a centered cloud of points, with 4 bright points outside of the central point. These 4 points represent the pattern noise you see in the image.

You will remove these points in the next few steps.

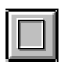
Continue to the next set of steps in this exercise: *Editing the Spectrum.*

■ Editing the Spectrum

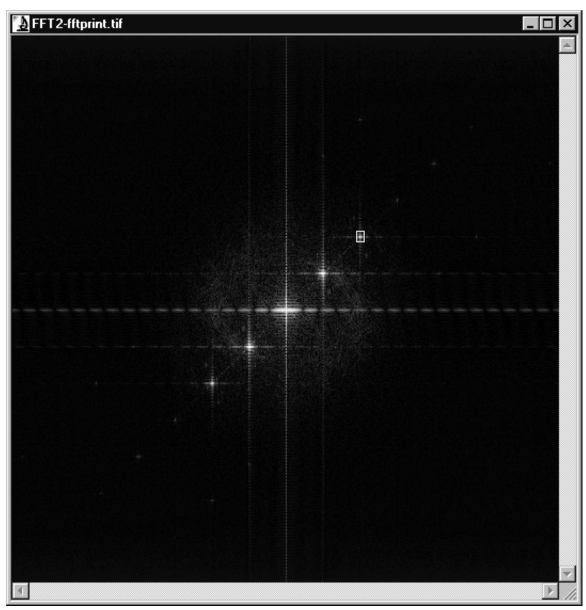
In this exercise, you will eliminate the anomalous frequencies (the 4 outside bright points) by defining an AOI around them and cutting the frequencies from the spectrum.

1. Click the *Spike-Cut* radio button in the *Filter* group box in the *FFT* window.

2. Select the AOI *Rectangle* tool from the Tool Bar.



3. Draw a small, rectangular AOI around the outermost bright point in the *upper-right* quadrant of the *FFT1—fftprint.tif* window.



Make the AOI as small as you can, to enclose just the bright point. If you can't remember how to define an AOI, refer to that exercise in the *Learning the Basics* section of this tutorial.

4. Click the *Apply* button in the *FFT* window.

The spectrum is filtered, and the selected frequencies are removed from it. This process may take a few moments.

Notice that the bright point, and its counterpoint (diagonally opposite) are gone. The frequencies represented by these points have been removed from the spectrum.

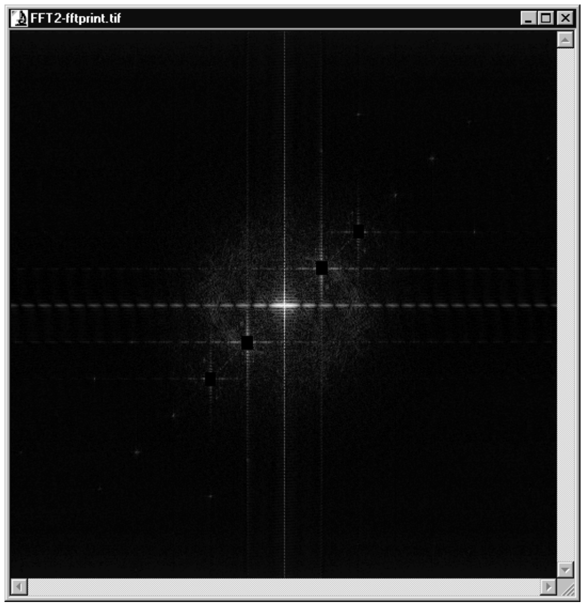
5. Click the *New AOI* button from the tool bar along the top edge of your screen.

The AOI you drew around the upper-right bright spot is cleared from the screen.

The ***New AOI*** tool removes the active AOI from the screen and allows you to define a new one.

6. Repeat steps *3* and *4* on the *innermost* bright point in the upper-right quadrant (i.e., define an AOI around it and cut it from the spectrum).

When this step is complete, the last pair of bright points will be gone.



The majority of the anomalous frequencies have now been removed from the image.

Note: *The remainder of the cross-shaped distribution around the points you just cut represent edge effects of the noise. As an additional exercise, you can go back and cut more of these edge effects using the AOI tools.*

7. Click on the AOI *Rectangle* tool to turn it off.

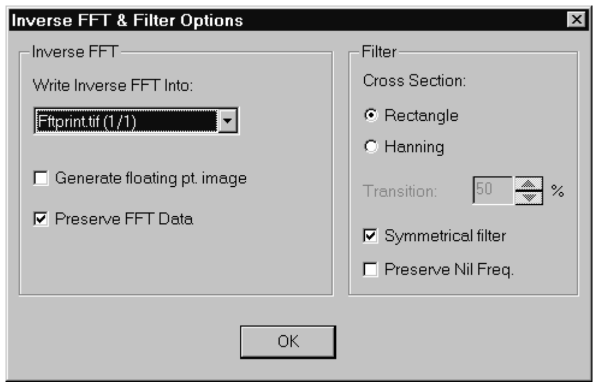
Continue to the next set of steps in this exercise: *Returning the Spectrum to Spatial Form.*

■ Returning the Spectrum to Spatial Form

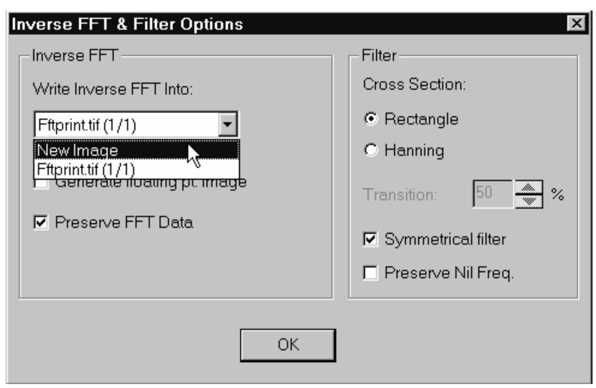
In this exercise, you will inversely transform the spectrum back into its spatial form. This will generate the original image, minus the frequencies that were edited-out in the last step.

1. Click the *Options* button in the *FFT* window.

The **Inverse FFT & Filter Options** dialog box appears.



2. Select the *New Image* option from the *Write Inverse FFT Into* combo box.



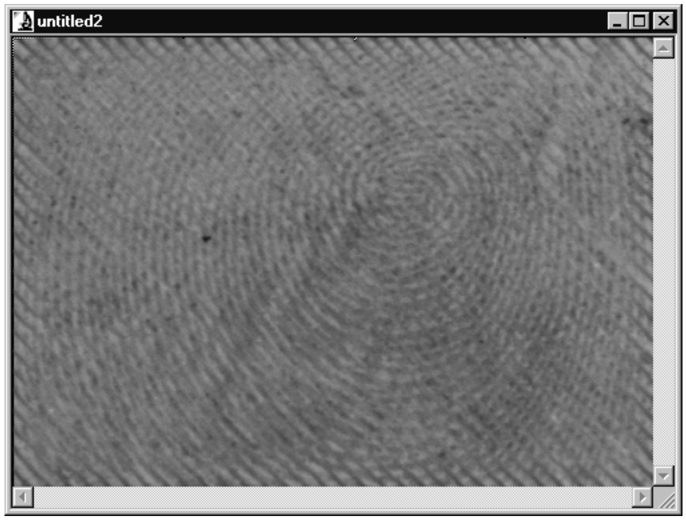
This directs *Image-Pro Plus* to write the results of the Inverse Transformation to a new image window. The original image window can also be selected here.

3. Click *OK*.

The **Inverse FFT & Filter Options** dialog box closes.

4. Click the *Inverse* button in the *FFT* window.

The FFT data is inversely transformed back into spatial form and placed in an untitled image window. This process may take a few moments.



Nearly every trace of the diagonal pattern has now been removed. The new image is the same size as your original, although the FFT image is larger. An FFT transformation requires sizes of powers of 2 (e.g., 16, 32, 64, 128, 256, 512...) for efficiency, and images that are not powers of 2 in size are expanded to the next larger power of 2 for processing.

5. Click on *Close* to close the *FFT* window.

More about... ***FFT filtering***

*Image-Pro Plus will transform an AOI, instead of the entire image, if an AOI is active when the **Transform** button is clicked.*

The spectrum window remains open even after you have performed an inverse transformation. You can now make additional edits to the spectrum if you are not satisfied with the results.

*In addition to the **Spike Cut** option, which was used in this exercise to eliminate a specific range of frequencies, the **Hi-Pass** and **Lo-Pass** options let you eliminate or attenuate all frequencies below or above a specified point. See the **FFT** command in the **Reference Manual** for more information about these options.*

*Image-Pro Plus will write the results of an Inverse Transform into an AOI if one is active in the designated destination window when the **Inverse** button is clicked.*

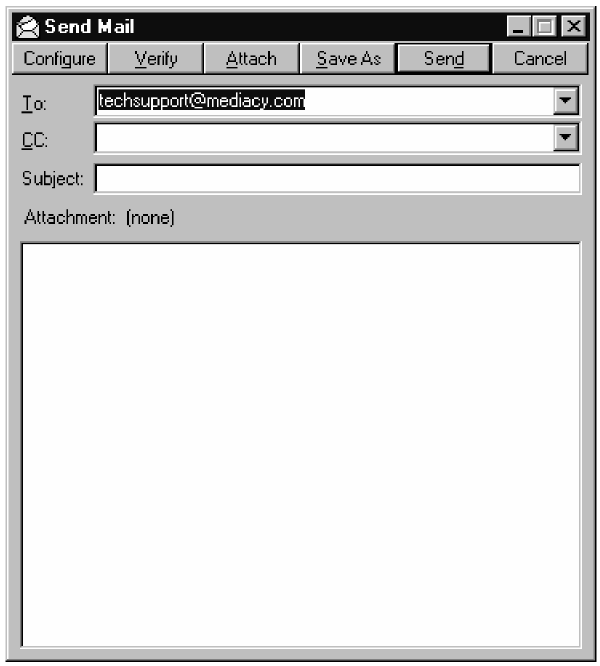
Continue to the next set of steps in this exercise: *Sending as an E-mail Attachment.*

■ Sending as an E-mail Attachment

In this exercise, you will learn how to send the modified image as an e-mail attachment.

1. Select the *Send Mail* option from the *File* menu.

The **Send Mail** dialog box appears.



2. Type the e-mail address you are sending the file to in the *To* combo box.

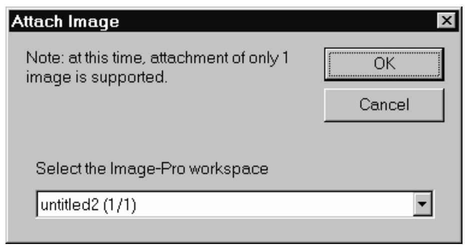
In this example, type the Media Cybernetics' Technical Support Internet address: `techsupport@mediacy.com` in the **To** field.

3. For this example, type "*Tax fraud suspect #1234ABCD*" in the *Subject* combo box.

4. **For this example, type "*Joe, Here is the suspect's fingerprint after a noise removal using Image-Pro Plus.*" in the message area.**

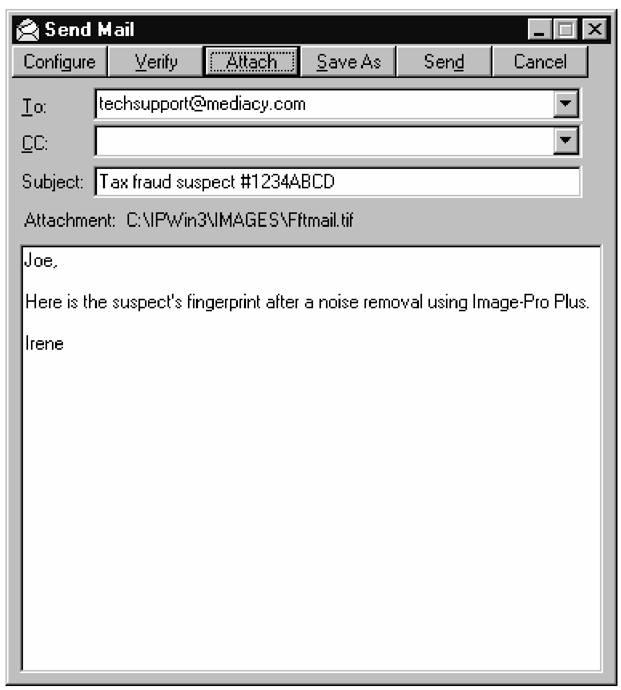
5. **Click the *Attach* button.**

   The **Attach** dialog box appears.



9. **Click on *OK*.**

   The **Attach** dialog box closes and the **Send Mail** dialog box contains the e-mail information, including the new attachment.



10. Prepare to send the e-mail message by clicking on *Send*.

For the purposes of this exercise, click ***Cancel*** and do **not** send this e-mail.

Normally, however, to send an e-mail, you would click ***OK***. *Image-Pro* would then directly connect to your e-mail software package so you could log in. After sending the file as an attachment, *Image-Pro* would immediately return back to the application window and close the **Send Mail** dialog box.

Continue to the last set of steps in this exercise: *Closing all the Windows and Exiting Image-Pro Plus.*

■ Closing all the Windows and Exiting *Image-Pro Plus*

Close all the windows by selecting the **CloseAll** button  from the *Image-Pro Plus* toolbar. You may continue working with *Image-Pro Plus* or exit *Image-Pro Plus* by selecting the *Exit* option under the *File* menu. Do not save any changes.

Continue to the next exercise: *Counting, Measuring, and Classifying.*

Counting, Measuring, and Classifying

The Tutorial in this section is designed to introduce you to *Image-Pro Plus'* automatic **Object-counting** and **Measuring** features. An **Object** is considered to be any contiguous group of pixels that share a specified range of intensity values. With the *Count/Size* command, these objects can be identified, counted, and measured. In this exercise you will:

- Perform manual gray scale thresholding of metal grains.
- Count the grains in an image and measure each grain's pore area, pore ratio, and the number of pores within each object.
- See the visual effects of filtering out (graphically excluding) objects from the count.
- Split clustered objects.
- Classify the measurement data.
- Color-code the objects by class.
- Store the measurement results to an Excel™ worksheet.
- Create a customized report using *Image-Pro Plus* and the *Image-Pro Report Generator*, as well as the template feature.

This exercise will take about 40 *minutes to complete.*

Setup: Start *Image-Pro Plus* by double-clicking on the *Image-Pro Plus* icon in your *Image-Pro Plus* folder.

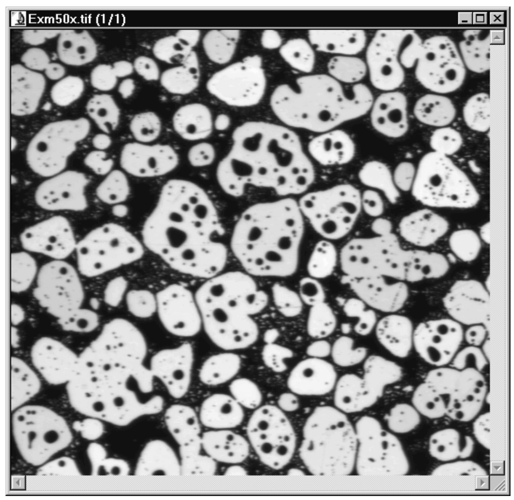
***Important**: Please check that your Image-Pro settings for this exercise **match** those in the dialog boxes shown in the tutorial. Image-Pro 7.0 retains the settings from previous experiments, which may not be the same as the ones used in this exercise.*

■ Perform Gray Scale Thresholding

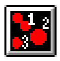
In this exercise you will use an image of a metal sample (captured from a microscope at 50x magnification) to analyze the grains. First, you'll use the manual thresholding feature to segment the image into objects and background. *Image-Pro* can detect objects automatically as well; however, this exercise will demonstrate the manual thresholding feature.

1. Load the *Exm50x.tif* file from the *Images/Objects within Objects* folder.

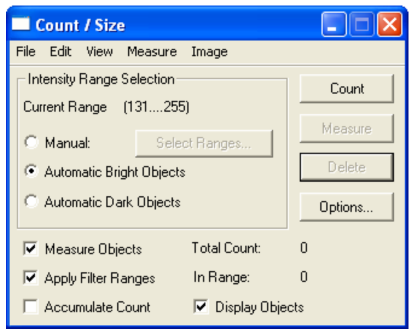
The **`Exm50x.tif`** image window is opened.



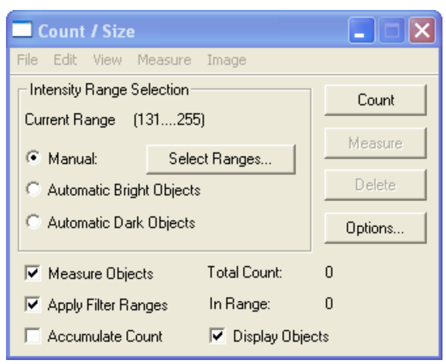
2. Select the *Count/Size* command from the *Measure* menu.



The **Count/Size** dialog box appears.



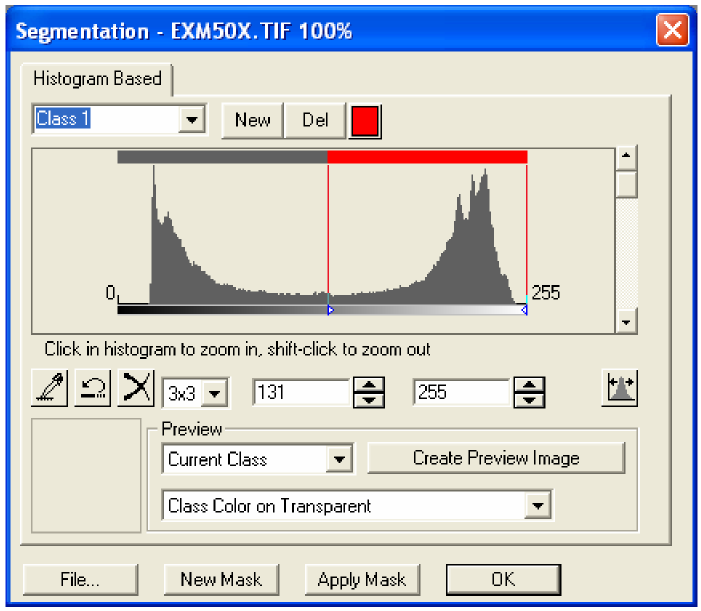
3. Click the *Manual* radio button in the *Intensity Range Selection* group box.



The **Select Ranges…** button is now accessible.

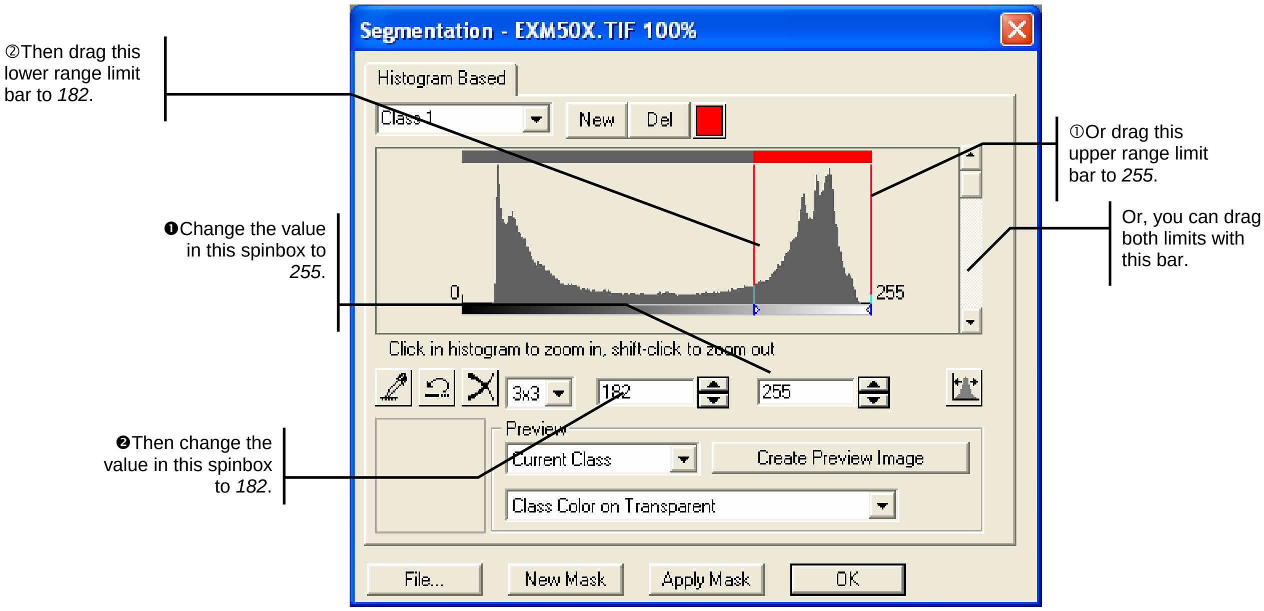
4. Click the *Select Ranges…* button.

The **Segmentation - exm50x.tif** dialog box appears.



5. Change the threshholds range from *182* to *255* to select the bright objects (holes) in the image.

First change the *upper* threshhold from 130 to 255 and then change the lower threshhold to 182. You can use the spin boxes (❶ and ❷) to change the values (type the values or use the spin arrows). - Or you can drag the range limit bars (① and ②) to the appropriate values, which are reflected in the spin boxes (see the diagram following).



As you change the thresholding values, you will notice an overlay on the image. This overlay defines the area you are thresholding, but does not effect or change your image data.

6. Click OK.

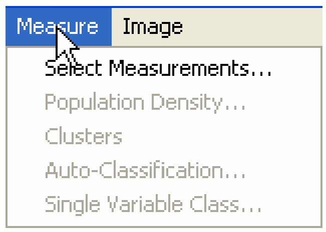
Image-Pro selects the intensity range of the objects to be counted and measured, and the **Segmentation - exm50x.tif** dialog box closes.

Continue to the next set of steps in this exercise: *Select Measurements.*

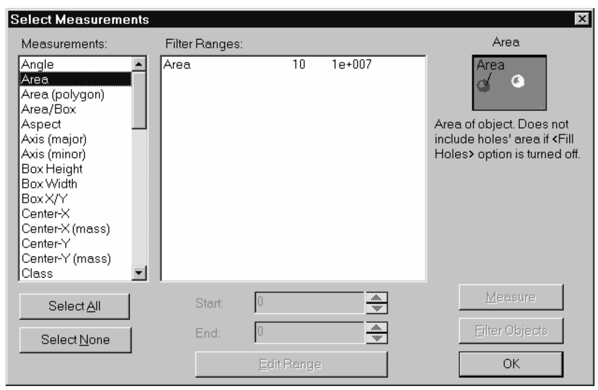
■ Select Measurements

In this exercise, you will choose which types of measurement data for *Image-Pro Plus* to record when the objects are counted.

1. From the *Measure* menu in the *Count/Size* dialog box, select the *Select Measurements* command.



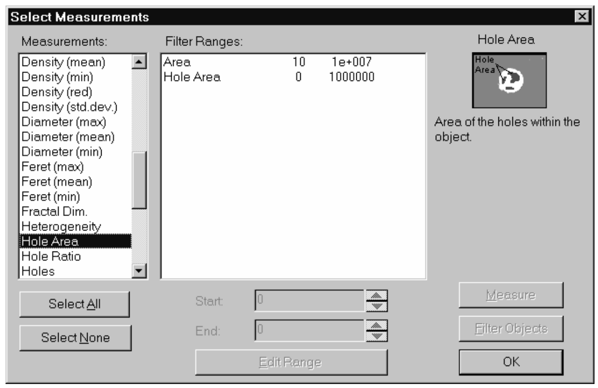
The **Select Measurement** dialog box appears. The **Area** measurement is the default.



In the following steps, add the measurement data relevant for the pores (**Hole Area**, **Hole Ratio**, **Holes,** and **Class**).

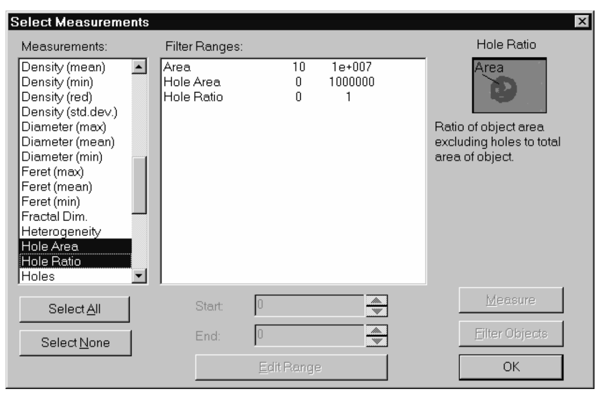
2. Add the *Hole Area* measurement to be included in the measurement statistics.

Scroll down the **Measurements** list box on the left and then click on **Hole Area**. **Hole Area** calculates the area of the holes within the object. The new measurement is included in the **Filter Ranges** list box on the right.



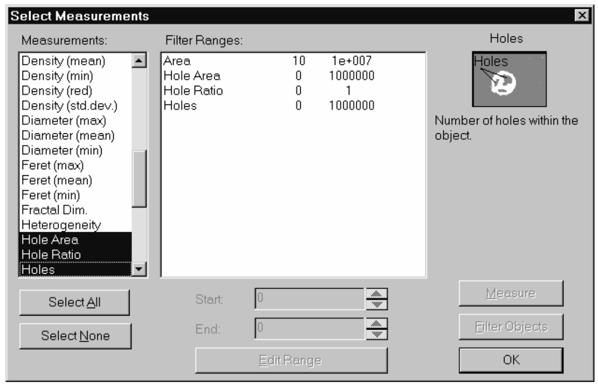
3. Add the *Hole Ratio* measurement.

Repeat the same procedure from step #2. **Hole Ratio** is the ratio of the object area (excluding holes) to the total area of the object.



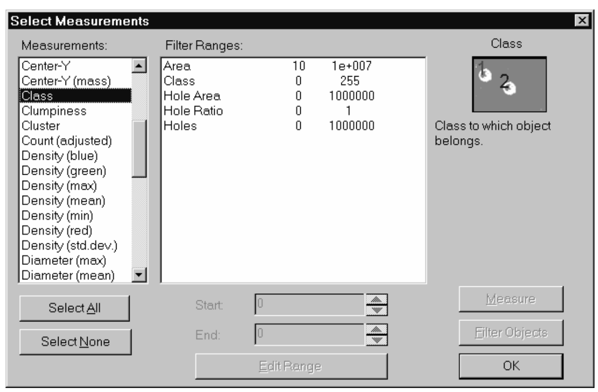
4. Add the *Holes* measurement.

Repeat the same procedure from step #2. **Holes** calculates the number of holes within an object.



5. Add the *Class* measurement.

Repeat the same procedure from step #2. **Class** groups counted objects by their class.



6. Click *OK*.

The **Select Measurement** dialog box closes.

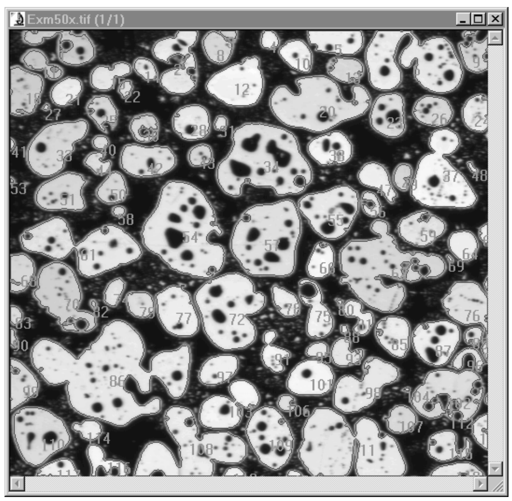
Continue to the next set of steps in this exercise: *Counting the Objects.*

■ Counting the Objects

In this exercise, you will count the grains and then modify the options relating to the way counted objects are identified and outlined.

1. Click the *Count* button in the *Count/Size* command window.

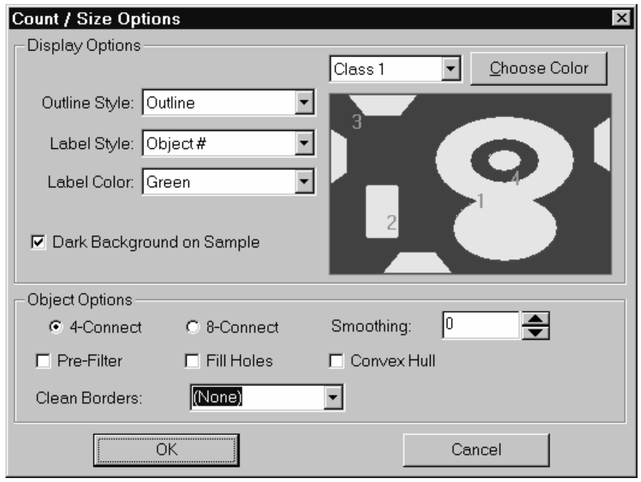
The image is analyzed, grains identified, counted, and measured. Object outlines and labels appear in the **`Exm50x.tif`** window.



Note that the pores or holes within the metal grains are not outlined in this image after the initial count.

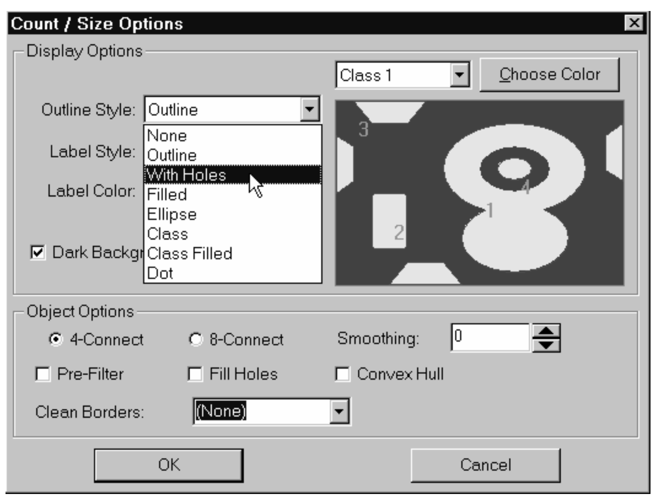
2. Click the *Options…* button.

The **Count/Size Options** dialog box appears.



3. Select the *With Holes* option from the *Outline Style* drop down list.

This option tells *Image-Pro Plus* to highlight counted objects around their perimeter **and** around any holes that are within the object. (Holes are defined as pixels within an object that are not of the specified intensity).

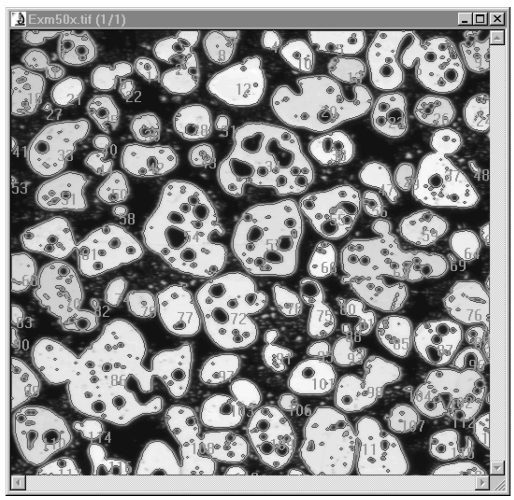


4. Make sure that the *Fill Holes* box is *unchecked*.

At this point, doublecheck to make sure that your settings for *Image-Pro* match the ones in the previous dialog box to continue with this exercise. (*Image-Pro* retains the settings from previous experiments, which may not be the same as the ones needed for this exercise.)

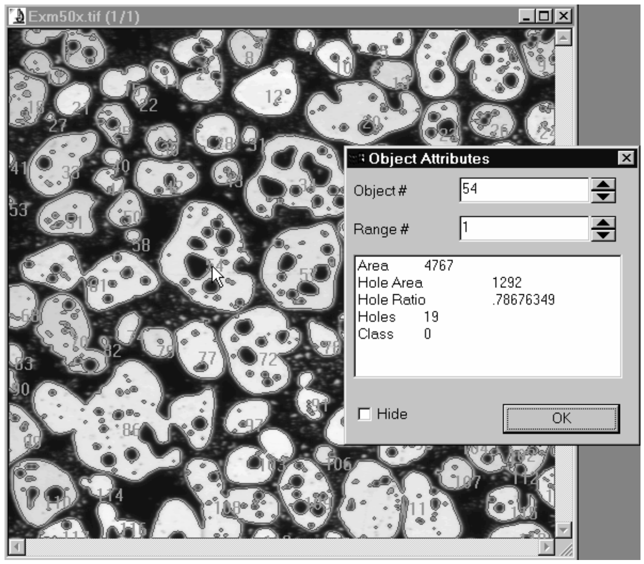
5. Click *OK*.

The pores are now highlighted.



6 Double-click on any object.

The **Object Attributes** window appears.



Measurements for a single object can be viewed by simply double-clicking on that object. The **Object Attributes** window presents all recorded measurements for the selected object.

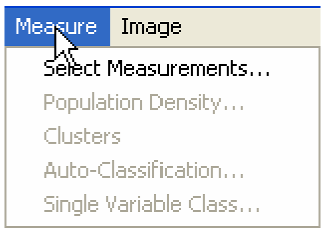
6. Click on *OK* to close the *Object Attributes* window.

Continue to the next set of steps in this exercise: *Graphical Exclusion of Ranges.*

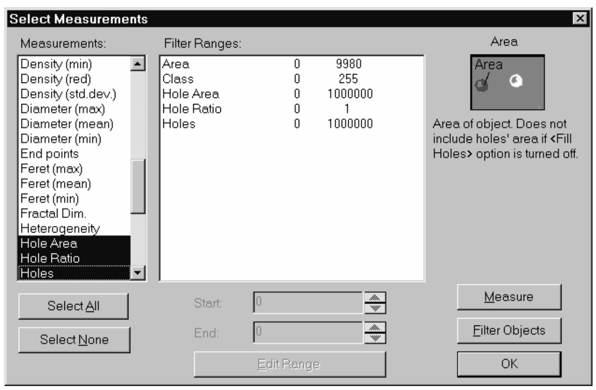
■ Graphical Exclusion of Ranges

In this exercise, we will demonstrate a method (graphical exclusion of ranges) for visualizing the effect of your filter ranges.

1. From the *Measure* menu in the *Count/Size* dialog box, select the *Select Measurements* command.



The **Select Measurement** dialog box appears.

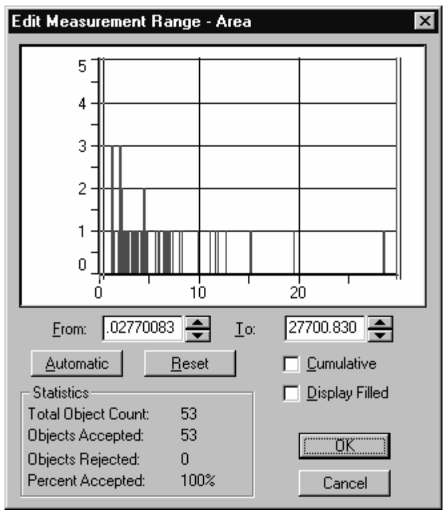


2. **Highlight the *Area* filter range.**

The **Edit Range** button is now undimmed.

3. **Press the *Edit Range* button.**

The **Edit Measurement Range - Area** graph appears. This is a graphical representation of the **Area** range shown in the **Select Measurements** dialog box in Step #1.

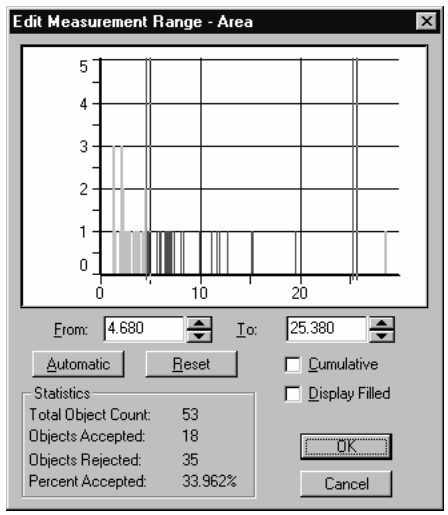


The x-axis represents the Area values for the individual objects, and the y-axis the number of objects with that area.

The **From** and **To** fields in this **Edit Measurement Range** dialog box correspond with the **Start** and **End** fields in the **Select Measurements** dialog box.

4. Drag the *left*-hand vertical marker to the right to view the outlines of the smaller areas disappearing in your image.

The smaller area outlines that are disappearing are the objects that you are filtering out as you drag the vertical marker (i.e., what you are graphically excluding as you increase the area values on the x-axis).



In this way, you can visually see the numerical values you are setting for the **Filter Ranges** in the **Select Measurements** dialog box.

5. To see the outlines of the larger areas disappear, drag the *right*-hand vertical marker to the left.

The larger area outlines that are disappearing are the objects that you are filtering out as you drag the vertical marker (i.e., what you are graphically excluding as you decrease the area values on the x-axis).

6. Click *Close* to return back to the *Select Measurements* dialog box.

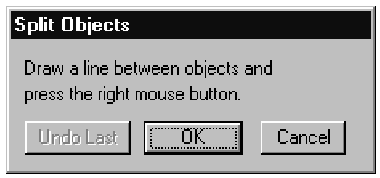
Continue to the next set of steps in this exercise: *Splitting Objects.*

■ Splitting Objects

If you examine the image, you will see a problem with the results of the count: Grain #61 (located on the left side of the image) is not a single object, but actually two individual objects counted as one. In the next few steps, you will correct this problem.

1. From the *Edit* menu in the *Count/Size* dialog box, select the *Split Objects* option.

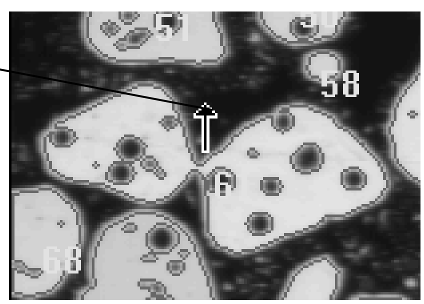
The **Split Objects** message box appears.



2. Place the outline cursor *above* object #*61,* as shown in the following illustration. Then click the *left* mouse button to begin drawing the line.

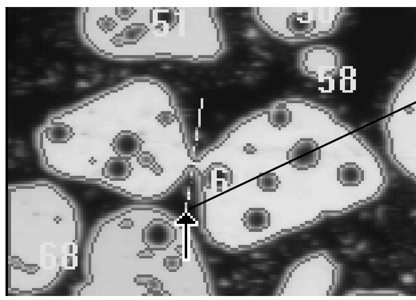
Be sure that the cursor is not touching the object when you click the mouse — the first point of the dividing line must lie outside of the object being split.

Place the cursor here.



3. **Move the cursor *below* the object, as shown below, and then click the *left* mouse button again to end the line.**

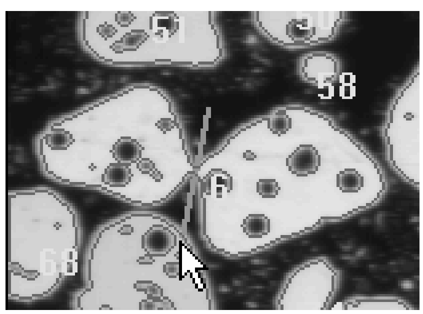
   Be sure that the cursor is well below the object when you click – the endpoint of the dividing line must be outside of the object being split.



Place the cursor here, and press the **left** mouse button again.

4. **Once the line is completed, click the *right* mouse button.**

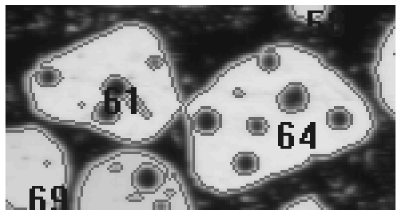
   A dividing line is drawn between the two objects.



Note: *Your cursor must be within the* **`Exm50x.tif`** *image window when you press the right mouse button; otherwise this action will have no effect.*

5. Click *OK* in the *Split* dialog box.

Image-Pro Plus reanalyzes the image, and splits object #61 into two new objects, now numbered **#61** and **#64**.



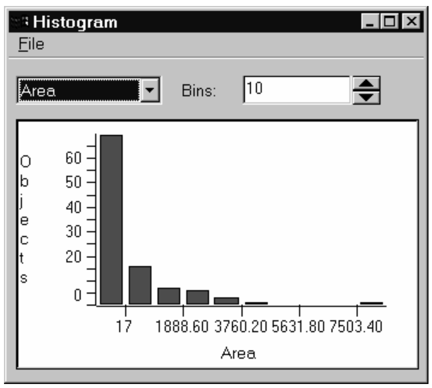
Continue to the next set of steps in this exercise: *Obtaining Measurement Distributions.*

■ Obtaining Measurement Distributions

Image-Pro Plus lets you obtain a size distribution curve for the measured objects. In this exercise, you will obtain a graphical representation of the area and hole ratio distribution..

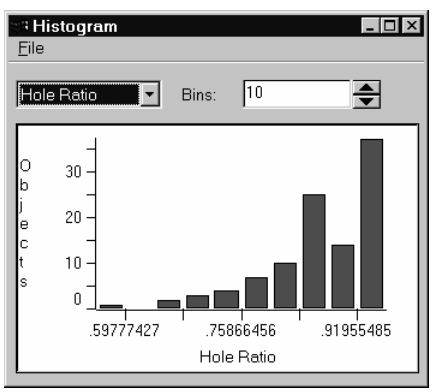
1. Go back to the *Count/Size* dialog box. From the *View* menu, select the *Histogram* command.

The **Histogram** dialog box appears, with **Area** as the default.



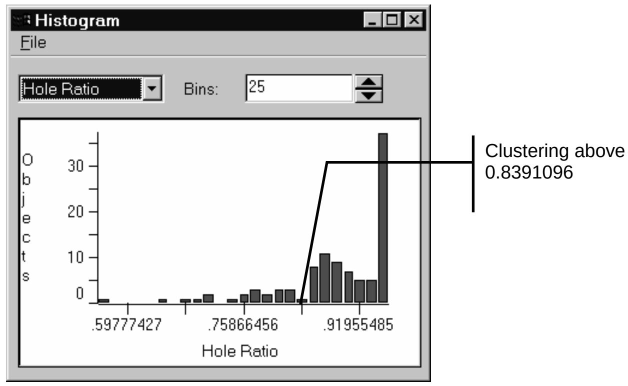
2. Change the *Area* option to *Hole Ratio* in the list box.

This displays the range of values the grains have for **Hole Ratio**.



3. Set the *Number of Bins* to *25*.

The default value of 10 is quite coarse. Raising the number of histogram bins will give a more detailed view of the data.



Look at the data for clustering. Note that there appears to be a cluster of objects with a hole ratio of 0.91955485 and above, a small gap in the data at 0.8391096, and a scattering of objects with lower hole ratios.

For the purpose of this exercise, you will divide the objects at **0.875**, based on the clustering observed in the histogram.

4. Close the *Histogram* dialog box.

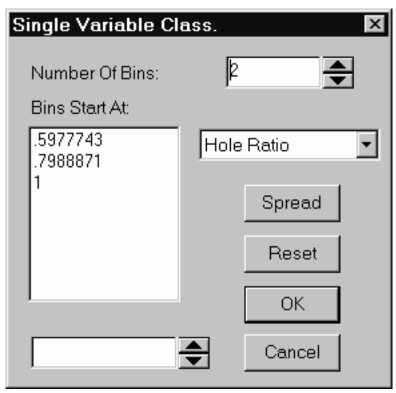
Continue to the next set of steps in this exercise: *Classifying the Measurement Data.*

■ Classifying the Measurement Data

Image-Pro Plus lets you take the measurement data you have collected about objects, and categorize them into ranges. In this exercise you will categorize the grains by the pore (hole) ratio. You will use *Image-Pro Plus'* single variable classification tool to define the classes.

1. Go back to the *Count/Size* dialog box. From the *Measure* menu, select the *Single Variable Class* command.

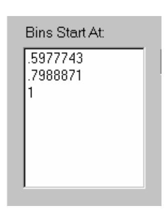
The **Classification** dialog box appears.



Make sure that **Hole Ratio** is displayed in the list box. This tells *Image-Pro Plus* that you want the objects classified by their **Hole Ratio** measurements.

2. Make sure the *Number of Bins* field is equal to *2*.

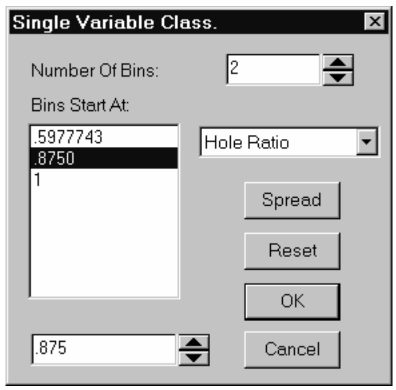
This instructs *Image-Pro Plus* to divide the hole ratio range (from the minimum value to the maximum value) into 2 intervals.



The beginning value of each interval is indicated by the first 2 values in the **Bins Start At** box. The 3rd value in the box reflects the maximum value in the range.

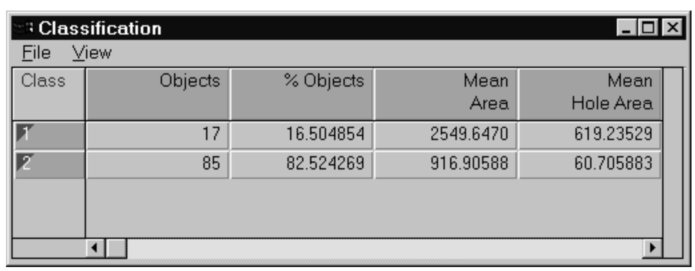
3. Change the second bin interval value to *.875*.

This is the value obtained from the previous histogram exercise.



4. Click *OK*.

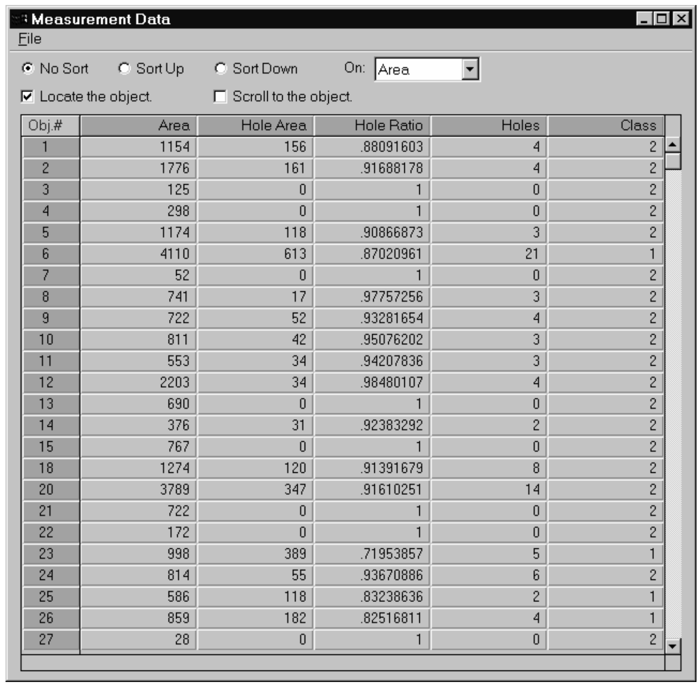
The **Classification** data window appears. This window lists the number of objects in each class, the percentage of objects in each class, the range values, and hole ratio measurements for each class.



Class	Objects	% Objects	Mean Area	Mean Hole Area
1	17	16.504854	2549.6470	619.23529
2	85	82.524269	916.90588	60.705883

Note: *You may need to enlarge the* ***Classification*** *window to view all of the measurement data.*

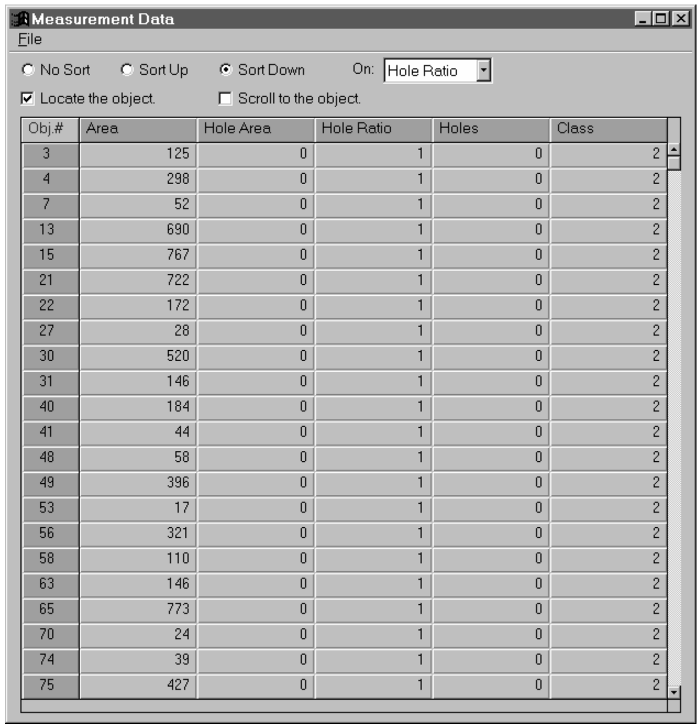
5. **From the *View* menu in the *Count/Size* dialog box, select the *Measurement Data* command.**



Obj.#	Area	Hole Area	Hole Ratio	Holes	Class
1	1154	156	.88091603	4	2
2	1776	161	.91688178	4	2
3	125	0	1	0	2
4	298	0	1	0	2
5	1174	118	.90866873	3	2
6	4110	613	.87020961	21	1
7	52	0	1	0	2
8	741	17	.97757256	3	2
9	722	52	.93281654	4	2
10	811	42	.95076202	3	2
11	553	34	.94207836	3	2
12	2203	34	.98480107	4	2
13	690	0	1	0	2
14	376	31	.92383292	2	2
15	767	0	1	0	2
18	1274	120	.91391679	8	2
20	3789	347	.91610251	14	2
21	722	0	1	0	2
22	172	0	1	0	2
23	998	389	.71953857	5	1
24	814	55	.93670886	6	2
25	586	118	.83238636	2	1
26	859	182	.82516811	4	1
27	28	0	1	0	2

Note: *You may need to enlarge the **Measurement Data** window to view all of the measurement data.*

6. Click on the *Sort Down* radio button to sort the list in descending order and change the drop list from *Area* to sort on *Hole Ratio*.



Obj.#	Area	Hole Area	Hole Ratio	Holes	Class
3	125	0	1	0	2
4	298	0	1	0	2
7	52	0	1	0	2
13	690	0	1	0	2
15	767	0	1	0	2
21	722	0	1	0	2
22	172	0	1	0	2
27	28	0	1	0	2
30	520	0	1	0	2
31	146	0	1	0	2
40	184	0	1	0	2
41	44	0	1	0	2
48	58	0	1	0	2
49	396	0	1	0	2
53	17	0	1	0	2
56	321	0	1	0	2
58	110	0	1	0	2
63	146	0	1	0	2
65	773	0	1	0	2
70	24	0	1	0	2
74	39	0	1	0	2
75	427	0	1	0	2

Image-Pro reorders the format of the *Measurement Data* table to correspond to the sort button.

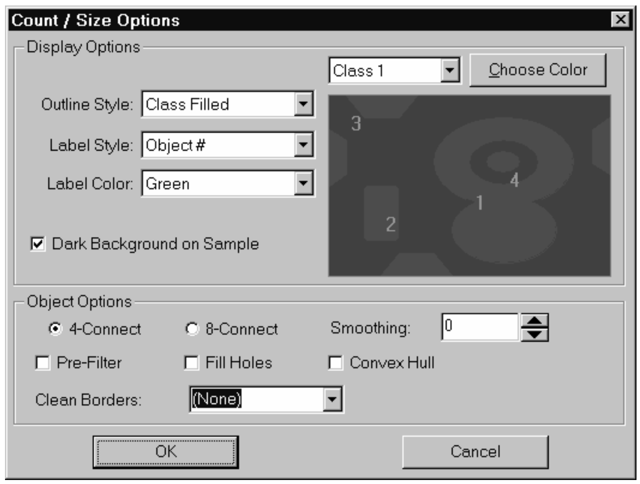
Continue to the next set of steps in this exercise: *Color-coding the Objects.*

■ Color-coding the Objects

In this exercise you will color-code the grains by their hole ratio class. Color-coding is accomplished using the *Class Filled* option in the **Count/Size Options** dialog box.

1. Go back to the *Count/Size* dialog box and click on the *Options* button.

The **Count/Size Options** dialog box appears.

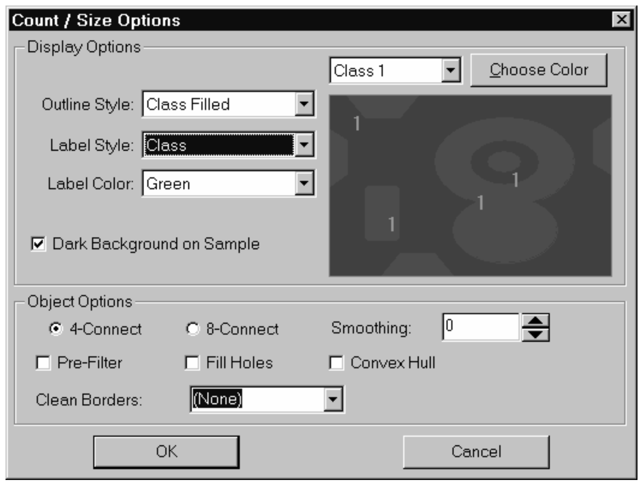


2. Make sure the *Class Filled* option is selected in the *Outline Style* drop-down list box.

This directs *Image-Pro Plus* to fill the objects with color, according to their class. In this exercise, *Image-Pro Plus* uses two colors, each representing one of the classifications you defined in the previous step.

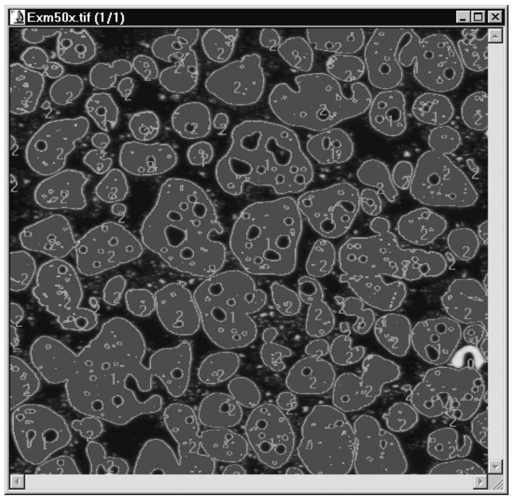
3. Select the *Class* option in the *Label Style* list box.

This directs *Image-Pro Plus* to label the objects according to the class to which they belong, e.g., objects falling into class 1, will be marked with a 1, those falling into class 2 will be marked with a 2, and so forth.



4. **Click *OK*.**

The grains appear color-coded and labeled according to their class.



*Note: Move the **Measurement Data** window if it is obstructing your view of the image window*

Continue to the next set of steps in this exercise: *Saving the Measurement Data.*

■ Saving the Measurement Data

In this exercise you will store the measurement results to a file.

Important: *Be sure the* **Measurement Data** *dialog box is active before beginning the steps below.*

1. **From the *File* menu in the *Measurement Data* dialog box, select the *Export Data* command.**

   Your data will be exported to an Excel spreadsheet automatically.

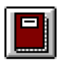
2. **Close the *Measurement Data* window.**

Continue to the next set of steps in this exercise: *Creating a Report.*

■ Creating a Report

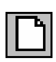
In this exercise, you will use the *Report Generator* to create a customized report based on the current *Image-Pro Plus* session: the **`Exm50x.tif`** image and the existing **Classification** table.

1. From *Image-Pro's Measure* menu, select the *Report* command.

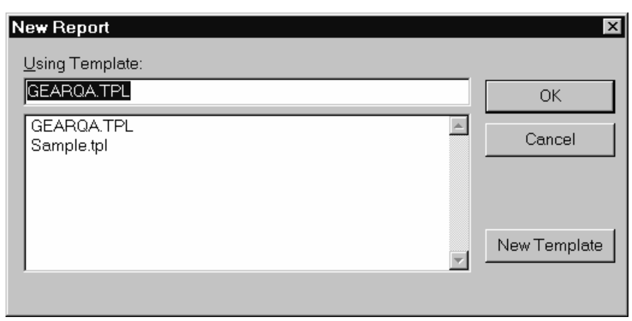


The **Report Generator** window appears.

2. From the *File* menu, select the *New* command.

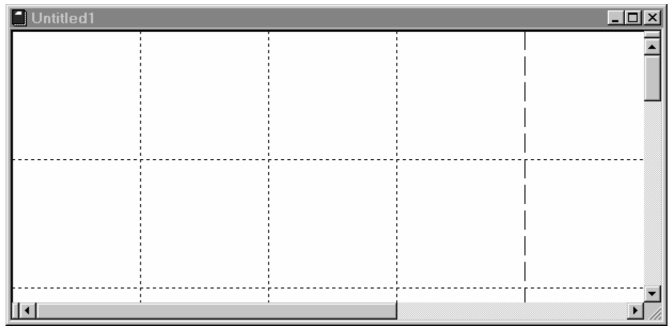


The **New Report** dialog box appears.



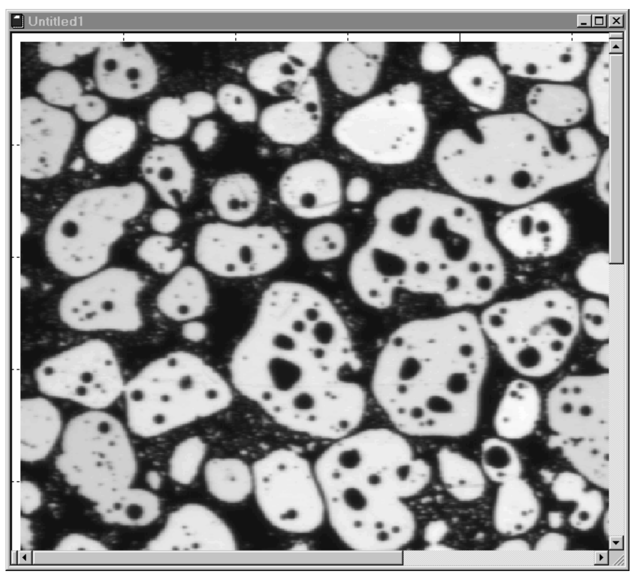
3. **Click on the *New Template* button.**

   The blank **Untitled1** window appears.

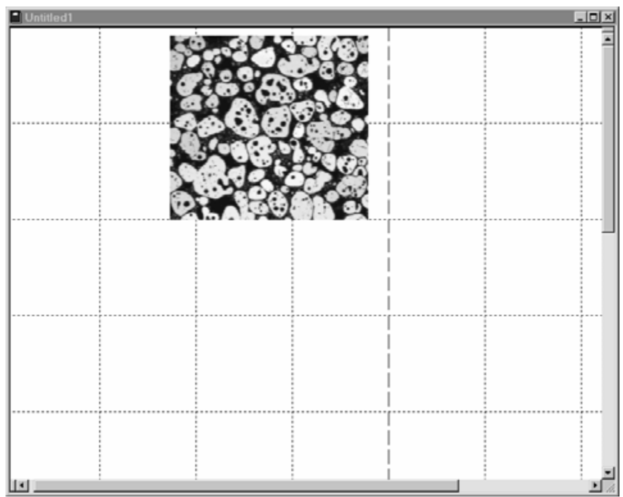
4. **From the *Insert* menu, select the *Image* command.**

   The original **`Exm50x.tif`** image appears.

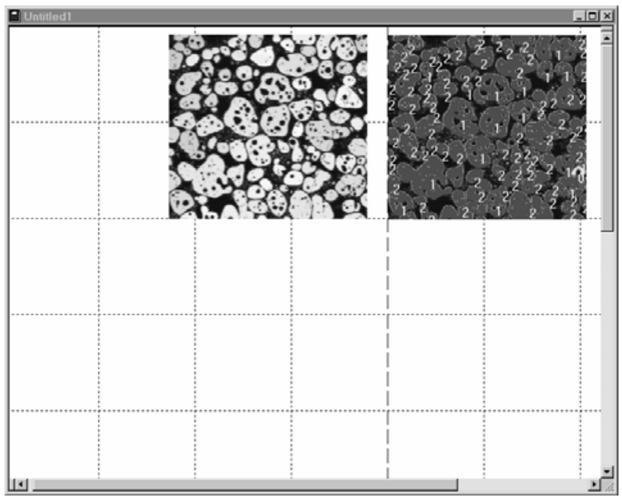
5. Reduce and then reposition the image to fit on the *New Template* window, as shown below.

Shrink the image by clicking and dragging the corner handles of the image window. Move the image to the new location in the top, middle portion of the **New Template** window by placing the cursor over the image and holding the left mouse button down.



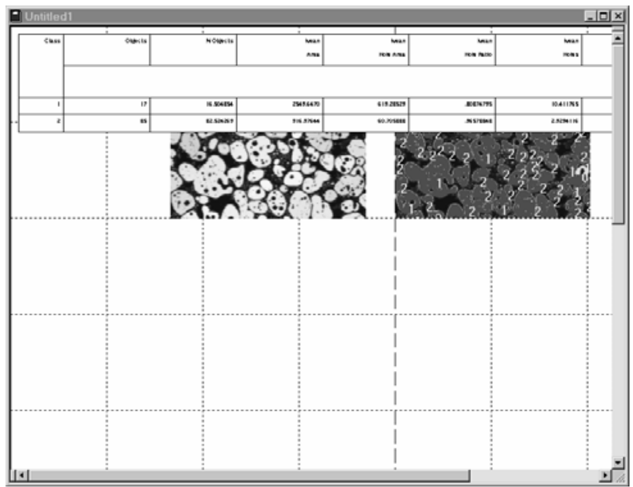
6. **From the *Insert* menu, select the *Image w/Overlay* command. Resize and reposition that image as well to match below.**

   The current **`Exm50x.tif`** image appears. Follow the instructions in Step #5 to resize and reposition this image, so that it is the same size as the first image and appears next to it (for comparison purposes), as shown below.



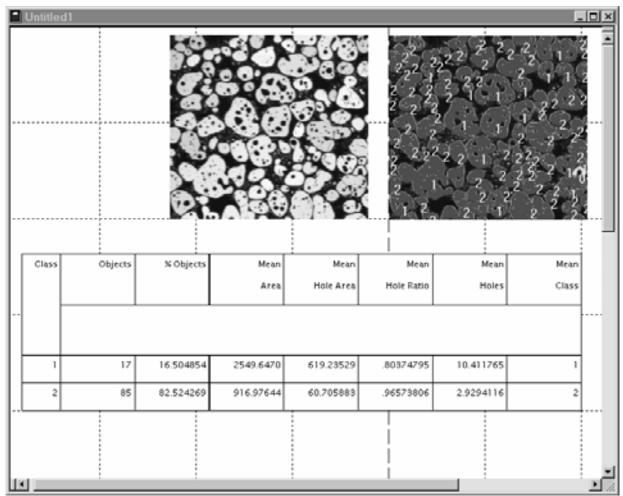
7. **From the same *Insert* menu, select the *Count/Size* sub-menu, and then select the *Classification Table* option.**

The **Classification** table appears overlaid on the image.



8. Move the *Classification* table to the bottom of the image.

Drag the **Classification** table by placing the cursor over the image and holding the left mouse button down. Enlarge it a bit so that it's easier to read.



Continue to the next set of steps in this exercise: *Using Templates.*

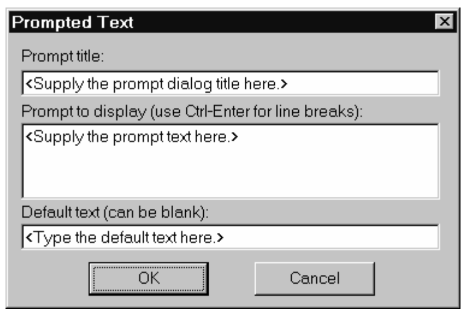
■ Using Templates

In this exercise, you will use the template feature in *Report Generator*. The template feature is useful if you are creating reports with the same type of data being entered for each report.

For other examples of using templates to generate standardized reports in *Image-Pro Plus*, open and run **`Gear.tmpl`** in the *Report Generator*.

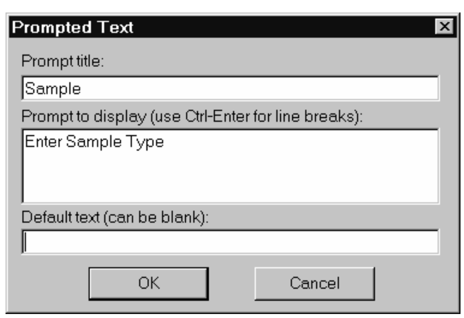
1. From the *Insert* menu, select the *Insert* sub-menu, and then select the *Prompted Text* option.

The **Prompted Text** dialog box appears.



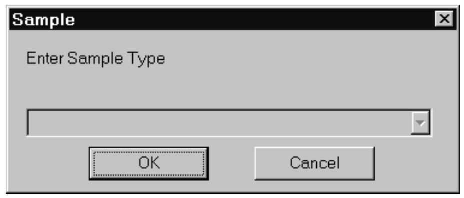
2. Enter "Sample" for the *Prompted* title, and then double-click to enter "Enter Sample Type" for the *Prompt to display* line.

"`Sample`" will be the name of the dialog box, with "`Enter Sample Type`" as the text for this template. Make sure that the **Default Text** line is blank (delete the existing test).

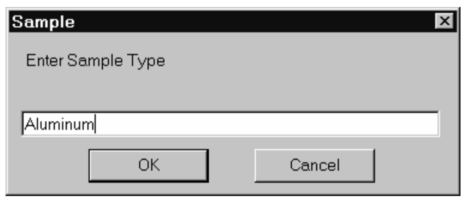


3. Click *OK*.

The newly created **Sample** dialog box with the text you typed above appears.

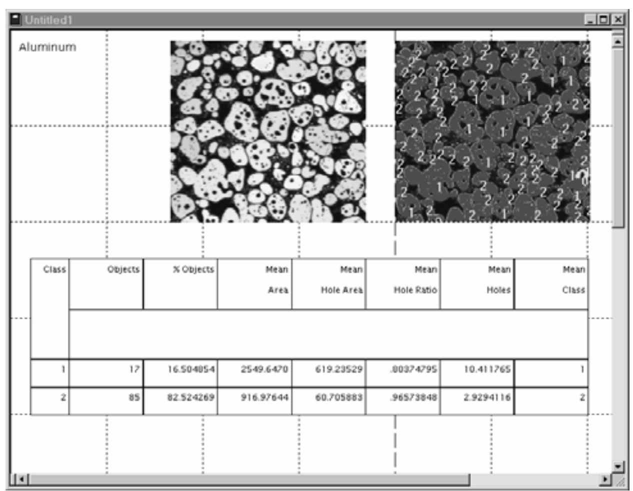


4. Enter *Aluminum* as the *Sample Type*.



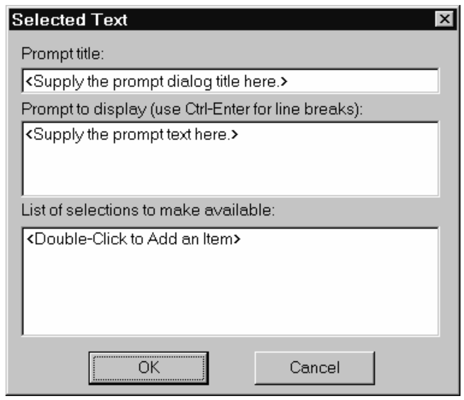
5. Click *OK*.

The information you were prompted for appears on the report.

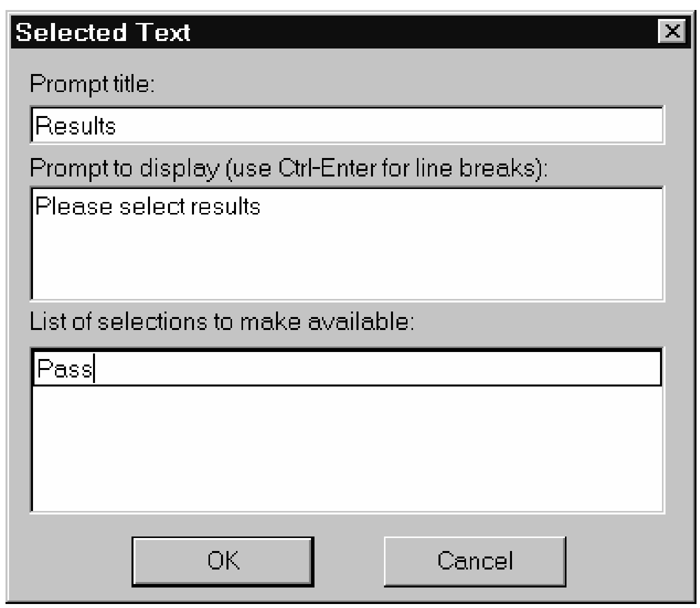


6. **Next, select the *Insert* sub-menu, and then select the *Selection Text* option.**

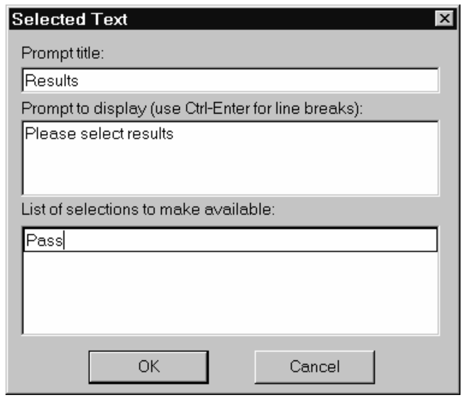
   The **Selected Text** dialog box appears.



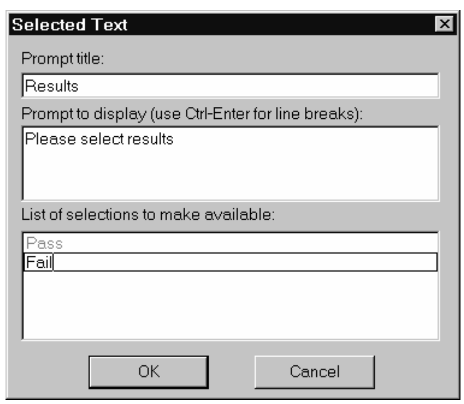
7. **Enter "Results" for the *Prompted* title and double-click to enter "Please select results" for the *Prompt to display* line.**



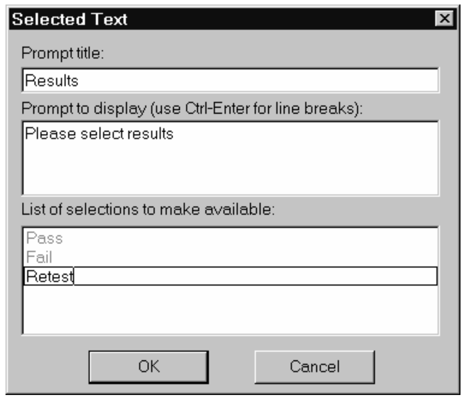
8. Double-click to enter "Pass" for the first entry of the *List of selections to make available*.



8. Double-click to enter "Fail" for the second entry of the *List of selections to make available*.

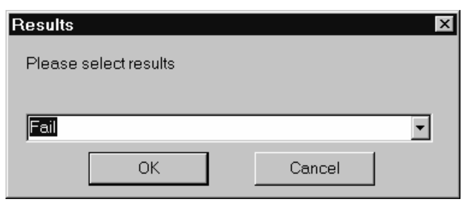


9. **Double-click to enter "Retest" for the third and final entry of the *List of selections to make available*.**



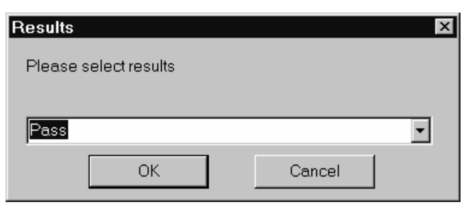
10. **Click *OK*.**

The newly created **Results** dialog box with the **Fail** option appearing in the drop down list box. *Image-Pro* lists the options you entered in alphabetical order.



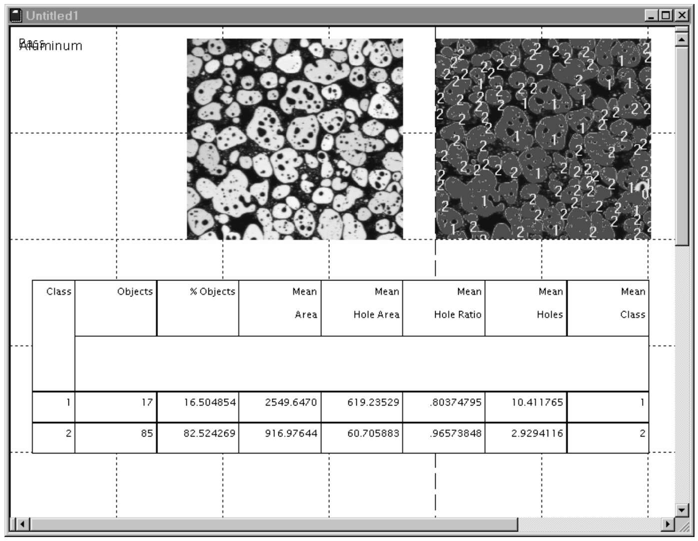
11. **Select *Pass* from the drop-down list.**

Click on the drop-down list to see the other choices (**Pass** and **Retype**), and select **Pass**.



12. Click *OK*.

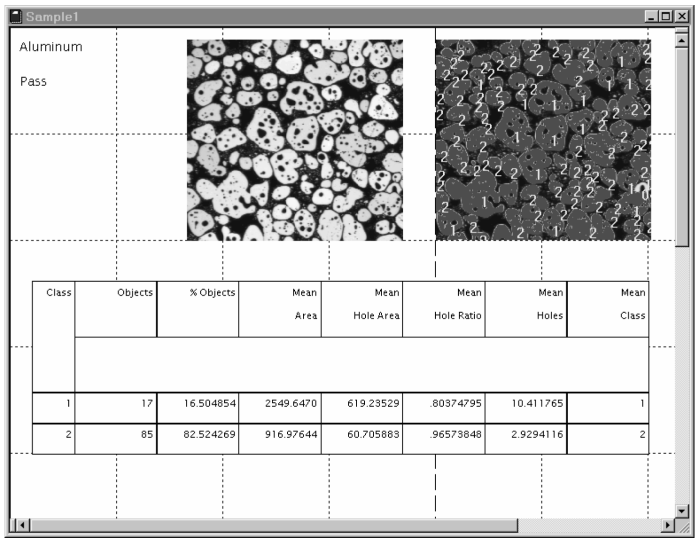
The newly created **Sample** dialog box with the text you typed above appears.



Unfortunately, the **Pass** text box overlaps the **Aluminum** text box.

13. Reposition the *Pass* text box.

Click and drag the **Pass** text box and move it below the text box created earlier, so that you can read both.

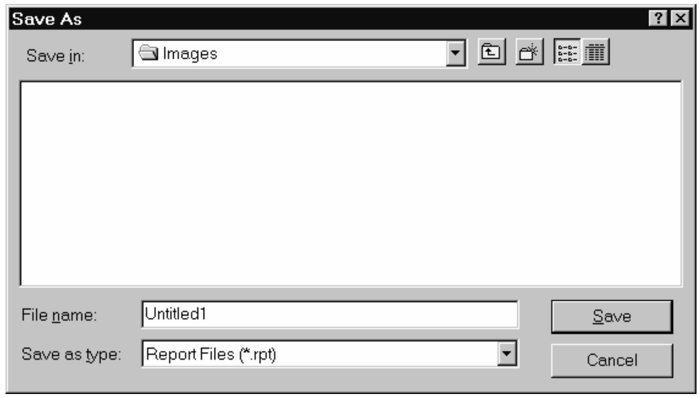


Note: For other examples of using ***templates*** *to generate standardized reports in Image-Pro Plus, open and run* **`GearQA.tpl`** *in the Report Generator.*

We will now save this report.

14. From the *File* menu in the *Report Generator* window, select the *Save* command.

The **Save As** dialog box appears.



15. Type: `Sample1` in the *File name* text box.

16. Click *Save*.

Image-Pro Plus saves your customized report as **`Sample1.rpt`** in the *Images* folder.

Note: *For other examples of using **templates** to generate standardized reports in Image-Pro Plus, open and run* **`Gear.tmpl`** *in the Report Generator.*

Continue to the last set of steps in this exercise: *Closing All the Windows.*

■ Closing All the Windows and Exiting *Image-Pro Plus*

Close all the windows by selecting the **CloseAll** button  from the *Image-Pro Plus* toolbar. You may continue working with *Image-Pro Plus* or exit *Image-Pro Plus* by selecting the *Exit* option under the *File* menu. Do not save any changes.

Continue to the next exercise**:** *Color Segmentation.*

Color Segmentation

The Tutorial in this section introduces you to the color segmentation options within *Image-Pro Plus*. Color segmentation separates objects or features from the background, based upon their color characteristics. In this exercise you will:

- Load an image of a cross-section of a tissue sample off a microscope.
- Enhance the image.
- Perform a color cube color segmentation.
- Count the nuclei contained within an AOI.
- Measure the percent area and view the corresponding statistical data.

This exercise will take about 25 minutes to complete.

Setup: If you have not yet started *Image-Pro Plus*, do so now by double-clicking on the *Image-Pro Plus* icon within the *Image-Pro Plus* folder. Once *Image-Pro Plus* is running, you may begin following the steps below.

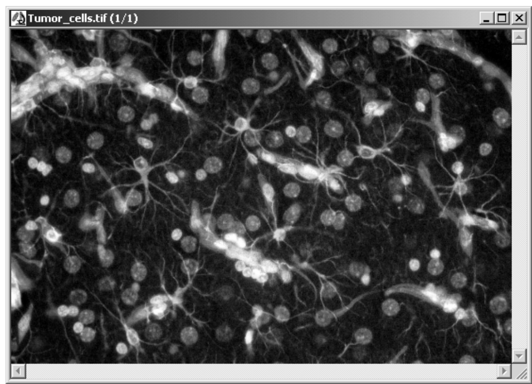
Important*: Please check that your Image-Pro settings for this exercise* ***match*** *those in the dialog boxes shown in the tutorial. Image-Pro 7.0 retains the settings from previous experiments, which may not be the same as the ones used in this exercise.*

Contrast Enhancement

In this exercise, you will use contrast stretching to enhance an image of a cross-section of a tissue sample from a microscopic slide.

1. **Load the *Tumor_cells.tif* file from the *Images/Color Extraction* folder.** (You will need to install the additional images on your Images CD to use this file.)

The **Tumor_cells.tif** image window appears.



2. **From the *Enhance* menu, select the *Equalize* sub-menu, and then select the *Best Fit* command.**

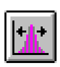
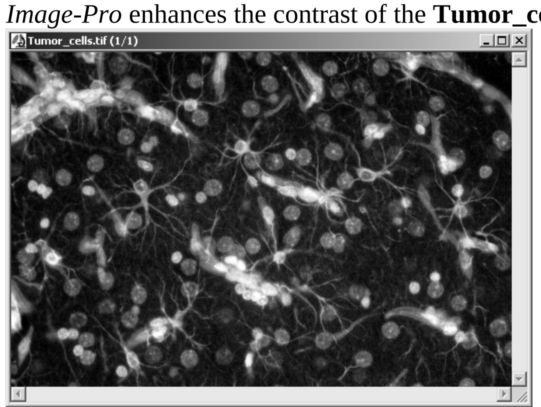


Image-Pro enhances the contrast of the **Tumor_cells.tif**.

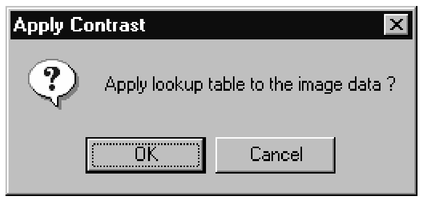


This operation maximizes the image contrast, taking the darkest portion of the image to black and the lightest portion of the image to white.

3. Apply the contrast changes to the image.

The contrast enhancement performed in the preceding step was recorded into a Lookup Table (LUT), through which the original image is interpreted. To permanently modify the image, the LUT must be applied to the image and then saved.

Select the *Apply Contrast* command from the *Enhance* menu. The *Apply Contrast* dialog box appears.



4. Click on *OK*.

Clicking **OK** uses the contrast enhancement to permanently modify your image bitmap.

Continue to the next set of steps in this exercise: *Counting the Nuclei Using Color Segmentation.*

■ Performing Color Segmentation

In this step you will count the nuclei by using the color cube method of separation.

1. Select the *Count/Size* option from the *Measure* menu.

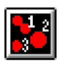
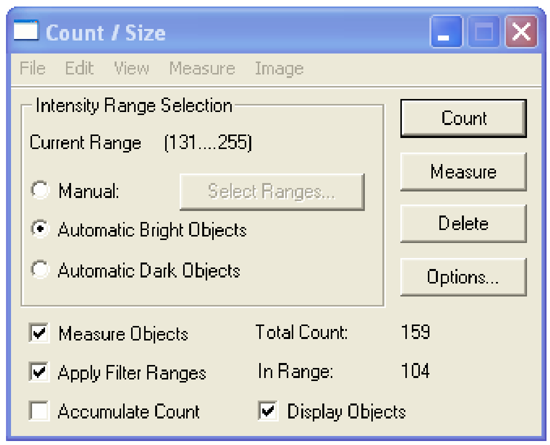


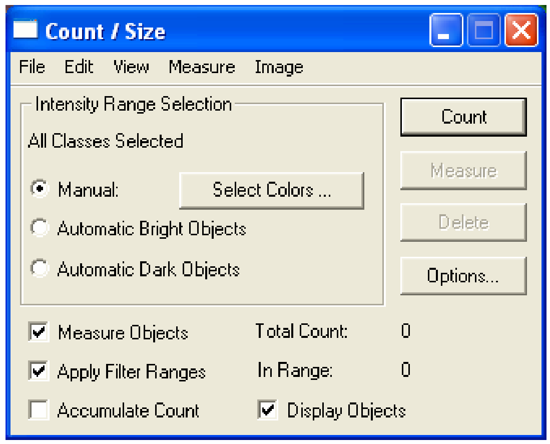
Image-Pro Plus opens the **Count/Size** dialog box.



Doublecheck to make sure that your settings for Image-Pro match the ones in this dialog box; e.g., **Accumulate Count** should be unchecked. (*Image-Pro* retains the settings from previous experiments, which may not be the same as the ones needed for this exercise.)

2. Click the *Manual* radio button.

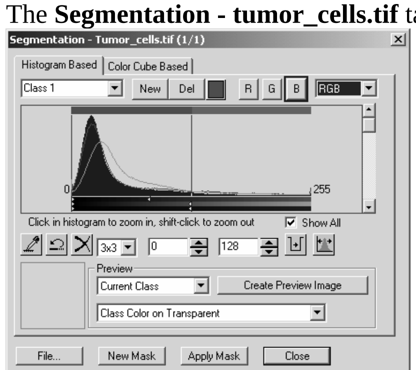
For this color segmentation, you will be defining the color range manually. The *Select Colors…* button is now accessible.



3. Click on the *Select Colors…* button.

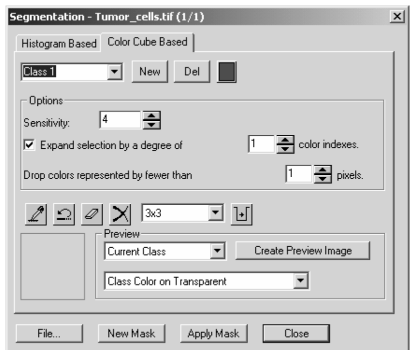
Now, specify the range of colors that define the objects in the active object.

The **Segmentation - tumor_cells.tif** tagged dialog box appears.



4. Click on the *Color Cube Based* tab.

For this exercise, you will use the color cube model to separate the objects that are to be counted and measured.



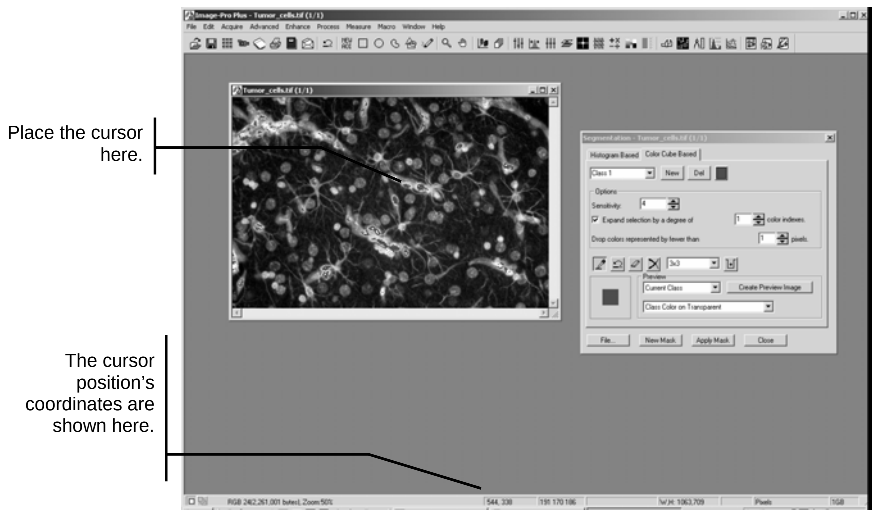
***Note:** The Histogram model provides an alternative color segmentation method; the best method to use will vary with the data.*

5. Click on the *Eyedropper* button in the *Select Image* group box.

Move the cursor on to the area in the open image where you want to perform segmentation. You will see the selected area in the box under the eyedropper.

6. Move the cursor to pixel location 544,338 on the image and then click the left mouse button.

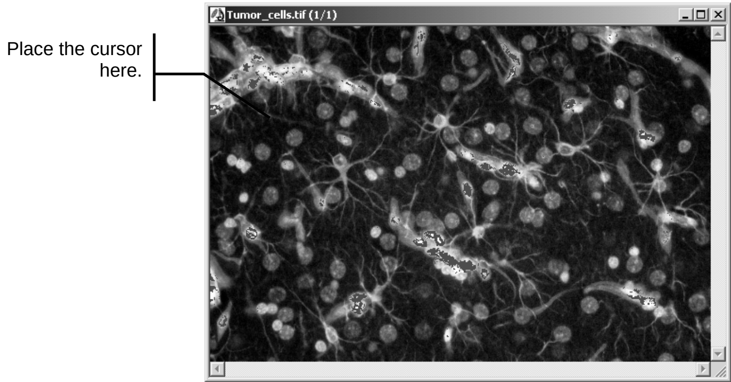
Here, you are selecting a specific color (corresponding to a 3x3 are around a pixel location) to segment. The exact location of the cursor is displayed on the bottom of the window in the status bar.



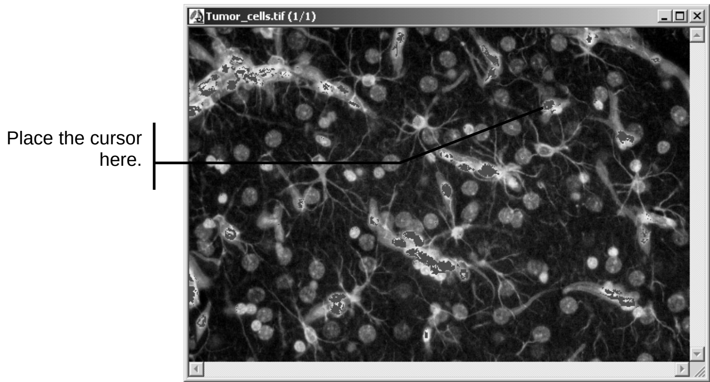
As you highlight areas to segment, *Image-Pro Plus* separates the selected color by overlaying it in red on your image. You are now adding additional colors to the range of segmented colors.

The eyedropper tool  adds colors, while the eraser tool  subtracts them. Use these tools to define the desired colors for segmentation in the next couple of steps.

7. Repeat step #6 for pixel location 90, 118.



8. Repeat step #6 for pixel location 722,165.



9. Click *Close*.

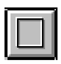
The **Segmentation-tumor_cells.tif** tagged dialog box closes. At this point, you have defined a color range for segmentation. In the next section, you will apply that range to separate or measure the nuclei.

Continue to the next set of steps in this exercise: *Counting the Nuclei.*

■ Counting the Spots

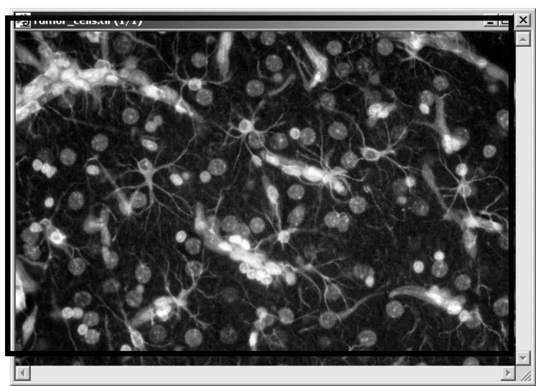
In this step you will define an Area of Interest (AOI) around portion of the tissue to count the nuclei using the color range you just defined, and view the resulting measurement statistics.

1. Select the *Rectangular AOI* tool.



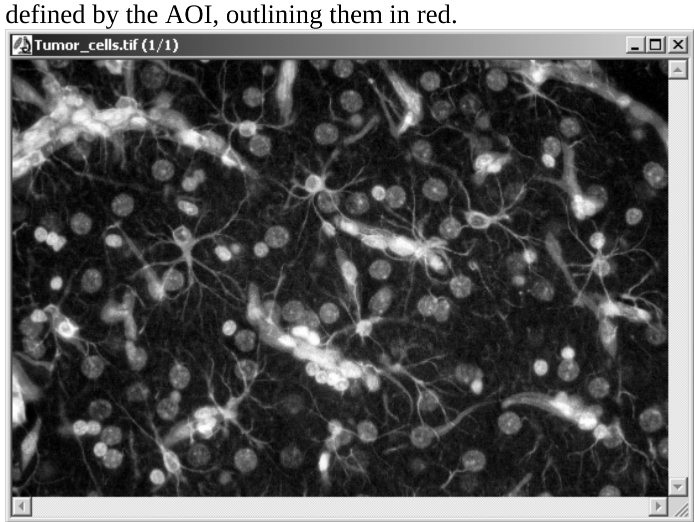
2. Define the AOI to cover most of the tissue section, as shown below in the *Tumor_cells.tif* image window.

The AOI is thickly outlined for emphasis in the image below. (It does not appear as such on your screen.)



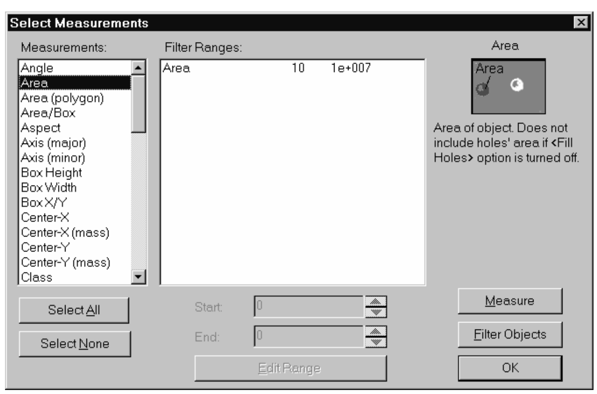
3. Click on *Count* in the *Count/Size* dialog box.

Image-Pro Plus counts the nuclei contained in the area defined by the AOI, outlining them in red.



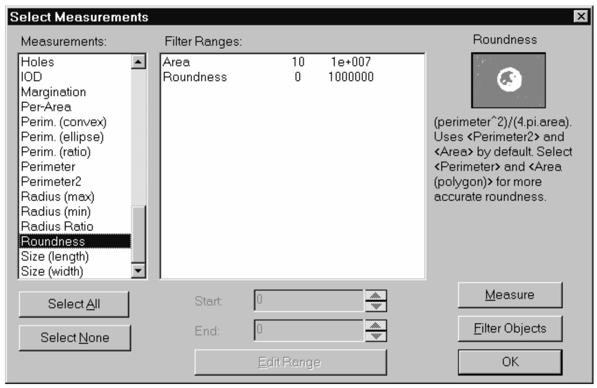
4. From the *Measure* menu in the *Count/Size* dialog box, select the *Select Measurements…* command.

The **Select Measurements** dialog box appears, with **Area** as the default.



5. Add the *Roundness* measurement to be recorded in the measurement statistics.

Scroll down the **Measurements** list box on the left and then click on **Roundness**. The new measurement is included in the **Filter Ranges** list box on the right.



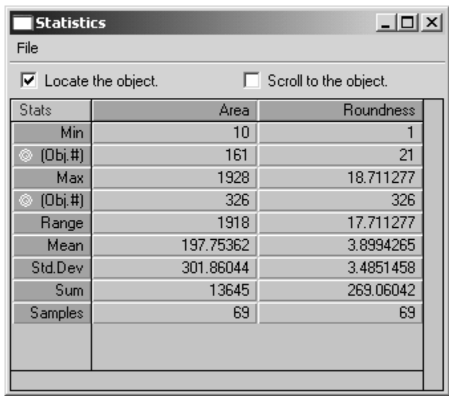
Roundness is a measure of how circular an object is (the expected perimeter of a circular object divided by the actual perimeter). A circular object will have a roundness of 1.0, while more irregular objects will have larger values.

6. Click on *Measure*.

Roundness is added to the measurement statistics.

7. From the *View* menu in the *Count/Size* dialog box, select the *Statistics* command.

The **Statistics** window appears with the statistic data sheet for the image, including the *Area* and *Percent Area* measurements. You may need to expand the size of the window to view all of the data in this table.



Stats	Area	Roundness
Min	10	1
(Obj.#)	161	21
Max	1928	18.711277
(Obj.#)	326	326
Range	1918	17.711277
Mean	197.75362	3.8994265
Std.Dev	301.86044	3.4851458
Sum	13645	269.06042
Samples	69	69

The following statistics in the **Statistics** data sheet are listed by row for each measurement you have made:

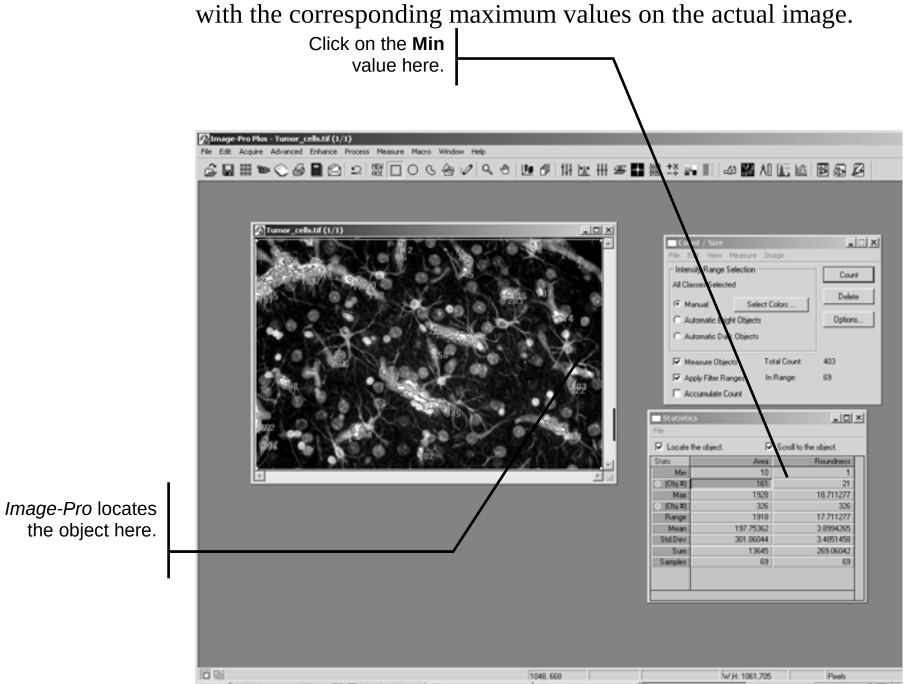
- **Min:** The smallest value for each measurement . The object possessing the minimum measurement is identified by number (**Obj.#**), below the stated minimum.
- **Max:** The largest value for each measurement. The object possessing the maximum measurement is identified by number (**Obj.#**), below the stated maximum.
- **Range:** The difference between the maximum and minimum values for each specified measurement.
- **Mean:** The average (mean) for each measurement.
- **Std. Dev:** The standard deviation for each measurement.

- **Sum:** The sum of the measurements for each measurement type (e.g., area, perimeter, etc.) you have specified.
- **Samples:** The total number of objects that have been included in the count from which these statistics were derived.

Note that the **Locate the object** check box is turned on. *Image-Pro Plus* can identify on your image any object that is the **Max** or **Min** object for a given statistic.

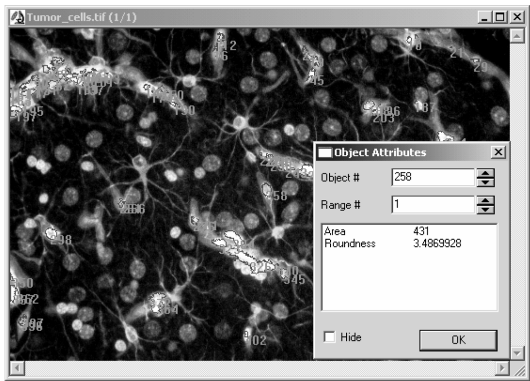
8. Click on the minimum (*Min*) value in the *Statistics* data table to see where that object is located on the image.

Click on the object number in the **Obj.#** row for the **Min** value. (**Obj.#** rows are flagged with a symbol — .) *Image-Pro Plus* uses the radiating signal to identify the object with the corresponding maximum values on the actual image.



9. Double-click on any individual object to see its statistical information.

Make sure that the *AOI* tool is turned ***off*** to see the statistical data. The **Object Attributes** dialog box appears with the statistical data corresponding to the individual object selected.



Continue to the next set of steps in this exercise: *Closing the Image Windows and Exiting Image-Pro Plus.*

■ Closing all the Windows and Exiting *Image-Pro Plus*

Close all the windows by selecting the **CloseAll** button  from the *Image-Pro Plus* toolbar. You may continue working with *Image-Pro Plus* or exit *Image-Pro Plus* by selecting the *Exit* option under the *File* menu. Do not save any changes.

Continue to the next exercise: *Calculating Population Density.*

Calculating Population Density

The Tutorial in this section introduces you to the **Population Density** features of *Image-Pro Plus*. Using Population Density analysis, you can measure the number of objects per unit of area. This feature is often utilized in cell analysis to determine the number of grains within a cell (grain-counting applications).

In this exercise you will use the **Population Density** command to calculate density of grains in a cell. You will:

- Load an image containing silver grains.
- Locate cell sites.
- Save site outlines to a file.
- Filter the grains in the original image to separate clustered grains.
- Count the grains.
- Load the cell site outlines over the grains and calculate their density.

This exercise will take about 25 minutes to complete.

Setup: Start *Image-Pro Plus* by double-clicking the *Image-Pro Plus* icon in your *Image-Pro Plus* folder. Once the *Image-Pro Plus* application window is active, you may begin following the steps below.

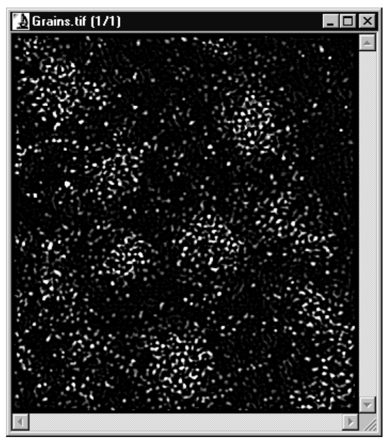
Important**: Please check that your Image-Pro settings for this exercise* ***match *those in the dialog boxes shown in the tutorial. Image-Pro 7.0 retains the settings from previous experiments, which may not be the same as the ones used in this exercise.*

■ Copying the Image to a New Window

In this exercise you will use a modified image of silver grains (attached to cells via a chemical process), and then copy the image into a new window.

1. Load the *Grains.tif* file from the *Images/Count and Size* folder.

The **`Grains.tif`** image window is opened.



2. Select the *Copy* command from the *Edit* menu.

The contents of the **`Grains.tif`** window are copied to the Clipboard.

Note: *Image-Pro Plus copies the contents of an AOI to the clipboard if one is active when the* ***Copy*** *command is selected. If no AOI is active, Image-Pro Plus copies the entire active image to the Clipboard (which is what you did in this step).*

3. Select the *Paste New* command from the *Edit* menu.

The copy of **`Grains.tif`** will be pasted into a new window. The new image is called **`Untitled1.`**

4. Save the *Untitled1* image as *IPpop1.tif*.

Select the *Save As…* command from the *File* menu and type **`IPpop1.tif`** for the filename.

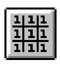
■ Identifying Cell Sites

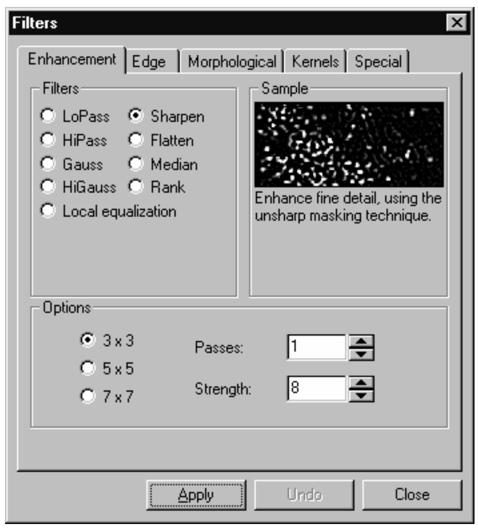
In this image, the individual grains are distinct, but there is no way to determine where the cell boundaries are located. There are actually 11 cells in this image. In this step you will identify the location of these cells by dilating the grains (using a morphological filter) until they form large clumps. The clumps will indicate where the cell sites are located.

Important: *Be sure the* ***IPpop1.tif*** *image window is active before beginning the steps below.*

1. Select the *Filters* command from the *Process* menu.

The **Filter** tabbed dialog box appears.





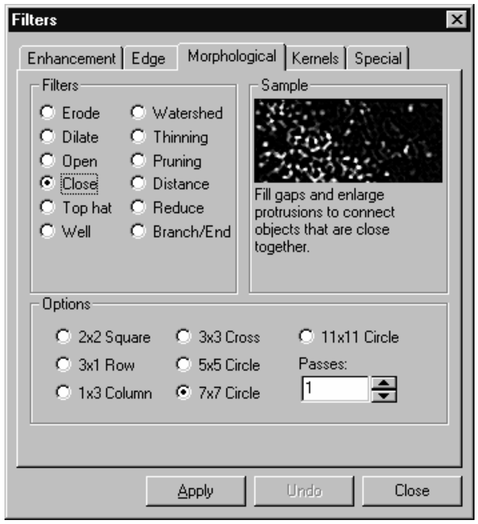
2. Click the *Morphological* tab.

In the following steps, *Image-Pro* will fill the gaps and enlarge protrusions to connect the objects that are close.

3. Click the *Close* radio button in the *Filters* group box.

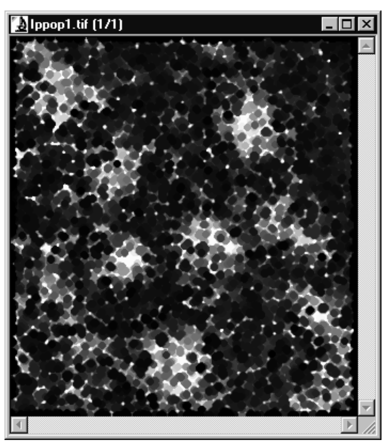
4. Click the *7 x 7 Circle* radio button in the *Options* group box.

5. Make sure that the number of *Passes* in the *Option* group box is 1.



6. Click the *Apply* button.

The **Close** filter is applied to the **`IPpop1.tif`** image. The image appears as follows:



The **Close** filter fills gaps between closely spaced bright spots. Because a rather large filtering kernel (**7 x 7**) was used, the filtered results are very coarse. However, the image is beginning to show concentrations marking where its cell locations are.

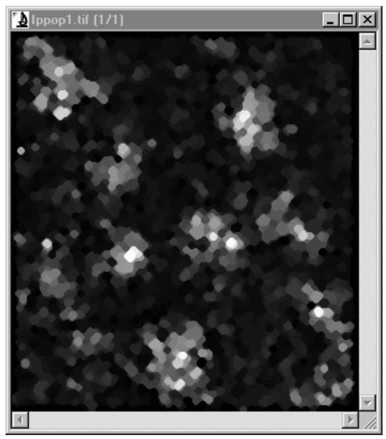
■ Removing Extraneous Grains

You will note 8 large, and fairly distinct patches of grains with many, many small spots surrounding them. In this step, you will eliminate these small spots using another morphological filter.

1. Click the *Open* radio button in the *Filters* group box of the *Morphological* tab.

2. Click the *Apply* button.

The **Open** filter is applied to the **`IPpop1.tif`** image. The image appears as follows:



The **Open** filter smoothes the contours of bright objects and removes minor protrusions. Because a rather large filtering kernel (**7 x 7**) was used, the filter also eliminated all small bright spots in the image.

Now the cell sites, as distinguished by concentrations of grains, can easily be identified. You'll note that while only 8 sites were apparent initially, a 9th site has been located with this filter.

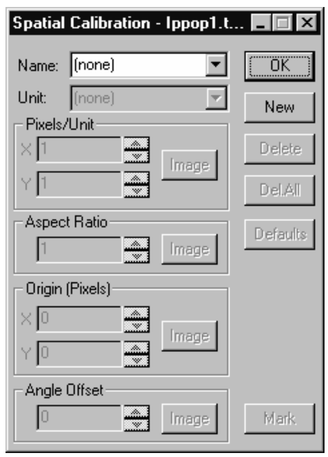
Continue to the next set of steps in this exercise: *Outlining the Cell Sites.*

Outlining the Cell Sites

In this exercise, you will count the cell sites using the *Count/Size* command, and outline each cell site with an ellipse. You will ultimately superimpose these elliptical outlines onto the **Ippop1.tif** image and use them to calculate grain density.

1. From the *Calibration* sub-menu under the *Measure* menu, select the *Spatial …* command and reset the calibrations, if necessary.

The **Spatial Calibration - Ippop1.tif** dialog box appears. Make sure the **Name** field selection is **(none)**.

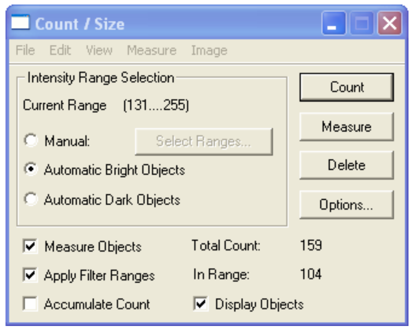


Since this image has not been spatially calibrated, marking “none” applies no calibrations.

2. Close the *Spatial Calibration - Ippop1.tif* dialog box.

**3.	Select the *Count/Size* command from the *Measure* menu.**

The **Count/Size** dialog box appears.



**4.	Click the *Automatic Bright Objects* radio button in the *Intensity Range Selection* group box.**

**5.	Click *Count*.**

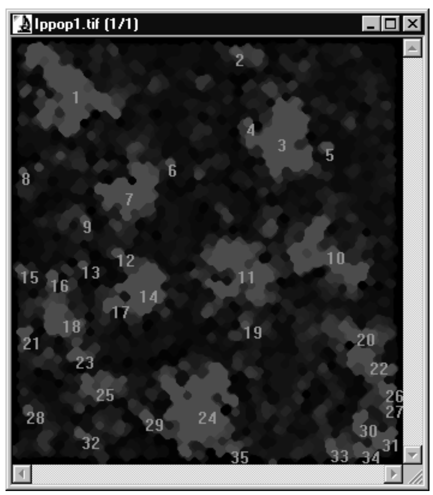
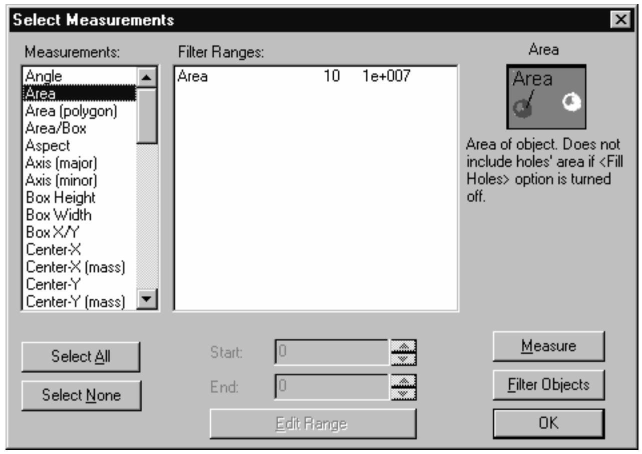


Image-Pro segments 35 objects. The next step is to select a subset of these objects, defining a valid size range.

6. **From the *Measure* menu in the *Count/Size* dialog box, select the *Select Measurements* command.**

   The **Select Measurements** dialog box appears.



7. **Select the *Area* selection.** Use your cursor to highlight the **Area** measurement in the **Filter Ranges** group box.

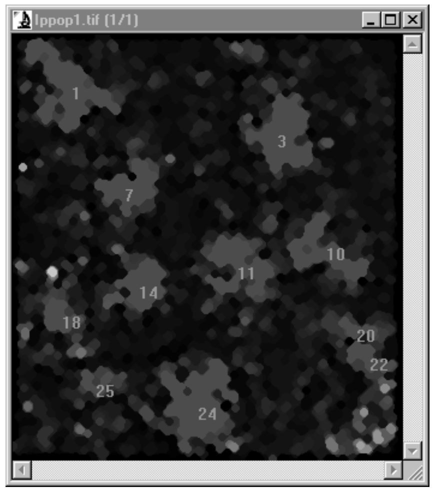
8. **Delete the old value in the *Start* field and type: *275* and click the checkmark.**

   This instructs *Image-Pro Plus* to ignore all objects with areas less than 275.

   ***Note**: The actual value you select is dependent upon your own data. In this case, 275 was selected to isolate large clusters of grains in this image.*

9. Click *Measure*.

The image is analyzed, and bright objects with areas greater than 275 are extracted, counted, and measured. Object outlines and labels appear within the **`IPpop1.tif`** image window.



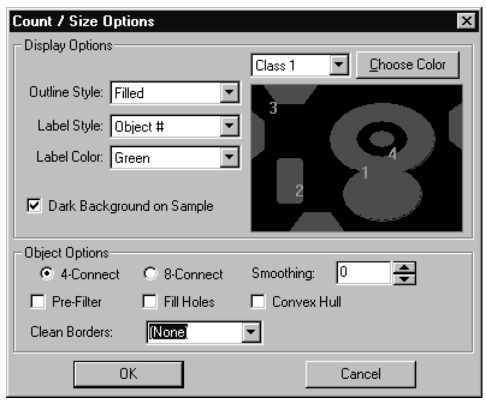
Continue to the next set of steps in this exercise: *Saving the Cell Outlines.*

■ Saving the Cell Outlines

In this step, you will change the outlines to **Ellipses**. Because the patches merely suggest the location of the cells and do not actually represent cell boundaries, you will, in this step, convert the outlines to elliptical form. This will more closely resemble the actual shape of the cells to which the grains were attached. Then, you will save the elliptical outlines to a file.

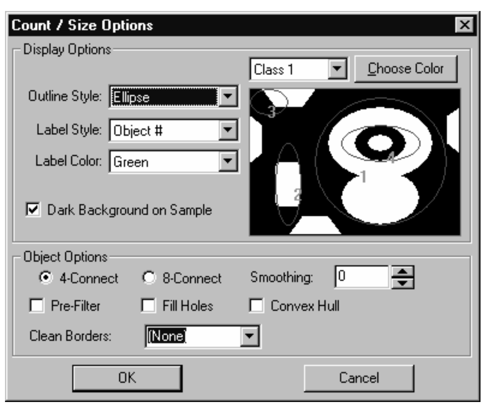
1. Click the *Options* button.

The **Count/Size Options** dialog box appears.



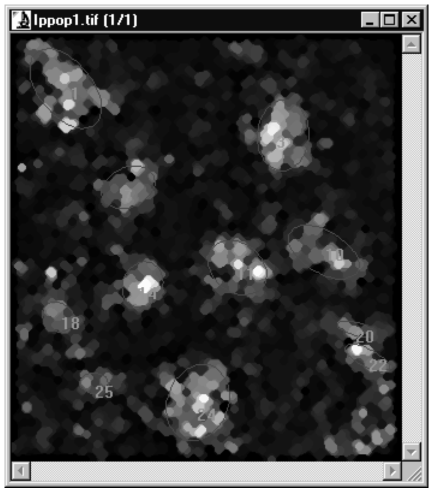
2. Select the *Ellipse* option in the *Outline Style* list box.

This instructs *Image-Pro Plus* to change the object outlines to ellipses with the same major and minor axes as the object outline.



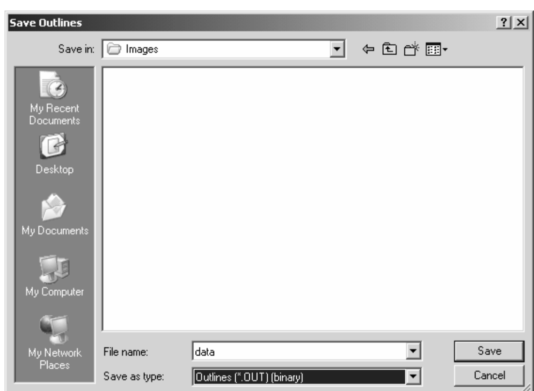
3. Click *OK* in the *Count/Size Options* dialog box.

The object outlines appear as ellipses.



4. From the *File* menu in the *Count/Size* dialog box, select the *Save Outlines* command.

The **Save Outlines** dialog box appears.



5. Type: `Cells` in the *Save Outlines* list box.

Image-Pro Plus will automatically assign the **`.OUT`** extension when the file is saved.

6. Click *Save.*

The outlines will be saved to file **`Cells.OUT`** in the *Image-Pro Plus* program directory. Outlines are saved in binary files containing a list of the polygons comprising the outlines.

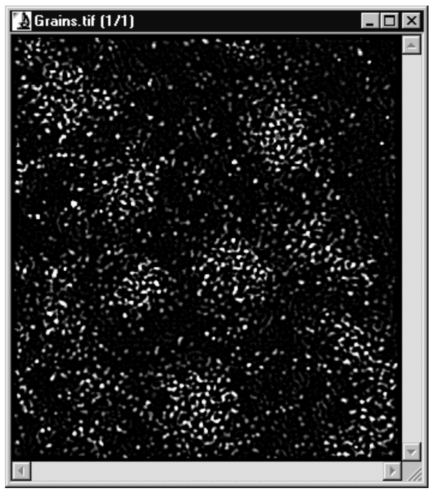
Continue to the next set of steps in this exercise: *Preparing the Grain Image.*

■ Preparing the Grain Image

In this exercise you will again work on the **`Grains.tif`** image window and filter it to make sure that any clustered grains are separated.

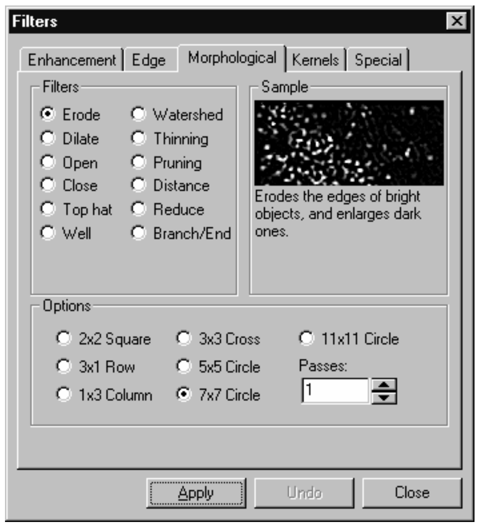
1. Click on the `Grains.tif` window to reactivate it as the "working" image.

The **`Grains.tif`** image window is selected.



2. Activate the *Filter* dialog box.

Note: *The* ***Filter*** *dialog box was opened earlier in this exercise, and should still be open on the screen. It may be hidden behind other open image or command windows, so you may have to move a few windows to locate it.*



3. Click the *Erode* radio button in the *Morphological* filters group box.

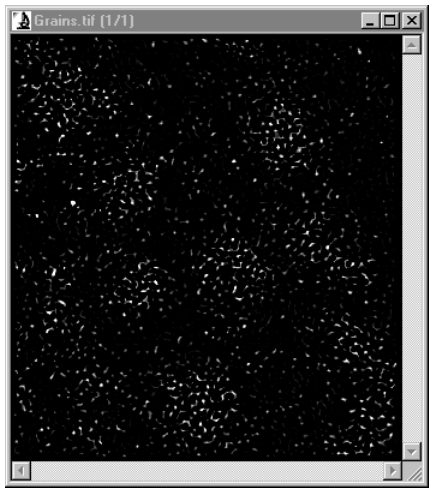
The **Erode** filter reduces the size of bright objects. This will shrink the grains in our image.

4. Click the *2 x 2 Square* radio button in the *Options* group box.

Because the objects in the image are quite small, the 2 x 2 kernel is used to produce a subtle effect.

5. Click *Apply*.

The **Erode** filter is applied to the **`Grains.tif`** image. This filtering ensures that the grains get counted as individual objects. The grains in the image appear smaller.



Continue to the next set of steps in this exercise: *Counting the Grains.*

■ Counting the Grains

In this exercise you will count the grains in this image. Because the grains are clearly distinguished against the dark background, you will use *Image-Pro Plus'* automatic object selection method.

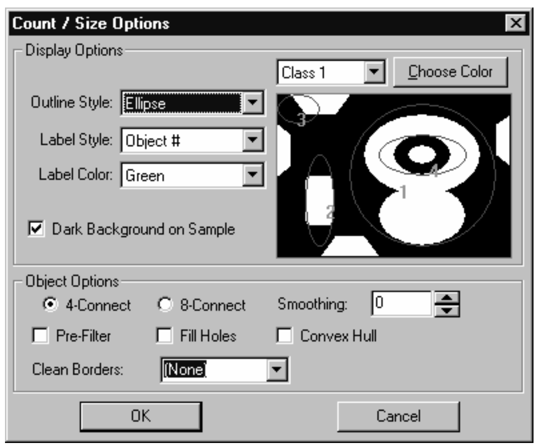
Since there are so many small objects, you will disable the object labeling feature. You will also eliminate all size selection criteria to ensure that all grains are included in the count.

1. Activate the *Count/Size* dialog box.

2. Click the *Automatic Bright Objects* radio button in the *Intensity Range Selection* group box.

3. Click the *Options* button.

The **Count/Size Options** dialog box appears.



4. Select *Outline* for the *Outline Style* list box.

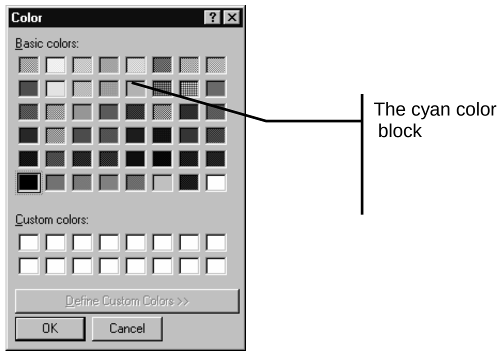
This directs *Image-Pro Plus* to highlight the counted grains with outlines, not the ellipses used in the previous exercise.

5. Select *None* for the *Label Style* list box.

This directs *Image-Pro Plus* to suppress labeling of the grains. Since there are so many small objects clustered together, labeling them would simply clutter the screen.

6. Click on the *Choose Color* button.

The **Color** dialog box appears.



7. Select the *cyan* color block.

The cyan block is on the second row down, fifth block from the left. This directs *Image-Pro Plus* to highlight the counted grains in cyan.

Note: *When measuring data, you can choose what color contrasts best with your image.*

8. Click *OK*.

The **Color** dialog box closes.

9. Click *OK*.

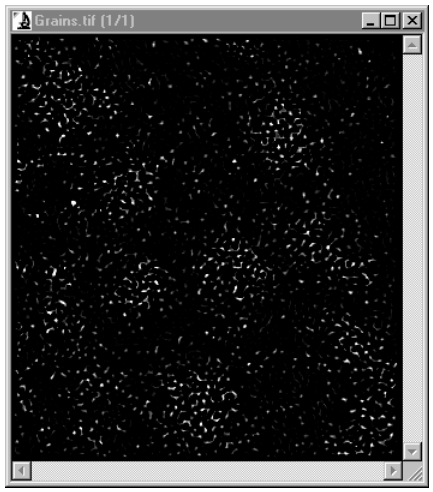
The **Count/Size Options** dialog box closes.

10. Click the *Apply Filter Ranges* check box to remove the checkmark from the box.

This action instructs *Image-Pro Plus* to ignore any selection criteria specified in the **Select Measurements** dialog box, and simply count **all** objects in the image.

11. Click the *Count* button.

The image is analyzed, and its bright objects (the grains) extracted, counted, and measured. Grains are colored cyan in the **`Grains.tif`** image window.



If you look in the lower right corner of the **Count/Size** dialog box, you will see that 1239 objects have been counted.

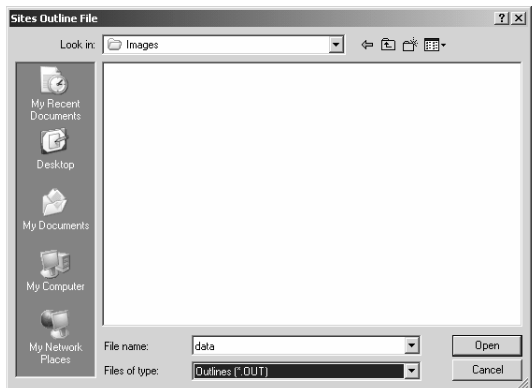
Continue to the next set of steps in this exercise: *Calculating Population Density*.

■ Calculating Population Density

In this exercise you will perform the population density analysis. This process will request the **`Cells.out`** file that you created earlier in this exercise. Those cell outlines will be superimposed upon **`Grains.tif`**, and the grain density within each site will be calculated.

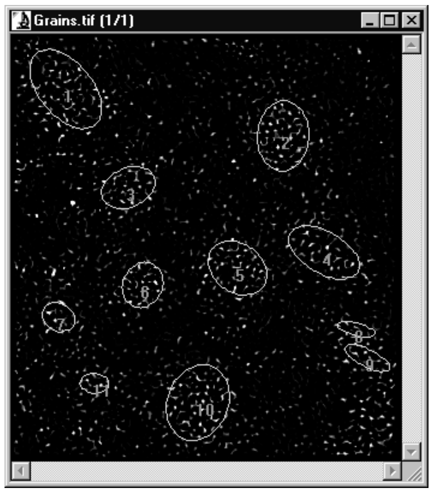
1. **From the *Measure* menu in the *Count/Size* dialog box, select the *Population Density* command.**

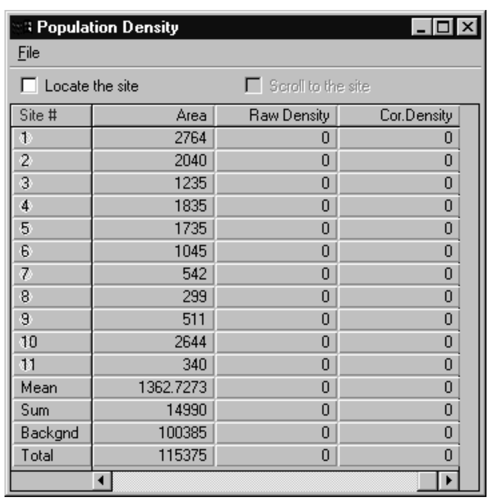
   The **Sites Outline File** dialog box appears.



2. Open the *Cells.OUT* file.

The yellow cell outlines appear in the `Grains.tif` image window overlaying the cyan-colored grains, and the **Population Density** data window appears.





Site #	Area	Raw Density	Cor.Density
1	2764	0	0
2	2040	0	0
3	1235	0	0
4	1835	0	0
5	1735	0	0
6	1045	0	0
7	542	0	0
8	299	0	0
9	511	0	0
10	2644	0	0
11	340	0	0
Mean	1362.7273	0	0
Sum	14990	0	0
Backgnd	100385	0	0
Total	115375	0	0

The **Population Density** data window contains the density calculations for each cell and other statistics. This data can be stored to a file using the *Data to File* option on the *File* menu in the **Population Density** window.

Summary of… The Population Density process

The population density process involves three essential steps:

1) *First, create a file that contains the outlines of the areas to be measured. In this exercise, you created the outlines by counting objects that were generated using morphological filters. In real life, you might create these outlines by counting objects that actually define the areas you want to measure. Or, by drawing the outlines using the* ***Draw/Merge Objects*** *or* ***Measurements*** *tools.*
2) *Next, count the objects making up the population for which you want to measure density. In this exercise, you counted the grains in the* **`Grains.tif`** *image.*
3) *Finally, use the* ***Population Density*** *command to load the outlines (created in Step 1), and calculate the object density within each.*

See the Population Density Analysis discussion in the ***Image-Pro Plus Reference Manual*** *for more information about this subject.*

Continue to the next set of steps in this exercise: *Closing all the Windows.*

■ Closing all the Windows

Close all of the open image and command windows using the **CloseAll**  button, and if you want, exit *Image-Pro Plus.*

Advanced Exercises

This section contains some additional exercises developed to demonstrate the new and improved features found in *Image-Pro Plus version 7.0.*

Using the Calibration Wizard

The *Calibration Wizard* is a new feature of *Image-Pro Plus v. 7.0*. It is designed to make it easier for you to calibrate a single image, or to develop a set of reference calibrations to be used when you are capturing images, or with *Stage-Pro* and *Scope-Pro*.

- The first part of this exercise shows you how to create a reference calibration.
- The second part shows you the two modified steps necessary to calibrate an active image

This exercise will take about 25 minutes to complete.

Setup: If you have not yet started *Image-Pro Plus,* do so now by double-clicking on the *Image-Pro Plus* icon within the *Image-Pro Plus* folder. Once *Image-Pro Plus* is running, you may begin following the steps below.

***Important**: Please check that your Image-Pro settings for this exercise **match** those in the dialog boxes shown in the tutorial. Image-Pro 7.0 now retains the settings from previous experiments, which may not be the same as the ones used in this exercise.*

■ Creating a Reference Calibration

In this exercise, you will create a reference calibration for your *Image-Pro Plus v. 7.0* system.

When you first select the *Calibration Wizard* from the *Measure* menu, you will have a choice:

- Create a reference calibration
- Calibrate the active image.

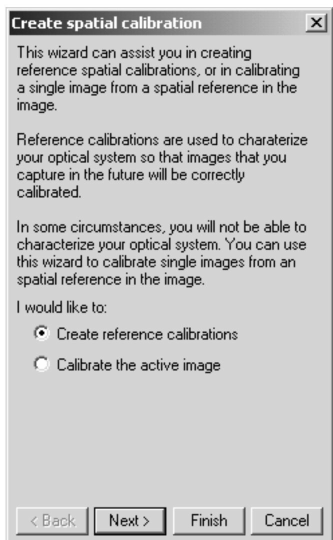
The following table indicates the steps required by both actions:

Step	Calibrate the active image	Create reference calibrations
1	**Welcome page**	**Welcome page**
2	**Name and Units page**	**Reference Process page**
3	**Image Calibration page**	**Capture Setup page**
4	**Finish Image page**	**Magnifier page**
5		**Objective page**
6		**Capture Preview page**
7		**Reference Calibration page**
		Finish Reference page

To create a reference calibration, follow the steps below:

1. Select the *Calibration* option from the *Measure* menu.

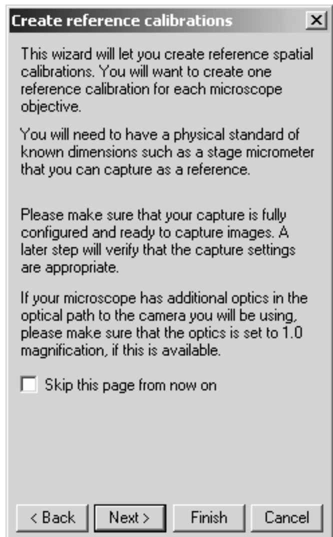
2. Then choose the *Spatial Calibration Wizard* from the *Calibration* sub-menu. You will see the Welcome screen.



3. Click the radio button next to Create Reference Calibrations.

4. Click Next.

5. You will see the Reference Process page.

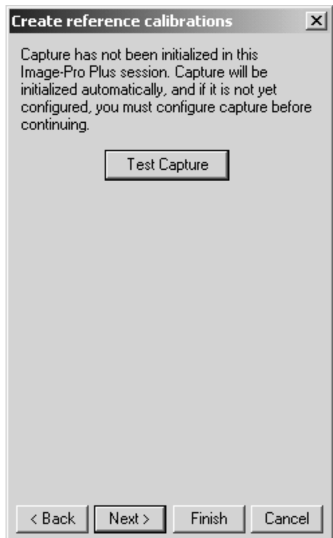


The **Reference** process page discusses the process of defining reference calibrations, what will be required to proceed, etc. It includes an option to skip this page in the future (in which case the *Wizard* will go to the **Capture Setup** page after the **Welcome** page). The **Back** button can still be used from the **Capture Setup** page to display the **Reference** process page.

Check the Skip…checkbox.

6. Click the Next button to continue.

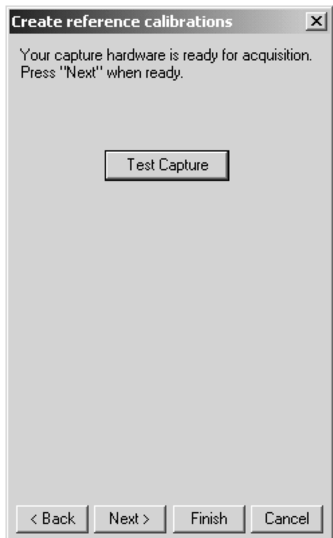
7. The next page is the Capture Setup Page.



Click the Test Capture button.

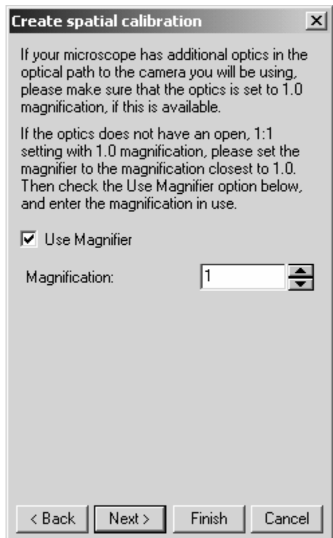
The **Test Capture** page is designed to ensure that your image capture settings are configured correctly. Clicking **Test Capture** snaps an image using the current capture settings.

When your **Capture** setup is ready to go, you will see the following message:



8. Click Next to continue.

9. You will see the Magnifier page.

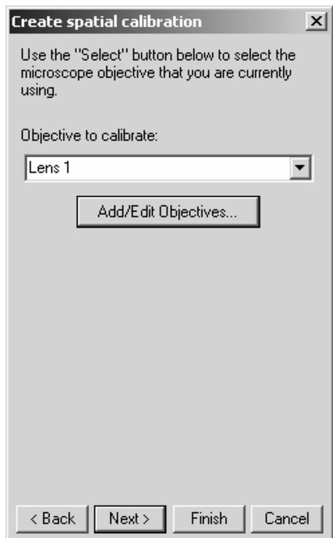


The **Magnifier** page allows you to use a magnifier that does not have any 1.0 magnification settings.

If your magnification changer does not have a 1.0 magnification setting, check the **Use Magnifier** box, and then enter the correct magnification setting.

10. Click Next to continue.

11. **The next page is the Objective page. You will need to select the microscope objective by the name of its lens file, or by entering the objective name.**



If you have already defined your lens using the **Edit Lens List** feature on the *Edit* menu, you can select the lens you want to calibrate from that list.

Otherwise, you can type the name of your objective into the space, or click the **Objective** button to select the correct **Lens Information File**. The name that you enter here will be used as the name of the **Reference Calibration** that your are currently creating.

12. **Click Next to continue.** *Image-Pro* will check to make sure that a calibration with the same name does not already exist.

The next page is the **Capture Preview** page.

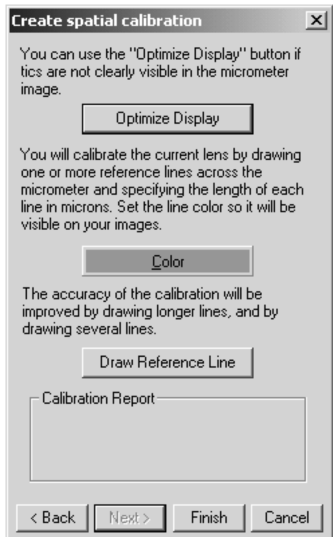
13. Click the Capture button.

The goal of the **Capture Preview** page is to get the stage positioned so as to capture the largest area of the micrometer possible, and to focus the image for the calibration capture. A live preview is started automatically. The **Stop Preview** button can be used to stop the preview, at which time the button changes to **Start Preview**, so the preview can be restarted.

When the **Capture** button is pressed, the preview is stopped, and an image is captured and displayed in an image workspace.

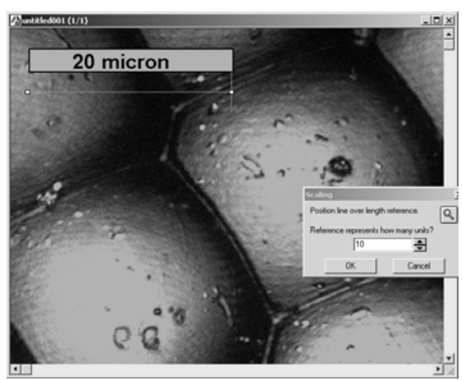
14. When you have captured a clear image of the micrometer, click Next to continue. You will see the **Reference Calibration** page.

15.



You will be asked to draw a calibration line on the image that you just captured. Click the Color button to select a color.

16. Click the Draw Reference Line button.



17. Position your reference line on the image, and indicate the number of calibrated units that it represents, then Click OK.

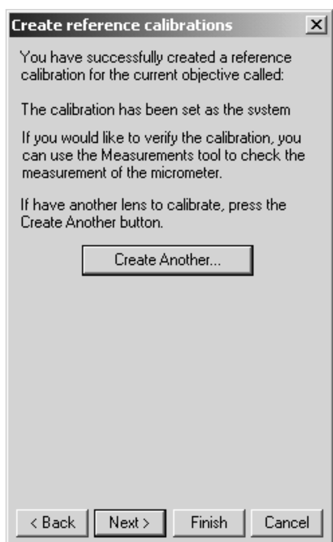
18. You can click on the magnifier button to open a **Local Zoom** window that displays the portion of the image that is under the cursor. This may help you to position the reference line more accurately.

You may draw more than one calibration reference line. As each reference line is drawn, the **Calibration Report** section is updated to indicate the calibration so far, including the average calibration and the calibration from the last reference line.

When you click **Next**, a reference calibration is created, using the name from objective selected on the **Objective** page, the calibration from this step, and using "um" as the units. The calibration is added to the reference calibration list, it is set as the system calibration, and it is applied to the active image

19. Click Next to continue.

20. You will see the Finish page.



You have successfully created a Reference Calibration. The **Finish** page verifies the creation of the calibration, and permits you to create more calibrations if you choose. All of the navigation buttons are enabled on this page. The **Create Another…** button will switch the wizard to the **Objective** page where the user can select the next objective to calibration.

To calibrate the active image, please follow the instructions in the next section.

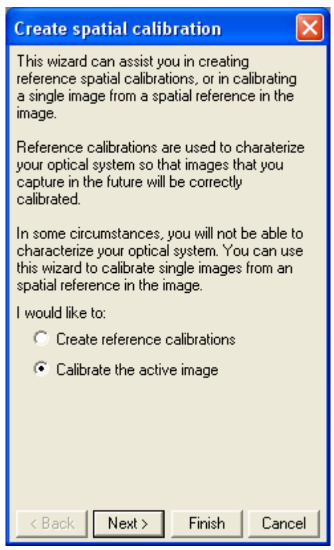
■ Calibrating the Active Image

In this exercise, you will calibrate an image in the *Image-Pro Plus 7.0* workspace.

1. Open an image in the Image-Pro Plus workspace.

2. Select the *Calibration* option from the *Measure* menu.

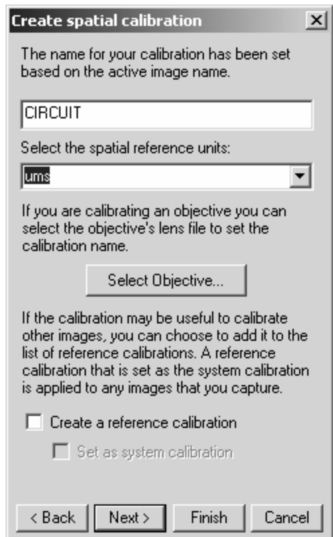
3. Then choose the *Spatial Calibration Wizard* from the *Calibration* sub-menu. You will see the Welcome screen.



4. Click the radio button next to Calibrate the active image.

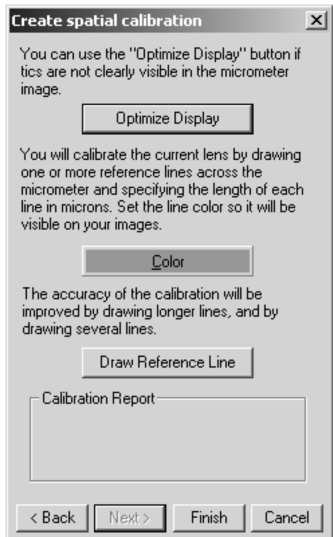
5. Click Next.

6. **The next step names your calibration.**



7. **Click Next to continue.**

8. You will see the Reference Calibration page.



You will be asked to draw a calibration line on the image that you just captured. **Click the Color button to select a color.**

9. Click the Draw Reference Line button.

10. Position your reference line on the image, and indicate the number of calibrated units that it represents, then Click OK.

11. Click Next to continue.

When you click **Next,** a calibration is created using a name derived from the active image's name, the calibration from this step, and using-your selected spatial reference units. The calibration is added to the reference calibration list, it is set as the system calibration, and it is applied to the active image

12. You will see the Finish page.

Now you have finished calibrating an image in *Image-Pro Plus*.

Tracking Objects

The *Tracking* feature of *Image-Pro Plus 7.0* enables you to follow an object as it moves through time and space. For example, a researcher could use it to follow the progress of a cell as it moved through the capillaries of the heart. In this part of the Tracking exercise, you will learn to add tracks to an image manually. You will also learn to save the tracking data and send it to an Excel spreadsheet.

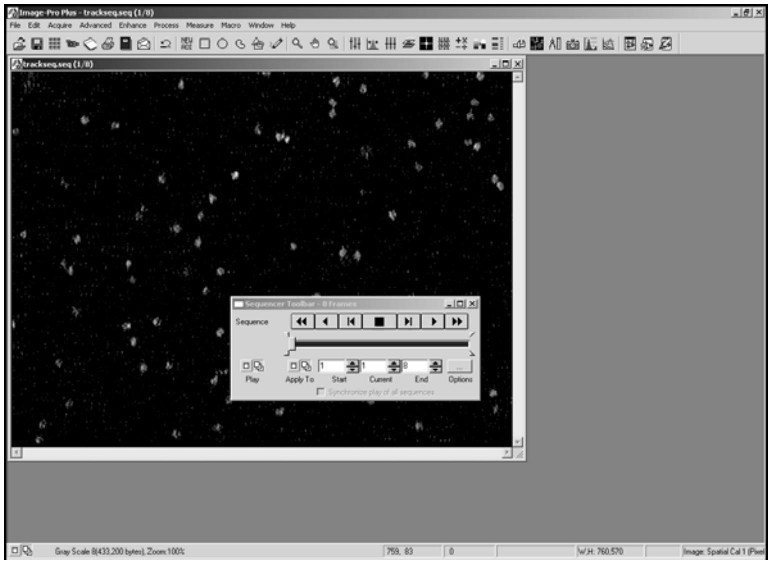
This exercise will take about 45 minutes to complete.

Setup: If you have not yet started *Image-Pro Plus*, do so now by double-clicking on the *Image-Pro Plus* icon within the *Image-Pro Plus* folder. Once *Image-Pro Plus* is running, you may begin following the steps below.

Important**: Please check that your Image-Pro settings for this exercise* ***match *those in the dialog boxes shown in the tutorial. Image-Pro 7.0 now retains the settings from previous experiments, which may not be the same as the ones used in this exercise.*

■ Manual Tracking

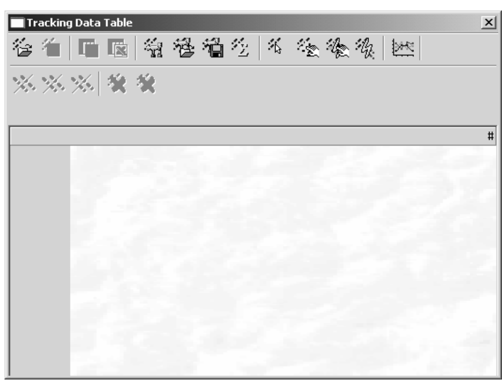
1. **Open the sample image Trackseq.seq from the Images/Tracking/2D folder**.
Open the entire sequence (all 8 frames).
The image workspace will look something like this:

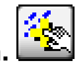


2. **Click the Play button on the Sequence toolbar to see how the objects move.**

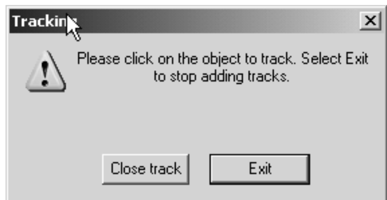
3. **Click the Stop button.**

4. **Select Track Objects from the *Measure* menu.** You will see the **Tracking Data Table**.

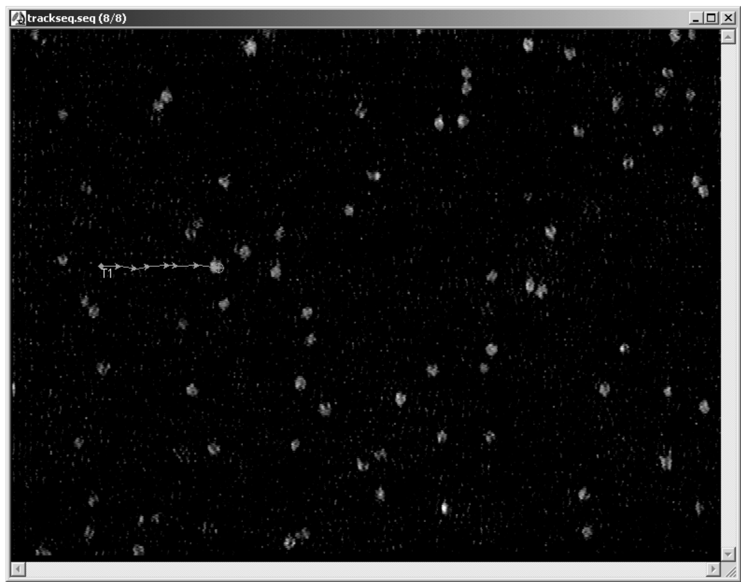


5. Click the Add Track Manually button. 

You will see a message asking you to click on the points to be tracked in the active image.



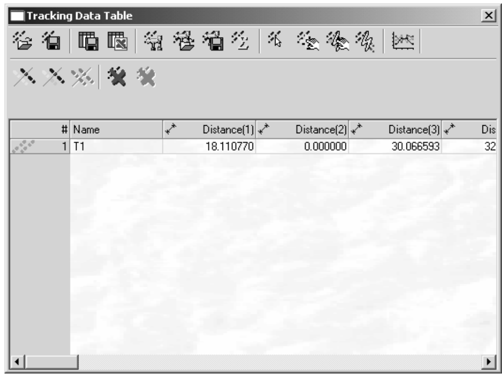
6. Click on a point on the active image. Continue to click on it as the frames advance, as shown here:



The *Tracking* module automatically draws a line showing the track you have selected. This is now **Track 1.**

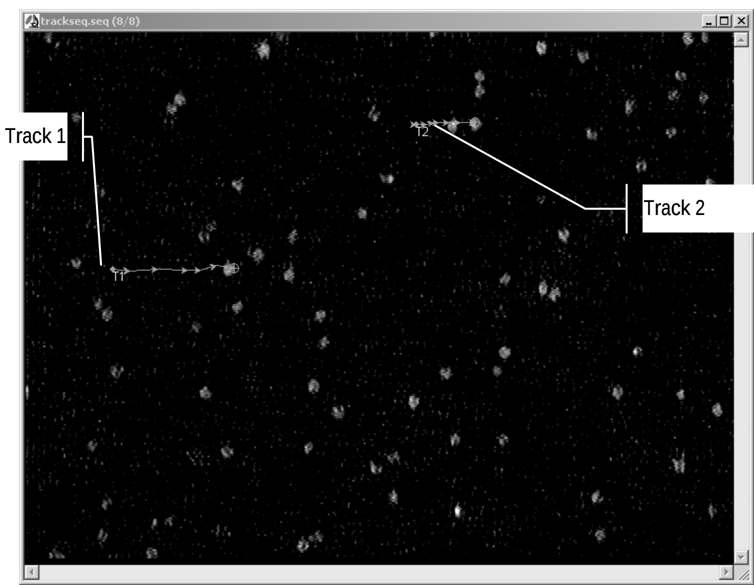
7. Click Close Track to stop adding points.

The *Tracking* module displays data and information about the track in the **Tracking Data Table**, as shown here:



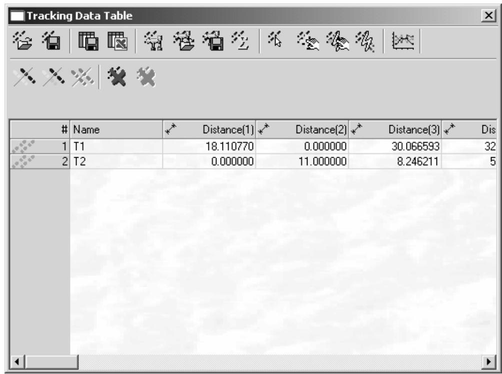
8. **Click the Add Track Manually button**  **again to add another track.** This will be **Track 2.**

9. **Click Close Track to stop adding points**. The image in your workspace should look something like this:

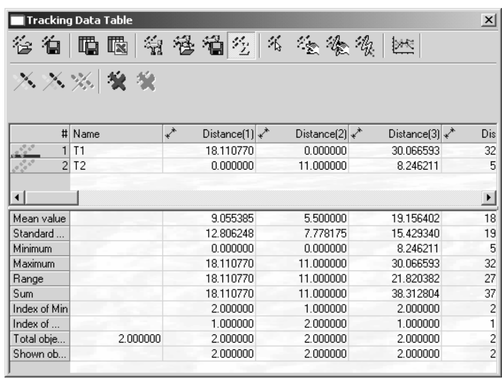


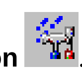
Each track on the active image is identified with a different color. You can change the color of the tracks using the **Tracking Options** button .

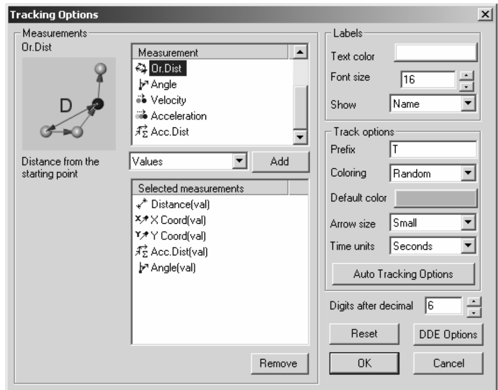
The **Tracking Data Table** displays information about the tracks recorded from your image, as shown here:



10. **To display the statistics for each track, click the Show Tracking Statistics button.**  The tracking statistics are shown in the bottom half of the **Tracking Data Table**, as shown here:

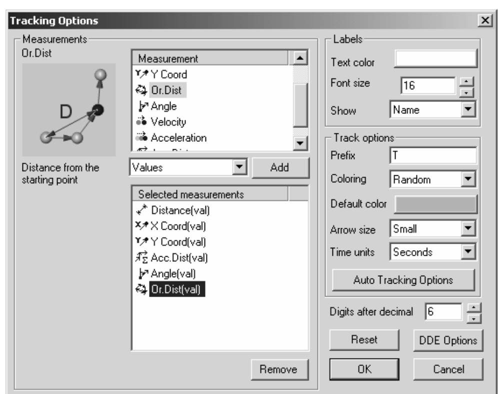


11. **Click the Tracking Options button** . You will see the **Tracking Options** dialog.



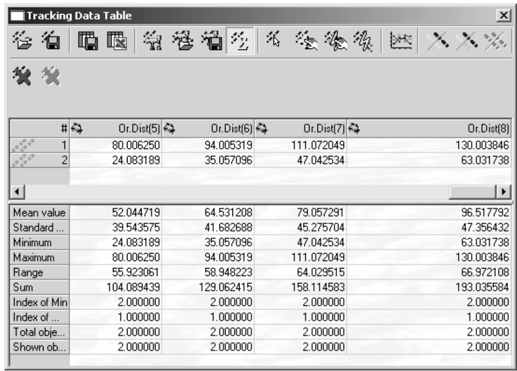
You can edit the list of measurements displayed in the **Tracking Data Table** by choosing items from the **Measurements** list box, and adding them to the **Selected Measurements** list.

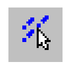
12. **Use your cursor to highlight the Or. Distance measurement. Then click the Add button**. The **Or. Distance** will appear in the list of **Selected Measurements**.

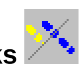


13. **Click OK**.

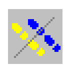
When you next look at the **Tracking Data Table**, the **Or. Distance** will appear in addition to the measurements already selected, as shown here:

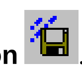


14. **Use the Select Track button**  **to select Track 2.**

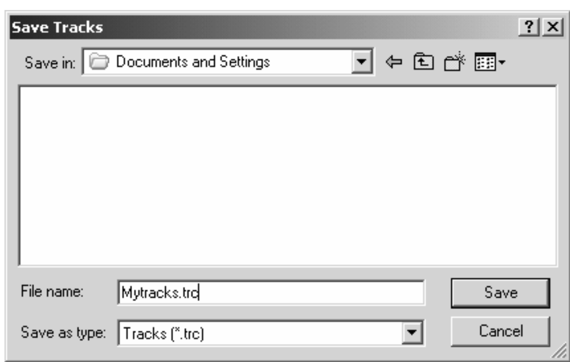
15. **Click the Hide Unselected Tracks**  **button to hide Track 1.**

    Only Track 2 will be displayed on the image.

16. **Click The Show All Tracks**  **button**. Now both tracks will appear on the image.

17. **Click the Save Tracks button** .

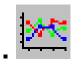
    You will see the **Save Tracks** dialog box.

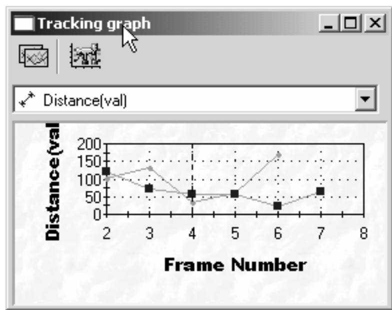


18. **Enter the file name as Mytracks.trc.**

19. **Click the Save button.**

Your tracking information is now saved in the **Documents and Settings** folder in *Image-Pro Plus 7.0*.

20. **Click the Show Graph button.**  You will see the **Tracking Graph** displayed, as shown here:



You can choose to display different values on the graph using the drop-down list box.

21. **Click the Send to Excel button** **.**

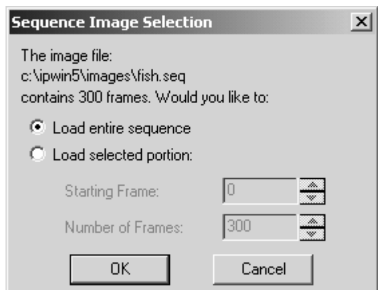
Your tracking information will be saved in an Excel spreadsheet.

■ Automatic Tracking

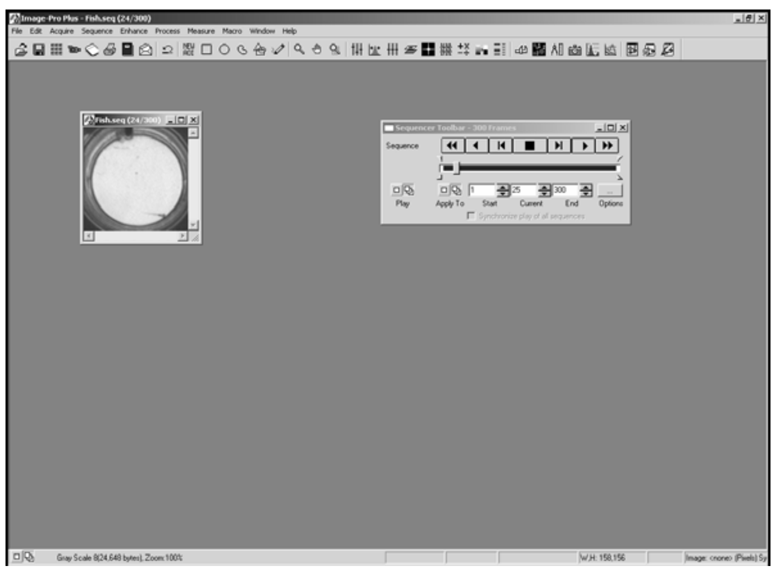
In this part of the tracking exercise, you will learn to use the automatic tracking feature in *Image-Pro Plus.*

1. Open the sample image Fish.seq from the Images/Tracking/2D folder.

2. Open the entire sequence (all 300 frames).

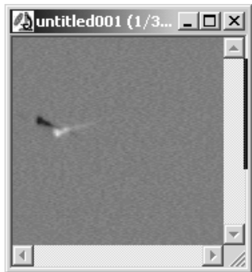


Your image workspace will look something like this:



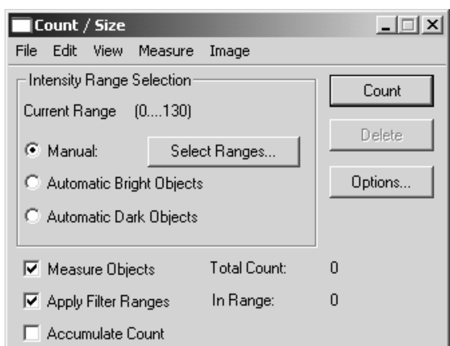
The fish in the image are not easy to see, so we will have to adjust the image.

3. **Select Difference from the Sequence menu**.
A new (untitled) image will appear in the workspace. This image has two fish, one dark and one light. We are going to track the lighter fish.



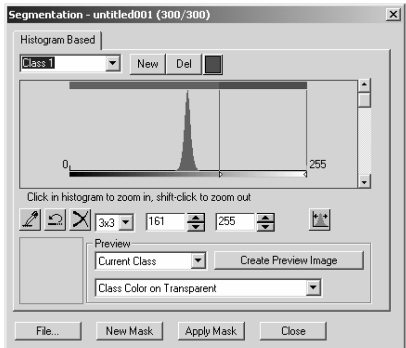
4. **Select Track Objects from the Measure menu**.

5. **Click the Find Tracks Automatically button**  **on the Tracking Data Table toolbar.** You will see the **Count/Size** dialog box**.**



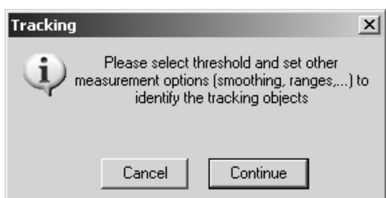
6. **Click the Manual radio button.**

7. **Click the Select Ranges button.**

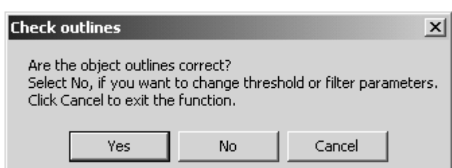


8. **Adjust the slider bars on the Segmentation dialog to show the one light fish as red against the background.**

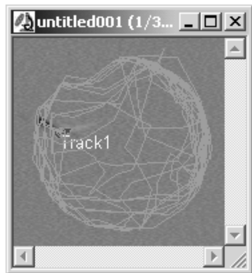
9. **Click Continue on the Tracking dialog.**

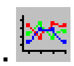


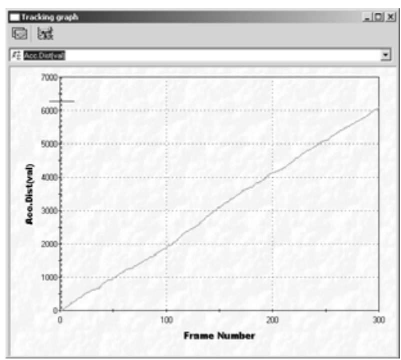
10. **Click Yes when Image-Pro asks if the outlines are correct.**



You will see the image with the one track highlighted.

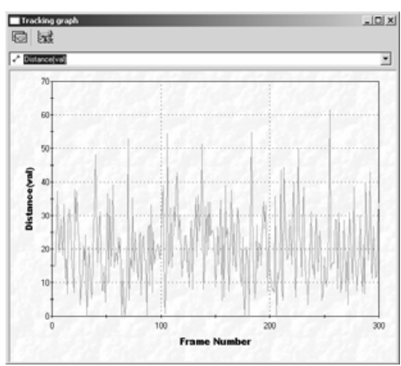


11. **Click the Show Graph button.**  You will see the **Tracking Graph** displayed, as shown here:



This graph shows the accumulated distance for the fish track.

12. **Use the drop-down list box to display a different measurement, such as the Distance**.



13. **Continue to the last set of steps in this exercise**: *Closing the Images and Exiting Image-Pro Plus.*

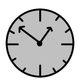
■ Closing all the Windows and Exiting *Image-Pro Plus*

Close all the windows and exit *Image-Pro* by selecting the *Close All* option under the *Window* menu. Do **not** save any files.

Note: *For more Tracking exercises, see the demo macros included in your Image-Pro Plus installation.*

Creating a Workflow Toolbar

The **Workflow Toolbar** is a way of saving and storing features of *Image-Pro Plus* that you use on a regular basis. You can create one toolbar, or several, to make the features that you use most often more easily accessible.



This exercise will take about 20 minutes to complete.

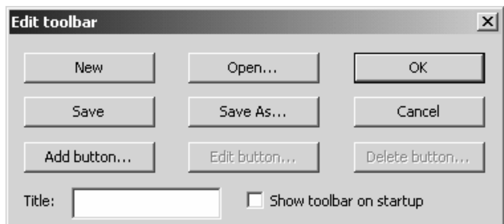
Setup: If you have not yet started *Image-Pro Plus*, do so now by double-clicking on the *Image-Pro Plus* icon within the *Image-Pro Plus* folder. Once *Image-Pro Plus* is running, you may begin following the steps below.

***Important**: Please check that your Image-Pro settings for this exercise **match** those in the dialog boxes shown in the tutorial. Image-Pro 7.0 now retains the settings from previous experiments, which may not be the same as the ones used in this exercise.*

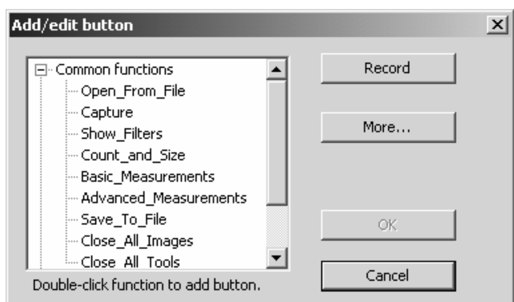
To create a *new* workflow toolbar, follow the steps below:

1. **Select the Edit Workflow Toolbar option from the Window menu.**

You will see the **Edit Toolbar** dialog.

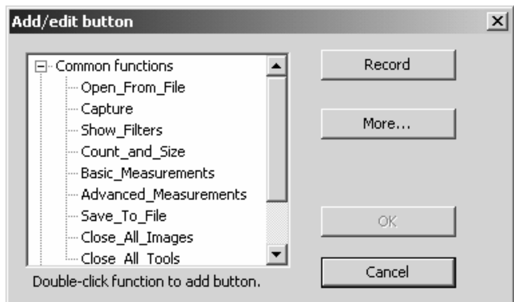


2. **Click Add button**… You will see the list of available buttons on the **Add/Edit button** dialog**.**

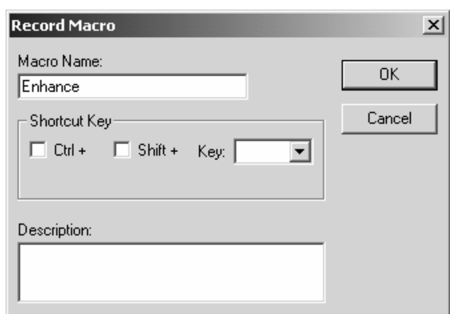


3. **Use your cursor to highlight Capture and double-click it.** A capture button will be added to the Workflow Toolbar.

4. Now, we need to add more buttons to the Workflow Toolbar. Some of the operations are not already saved. **Click the Record button before continuing.**

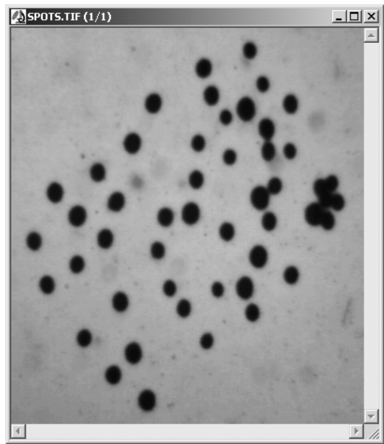


5. **You will see the Macro Recording dialog. Name your new macro Enhance, and click OK.**



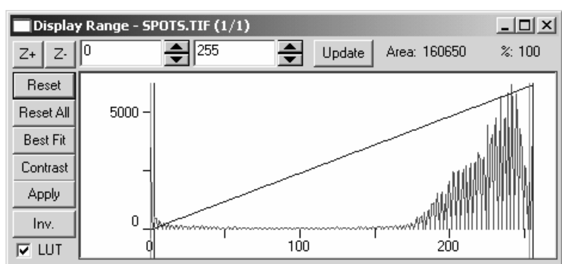
Now, all your subsequent actions will be recorded for the Workflow Toolbar.

6. Capture (or open) the image called Spots.tif.



Some of the spots look a little fuzzy. We will enhance the contrast using the **Best Fit** display range.

7. Choose Display Range from the Enhance menu. Click the Best Fit button. The image contrast will be automatically adjusted, and the histogram will reflect this:



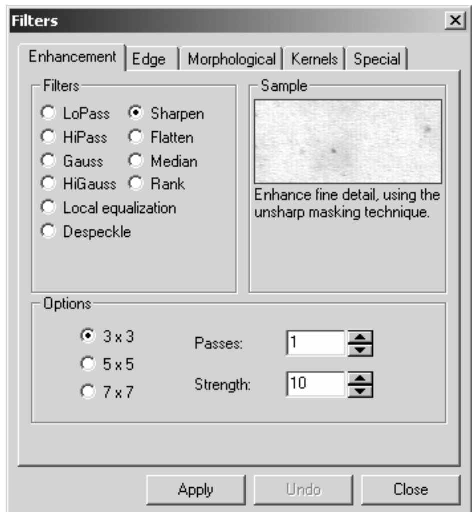
Note that there is now an Enhance button in the Workflow Toolbar.

8. Click Stop Recording to end the macro recording.

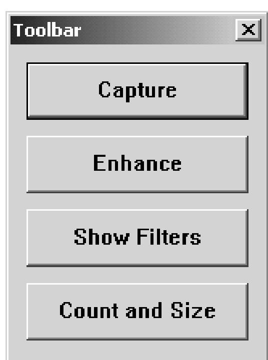
Now we will add the button called **Show Filters** to the Workflow Toolbar.

9. Use your cursor to highlight Show Filters on the Add/Edit button dialog, and click OK.

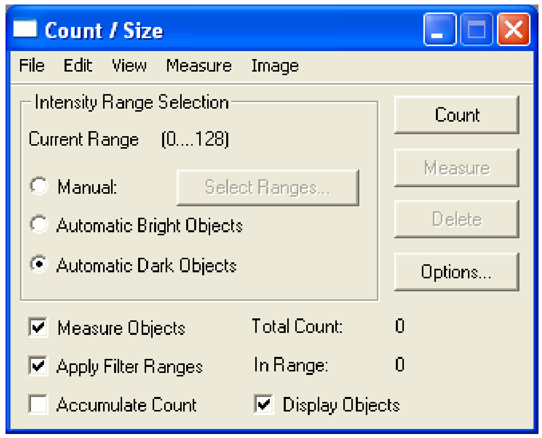
10. Use the Filters dialog to apply a Sharpen filter to the spots image.



11. Next, we want to count all the spots in the image. Use the Add/Edit button dialog to add the Count and Size button to the Workflow Toolbar.

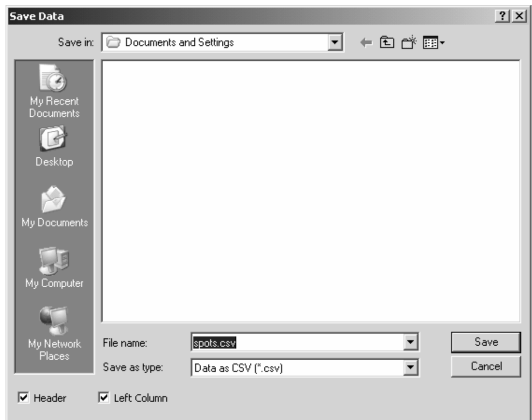


12. Choose Automatic Dark Objects for your count.



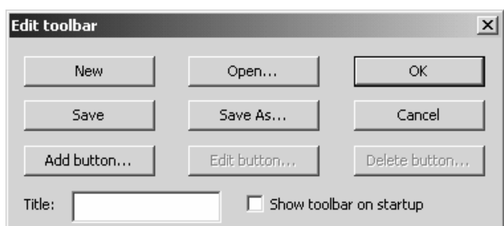
13. Use the Add/Edit button dialog to add the Save to File button to the Toolbar.

14. Save your count in a file called spots.csv

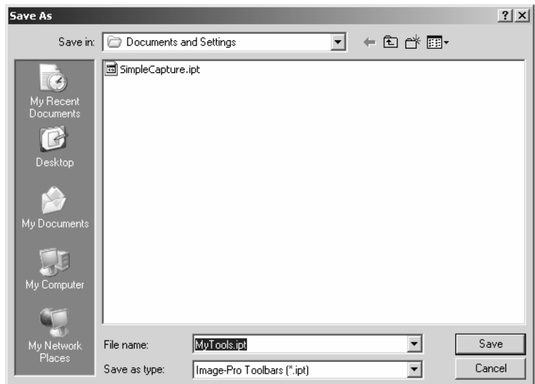


15. Now, you need to save your Workflow Toolbar.

Click the Save As button on the Edit Toolbar dialog.



16. Name your Workflow toolbar MyTools.ipt.



17. Click the Save button. Your toolbar is now saved in Image-Pro Plus 7.0.

■ Closing all the Windows and Exiting *Image-Pro Plus*

Close all the windows by selecting the *Close All* option under the *Window* menu or clicking the **Close All**  button. Do **not** save any files. You may exit *Image-Pro Plus* or continue working.

Note: *For more exercises, see the demo macros included in your Image-Pro Plus installation.*

Using AFA

AFA is a wrapper that ties together the functionality of the *Scope-Pro/Stage-Pro* plug-in and the capture module. To stream line the *AFA* interface, there are a number of places where *AFA* has been designed to reference a settings file for one of these modules, rather than replicate the controls from that module. If you have not already done so, please refer to the *Installation* instructions in order to install *AFA* and *Scope-Pro*.

There are a number of prerequisites that are required or recommended before *AFA* can successfully start and acquire image sets. The following is a brief listing and discussion of the files *AFA* needs. The *AFA* wizard can assist you in defining all of these files, once *Scope-Pro* and *Stage-Pro* have been configured.

1. *Scope-Pro and/or Stage-Pro* must be installed and configured. This means that you can start either or both interfaces, and access all of the tabs. Please see your *Scope-Pro User Guide* for information about configuring *Scope-Pro* and *Stage-Pro*.
2. *Calibrations*, *Dye files* and *Lens files*
    a. Calibrations allow *AFA* to move your stage accurately and enable accurate post acquisition measurements in real spatial units.
        - *Scope-Pro* and *Stage-Pro* associate an *Image-Pro reference calibration* with each lens.
        - *Stage-Pro* requires a calibration for each lens that you will use before it will allow access to the majority of its tabs.
        - If you have a motorized nose piece, *Scope-Pro* will allow you to associate a capture driver and set of calibrations with each available light path.

Note: Even if you do not have a motorized nose piece, allowing *Scope-Pro* to manage your calibrations dramatically streamlines the setting of the proper system calibration. We recommend that you install the manual objectives component in *Scope-Pro* and follow the instructions in the *Scope-Pro User Guide* for configuring *Scope-Pro's* calibrations.

    b. *AFA* associates a dye file with each channel. These files specify data about your dyes that are useful in later post acquisition processing steps.
    Image-Pro Plus comes with a default set of common dye files, but you should make sure that there are dye files for the dyes you will be using. You can create your own dye files by choosing **Edit: Dye List** and clicking the **New** button.
    c. *AFA* associates a lens file with each channel. These files specify data about your optical system that are useful in later post acquisition

processing steps. You will have to create one lens file for each objective you plan to use with *AFA*.

3. *Scope-Pro* and *Stage-Pro* settings

a. *Scope-Pro* settings files `(*.scp)` allow *AFA* to control the configuration of your microscope during acquisition. You should have one `*.scp` fie for each channel that you plan to acquire with *AFA*.

Note: If you match the names of your Scope-Pro settings files to the Dyes that you will be using, AFA can automatically select your Scope-Pro settings file when you select the Dye as you define a new channel.

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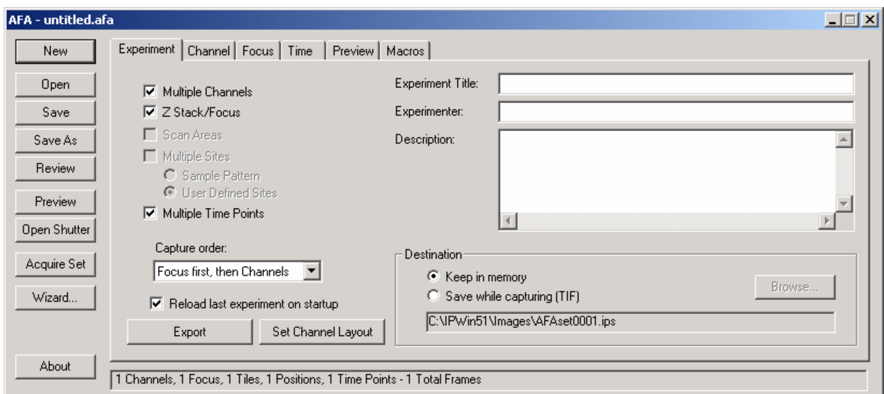
b. *Stage-Pro Scan Area* settings `(*.scn`) and *Stage Pattern* settings `(*.ptn)` files are used by *AFA* to control the movement of the X/Y stage. You may or may not need these files depending on your particular configuration and acquisition plans.

4. *Capture settings*. *AFA* directly controls the exposure time, and provides auto exposure controls. For all other capture settings, AFA relies on capture settings files `(*.vpf).`

How is Information Organized?

Information in AFA is organized by capture set, also known as an acquisition set. A capture set consists of a number of different channels — each with a possible different microscope, capture settings, position and time settings, as well as additional information such as color composite generation and the data storage options.

AFA saves these parameters in a settings file, with the extension .AFA (via the Save As button). An AFA settings file retains set acquisition information, while also linking to the Scope-Pro and Capture settings files with which it is associated.



Capture sets can be acquired, displayed, and stored by a single operation.

Overview of AFA

AFA represents an extension of *Image-Pro Plus'* capabilities. *Scope-Pro* controls the physical configuration of microscopes, filter wheels, and optical components. *Capture* controls the cameras and frame grabber boards.

AFA coordinates these functions, enabling complex captures of sets of images, multiple channels, each with distinct optical configurations, different Z positions, different time points, varied scan area positions, and different sampling positions (wells, slides, or user-defined positions) — with a single button press. *AFA* enables the integration of capture and viewing of data in a way not previously available with *Image-Pro Plus*.

AFA General Workflow

Load an AFA environment and check its settings or create a new AFA environment by following this procedure:

1. **Select your acquisition settings**. This sets which tabs are visible.
2. Configure channels with microscope settings (At least one channel is required).
3. Set exposure times and desired previews.
4. Configure the XY scanning pattern (Optional).
5. Configure XY position or well movement (Optional).
6. **Configure Z stack acquisition**, possibly including calibration (Optional).
7. Configure time lapse settings (Optional).
8. Save AFA environment (Optional).
9. Capture image set(s).
10. Save the set(s) to file.
11. Post-process as desired (Optional).

The *Capture, Scope-Pro, Stage-Pro,* and *Color Composite* dialogs may be invoked as part of the setup procedure.

The AFA Setup Wizard

The *AFA Setup Wizard* is designed to help you work with *AFA* more successfully, by ensuring that certain conditions and prerequisites are met before you start your *AFA* session. The wizard checks for:

- Objective definitions/lens files (`*.ipl` file). You should define at least one lens file for each objective.
- Capture settings (*.`VPF` file). You should have defined at least one capture settings file.
- Dye files. You must define at least one dye file`,(*.ipd` file) and if you are doing a multi-channel acquisition, you must define one dye file for each channel.
- *Scope-Pro* settings files (`*.scp` file). You must define at least one *Scope-Pro* settings file, and if you are doing a multi-channel acquisition, you will probably need to define one *Scope-Pro* settings file for each channel
- *Stage-Pro* Scan Area files (`*.scn` file) must be defined if you have selected X/Y Scan Areas for acquisition.
- *Stage-Pro* Sample Pattern files (`*.ptn` file) must be defined if you have selected Multiple Sites for acquisition, and the site type is set to Sample Pattern.

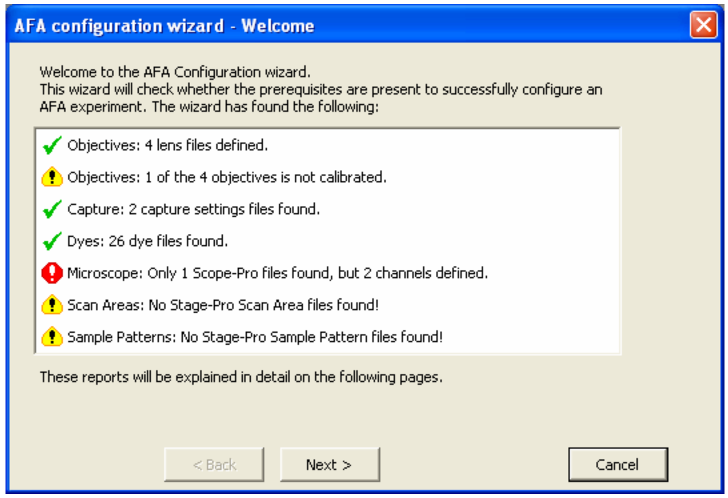
The purpose of the wizard is to help ensure that everything is properly configured prior to acquisition. The wizard will be displayed automatically when you start *AFA* for the first time in each *Image-Pro* session if there are any warnings or errors due to missing prerequisites. Canceling the wizard will not cancel *AFA* startup or the acquisition. Once your system is configured, the wizard will only appear under the following conditions:

- If it is invoked from the **Wizard** button on the *AFA* dialog
- If prerequisite files are moved or deleted
- You click **Acquire** and there are problems that prevent *AFA* from acquiring images using the current *AFA* settings.

The following pages are always shown in the following order:

Welcome Page

The first screen you will see is the **Welcome** page of the *Setup Wizard*:



The results of the prerequisites testing and checking are displayed in the list. The list may have a vertical scroll bar if necessary to display all of the results. All items with a satisfactory status receive the green check mark. Warnings are displayed with the yellow exclamation point, while critical errors receive the stop sign.

The warnings indicate items that may or may not cause problems during acquisition. Whether or not you encounter these problems will depend on how you configure the experiment. For example, the screen above indicates that one of the four objectives is not calibrated. As long as none of your experiments use that objective, you will experience no problems. However, it would be a good idea to resolve as many of the warnings as possible during your initial set up, so that you do not have to pause in the middle of an experiment to set up a new configuration.

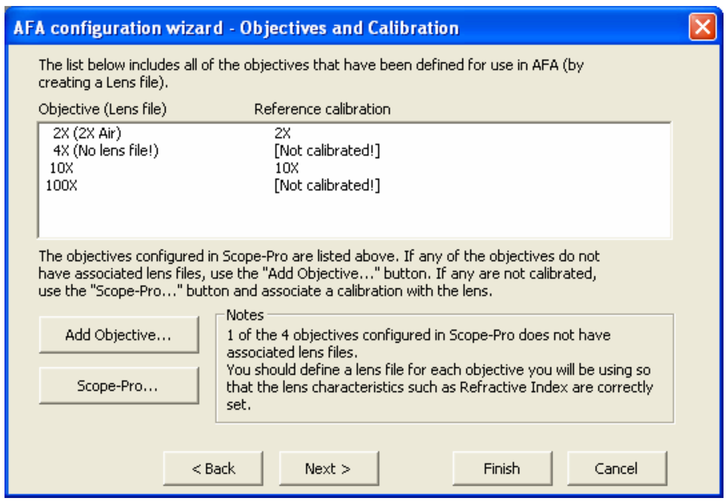
Depending on the parameters of your work, the `*.vpf` files may need to be updated for each experiment. The scan area and sample pattern files may be unique to each experiment or type of experiment, and may need to be updated also.

Critical errors are items which will prevent *AFA* from acquiring any images. For example, *AFA* identifies the different channels based on the dye file assigned to each channel. Not having at least as many dye files as there are channels means that *AFA* will not be able to identify each individual channel.

If no critical errors are found, the **Finish** button (shown in the figure for the **Objectives and Calibration** page) will be shown and you will be able to **Finish** or **Cancel** the wizard. Canceling the wizard with critical errors will result in a prompt asking if you really wants to exit the wizard. Click **Next** to continue.

Objectives and Calibration Page

The **Objectives and Calibration** page of the *Setup Wizard* will change its reporting format depending on whether *Scope-Pro* is controlling the objectives or not. The example below shows that *Scope-Pro* is controlling the objectives (the recommended configuration):



As noted previously, the objectives that are currently configured in *Scope-Pro* are checked for corresponding lens files. Exact matches are not shown, but a near match will be shown in parenthesis, as illustrated by the 2X objective. Objectives without a corresponding lens file will be noted (as with 4X in the illustration), as will objectives that do not have associated reference calibrations.

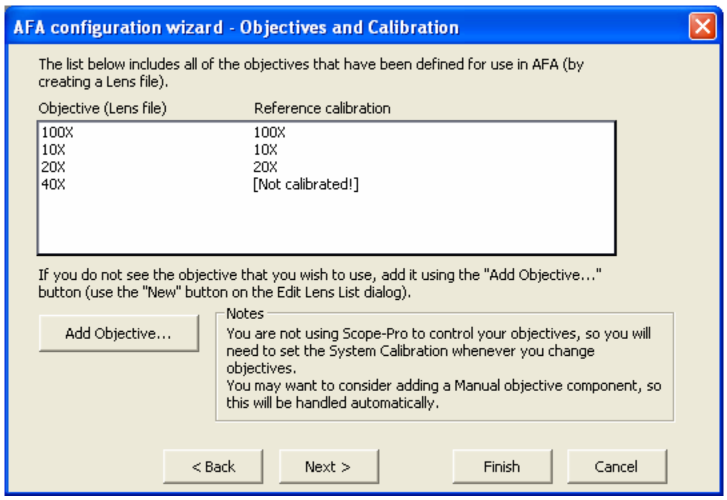
Click **Add Objective** to display the **Edit Lens List** dialog, and define new lens files or edit existing lens files.

Click **Scope-Pro** to open *Scope-Pro* so that you can associate existing calibration files with the lenses in *Scope-Pro*.

If you need to create new calibrations, choose *Measure:Calibration: Spatial Calibration Wizard* from the *Image-Pro* menu bar. The *Spatial Calibration Wizard* will walk you through the process of creating the reference calibrations needed by *Scope-Pro* and *AFA*.

The **Notes** group can only report one problem at a time, so it checks first to see if the objectives have lens files, and second, to see if *Scope-Pro* has a calibration associated. The **Notes** group may be blank if no problems are found. Click **Next** to continue

When *Scope-Pro* is not controlling the objectives, *AFA* can only check for lens files, and reference calibrations with similar names:



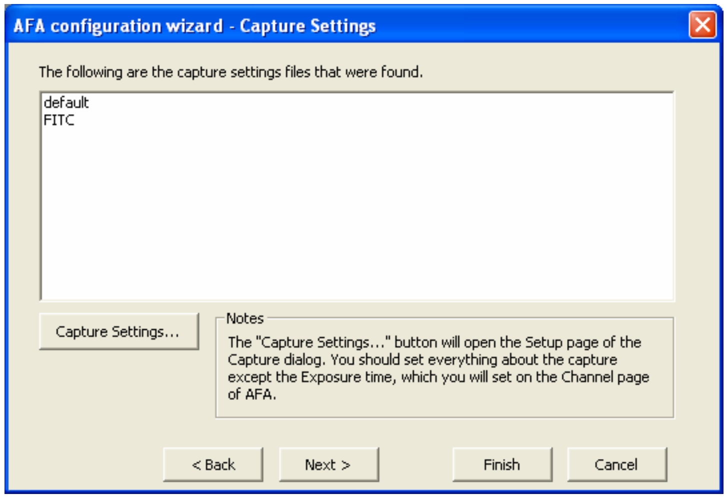
The results of the objectives check are displayed in the list. The objectives listed are the objectives that have lens files defined for them.

When *Scope-Pro* is not controlling the objectives, the warning that is displayed under **Notes** appears to tell you about the lack of an automatic system calibration. *AFA* will consider that a reference calibration for a given lens exists when a reference calibration is found whose name includes the full name of the objective. Click **Next** to continue.

Even if you do not have a motorized nose piece, you can simulate it with using *Scope-Pro's* manual driver, which displays a prompting dialog when asked to change a component. This is recommended because it provides *Scope-Pro* and *AFA* with much better control of spatial calibration.

Capture Settings Page

The following is the **Capture Settings** page of the *Setup Wizard*:

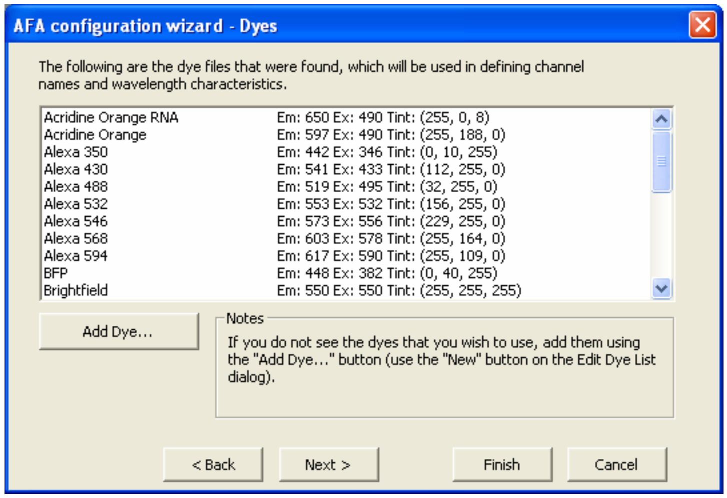


Click the **Capture Settings** button to display the **Capture** dialog. This dialog permits you to adjust and save your capture settings. Although *AFA* can acquire images based on the current capture settings, this is not the recommended method. Saving a capture setting file for *AFA to* use insures that you are working with a known capture environment.

The **capture** settings found by the wizard are displayed in the list. Click **Next** to continue.

Dyes Page

The following is the **Dyes** page of the *Setup Wizard*:

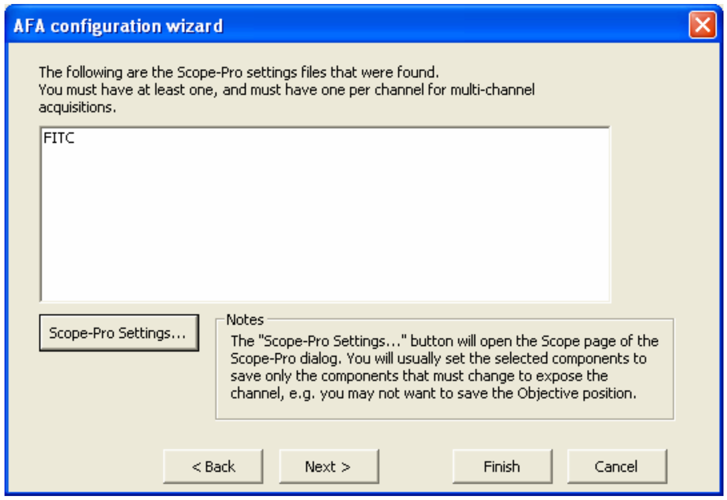


Dye files are necessary because they provide the names for the channels in *AFA*. They also provide data about how the images were acquired. This data may be useful in post-acquisition processing. If this list does not contain the specific dyes that you will be using, you may create and edit your own files by clicking the **Add Dye** button.

The **dye** files found by the wizard are displayed in the list, along with the **Emissions wavelength** (Em), **Excitation wavelength** (Ex) and the current tint. Click **Next** to continue.

Scope-Pro **Settings Page**

The following is the ***Scope-Pro* Settings** page of the *Setup Wizard*:

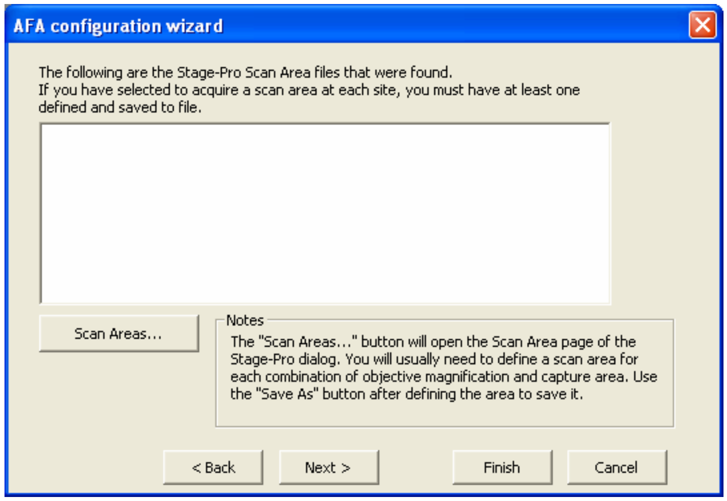


Scope-Pro settings are important in *AFA* because they provide a means for *AFA* to configure each channel for your microscope correctly.

The *Scope-Pro* settings files found by the wizard are displayed in the list. Click **Next** to continue.

Stage-Pro Scan Areas Page

The following is the ***Stage-Pro*** **Scan Area** page of the *Setup Wizard*:

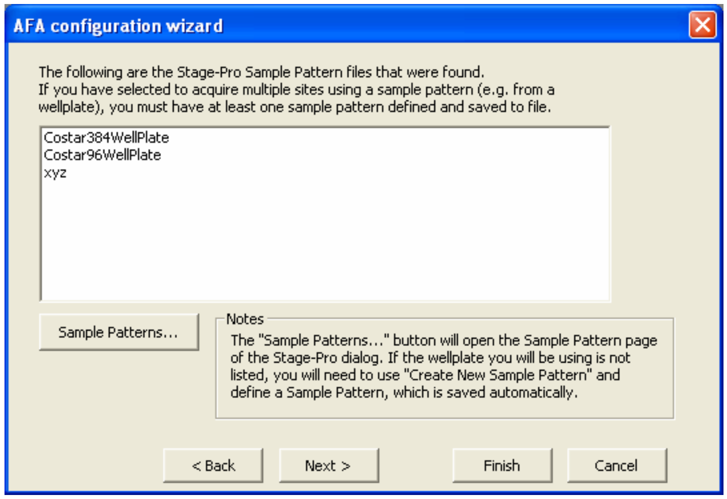


Scan area settings files are created by the ***Scan Area*** tab in *Stage-Pro*. They define the number and arrangement of images to be scanned at each user-defined or well position.

The ***Scope-Pro*** **Scan Area** files found by the wizard are displayed in the list. Click **Next** to continue.

Stage-Pro Sample Patterns Page

The following is the ***Stage-Pro* Sample Patterns** page of the *Setup Wizard*:

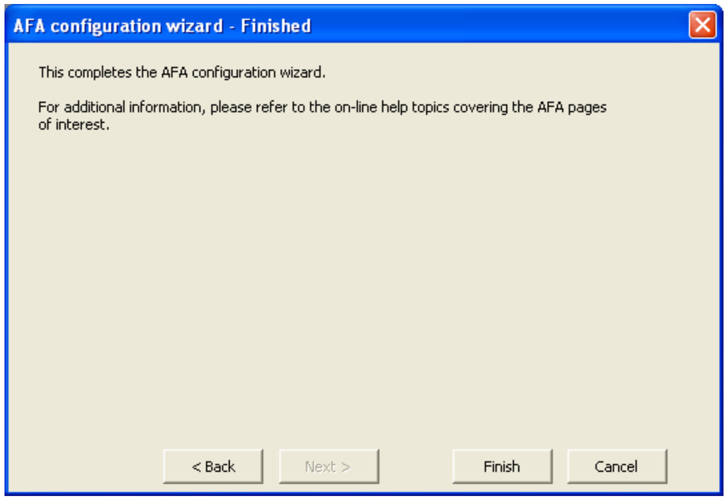


Sample pattern files define regular arrays of points to be visited during the acquisition process. They can be used in combination with scan area files to capture images of more than one field of view at multiple points on a well plate or slide.

The ***Stage-Pro* Sample Pattern** files found by the wizard are displayed in the list. Click **Next** to continue

Finish Page

The following is the **Finish** page of the *Setup Wizard*:

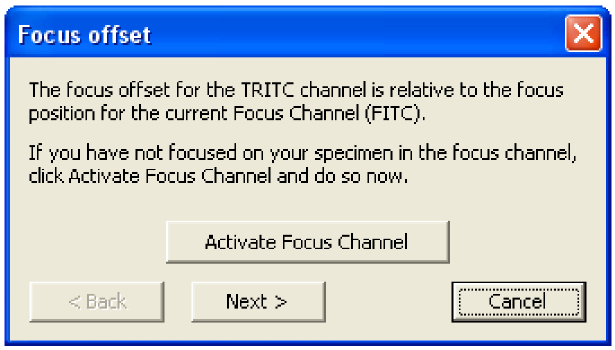


Click **Finish** to go to the main *AFA* page.

Focus Offset Wizard

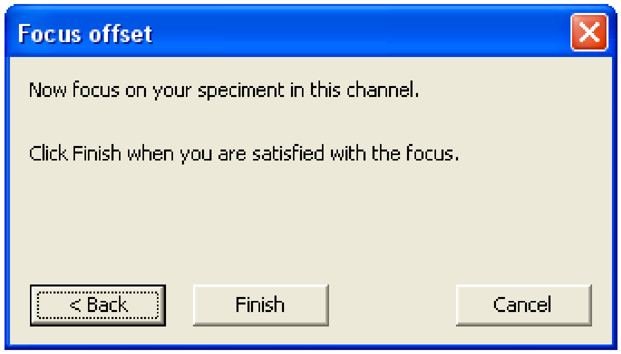
Focus Offset Wizard: The *Focus Offset Wizard* is used to set the focus offset for a channel. The focus offset is relative to the current focus channel, whose offset is fixed at 0.0 (and is therefore not displayed on the ***Channel*** page). The wizard is intended to help with chromatic changes in the focus level, or where the objects of interest are at different depths, as with nuclei and cellular attachment points.

The first page of the wizard is illustrated here:



This page of the wizard introduces the concept of focus offset, and allows you to adjust the focus of the **Focus Channel** . This will become the reference for the focus positions for all other channels. If you click **Activate Focus Channel**, the focus channel is previewed, just as it would on the **Preview** page. Then you can adjust the focus of the focus channel prior to clicking **Next >.**

When you click **Next >**, the Z focus position for the focus channel will be updated, if you chose to **Activate Focus Channel**. The preview will be switched on if it is not already on, and switched to the currently selected channel, and the second and last page of the wizard will then be displayed:



You will be prompted to adjust the focus for the current channel. When you click **Finish**, the Z focus position is captured and the difference between the current channel's focus position and the **Focus Channel's** focus position calculated. This delta value will be used as the focus offset for this channel. The dialog will close and the focus offset for the selected channel will be updated on the **Channel** page.

AFA Sample Sessions

This section includes:

- Sample sequence sessions for using *AFA*
- A flowchart explaining how *AFA* image acquisition is done
- A brief explanation of the three demonstration macros included with *AFA*.

Sample Session #1: Multi-Plane and Multi-Channel Sequence

1. Configure *Scope-Pro.*
2. Configure *Stage-Pro.*

Note: Specify filters, stage calibration, shutters, motors and other components.

3. Create *Scope-Pro* configurations for various modes of observations. Each will represent a microscopy mode, such as brightfield, DIC, or various fluorescent filters
   - Create configurations for different dye colors, different filters, different lenses, etc.
   - Save these with understandable file names (e.g. for a channel using the FITC dye, name your settings file `FITC.scp`).
4. If desired, do the same with **Capture** settings.
   - Change whatever non-exposure settings desired.
   - Save these as camera configuration files (**`name.VPF`**).
   - Note that the image size must be the same for each capture area used, so be careful, if you are selecting a sub-region, to use the same region for each `.VPF` file.
5. Start *AFA*

Note: *AFA* will initialize *Scope-Pro* and *Stage-Pro* upon startup. If neither is installed, *AFA* will not launch. If either is not configured, *AFA* will prompt you to configure them.

6. Configure the various acquisition dimensions. For this example, select **Multiple Channels** and **Z Stack/Focus** on the *Acquire **tab***
7. Add channels from the ***Channel tab***.

8. Click on the ***Focus*** tab.
9. Click on the range to include the number of planes and micron spacing desired for a Z Stack.

Note: This can be set explicitly or you can use the **Set Range** button to cover the distance. For the purposes of this experiment, set your range to 3-4 planes at a reasonable spacing. Select **Retain full stack** and do not focus while acquiring.

10. Click on the ***Channel tab***. Use the table in this tab to set the exposure options.
11. Click on **Acquire Set**
12. Open *Advanced: Set Navigation*. In *Set Manager*, you will be able to browse the set. *Set Manager* opens automatically after the set is acquired.
13. Save the set using the *Set Manager Info:File* page and save the *AFA* settings using **Save As** in the **AFA Dialog** box.

Note: if you click on the **Review** button, it will list the parameters of acquisition to the output window.

Note: The default order of acquisition is channels, focus, scan area, samples/wells and time. This can be changed on the ***Acquire tab*** to **Focus first** if you wish higher accuracy in focus (less time for drift) or if you are using a manual filter changer. **Channels First** provides better color registration. Depending on the order and the microscope used, one method may be faster than the other. You should take this into consideration when performing time-lapse experiments.

Sample Session #2 - Time Lapse Acquisition

If you would like to perform *Time Lapse Acquisition* add the following steps to Sample Session #1 in chapter 2.

1. Select **Multiple Time Point**s on the ***Experiment tab***.
2. On the ***Time*** tab, select the number of time points you need.
3. Set the interval between time points.
4. Optionally, test the timing to see if the time interval is sufficient.
5. Optionally, add an additional phase with different time points.
6. When you acquire, you will do an acquisition at each time point. Add other dimensions, or acquire and save as described in Sample Session #1, steps 11 through 13.

Sample Session #3 - Multiple Sampling Positions – User-defined Positions

If you would like to acquire at multiple user-defined sampling positions, add the following steps to Sample Session #1, steps 1 through 5.

1. Select Multiple Sites on the ***Acquire tab,*** then select User-Defined Sites.
2. Click on the ***Stage*** tab.
3. Move stage (while using Capture Preview or the eyepiece to view your sample) to the desired locations.
4. Add each desired location to the list.

Note: You can use the scroll buttons to review positions.

5. When you acquire, you will do an acquisition at each of these sampling positions. Add other dimensions, or acquire and save as described in Sample Session #1, steps 11 through 13.

Sample Session #4 - Multiple Sampling Positions – *Stage-Pro* Sample Pattern

If you would like to acquire at multiple positions defined by a *Stage-Pro* sample pattern, add the following steps to Sample Session #1, steps 1 through 5.

1. Select Multiple Sites on the ***Acquire tab,*** then select Sample Pattern.
2. Click on the ***Stage*** tab.
3. Select a pattern from the combo box. If you do not see the sample pattern you want to use, you can click the Sample Setup button to define a pattern in *Stage-Pro*.
4. When you acquire, *AFA* will do an acquisition at each of the sampling positions defined by the sample pattern. See the *Stage-Pro* section on Well Plate Analysis.
5. Add other dimensions, or acquire and save as described in Sample Session #1, steps 11 through 13.

Sample Session #5 - Multiple X/Y Locations – Stage-Pro Scan Area

If you wish to traverse a scan area at each sampling position, add the following steps to Sample Session #1, steps 1 through 5.

1. Select Scan Areas on the ***Experiment tab.***
2. Click on the ***Stage*** tab.

Note: If the object you're looking at is bigger than the field of view, you may want to acquire multiple tiled images at each location to capture the entire object.

3. Select Scan Area for the area you wish to capture.

Note: If you do not see the scan area you want to use, you can click on the Scan Setup button to define a scan area in *Stage-Pro*.

4. To tile these images and create a single large image at each sampling position, select the Tile Image check box.
5. When you acquire, *AFA* will do an acquisition at each frame of the scan area. Add other dimensions, or acquire and save as described in Sample Session #1, steps 11 through 13.

Note: The scan pattern must be created for the objective that will be used. Otherwise, the calibrated distance between the tiles will be incorrect.

Scope-Pro And Stage-Pro

Scope-Pro (the *Image-Pro® Plus* Microscope Controller Module) lets you use *Image-Pro Plus* to control and program the movement of your microscope. *Scope-Pro* also allows you to install and configure various components to control shutters, sliders, filter wheels, lamps, apertures, and other microscope hardware.

Scope-Pro incorporates the *Stage-Pro* functions, which allow you to control the movement of your trinocular microscope's motorized stage. It provides an environment in which your microscope can be completely controlled from the PC — virtually eliminating the need to put your eye to the ocular.

Moreover, repetitive stage movement can be automated by recording the motion in a macro. You can even use this capability to create a large, high-resolution image from a series of "tiles" collected from a single specimen. The *Stage-Pro* component, (which can be configured at the same time or anytime thereafter) permits you to control the motorized stage and Z focus controllers.

Repetitive movements for both microscope and stage can be automated by recording the motion in a macro.

If you configured an X/Y stage, ***Stage-Pro*** is installed and you will need to refer to the section titled *Working with Stage-Pro*.

If you configured any non-stage hardware, or only configured a Z-focus component, ***Scope-Pro*** is installed and you should refer to the section titled *Working with Scope-Pro*.

You will want to review both sections if your configuration installed both programs.

Product Features

Your *Scope-Pro* module provides:

- Support for the industry's leading microscopes.
- Easy and precise control of the objectives, filter wheels, shutters, lamps, and other microscope hardware.
- Support for the auto-focus process on hardware equipped with auto-focus circuitry.
- A set of function calls that can be used to drive the microscope from an *Image-Pro Plus* macro or a Visual Basic™ program.

System Requirements

- To install and work with *Scope-Pro/Stage-Pro*, you will need the following equipment and software:
- Pentium III CPU system (running at 1 GHz or higher), a screen resolution of 1280 x 1024 or higher and *Image-Pro Plus* version 7.0 or later.

A motorized microscope, and/or some of the following hardware controllers:

— EcoStep stage controller
— Leica DM RXA
— Leica 2 Series (DM-RXA2, DM-RA2, DM-LA, and DM-IRE2)
— Leica DM 5000B/DM 6000 B
— Ludl Biopoint stage controller
— Ludl Mac 2000, 2002, or 5000 controller for stages, filter wheels, and shutters
— Media Cybernetics stage controller
— Märzhäuser ITK Multicontrol 2000 and LStep stage controllers
— Nikon E1000/E1000M microscope
— Nikon TE2000 microscope
— Nikon RFA focus accessory
— Olympus Provis AX-80

- — Olympus BX-61
- — Olympus IX-81
- — Prior Proscan H128, H129, or H130 series stage controller
- — Prior Optiscan stage controller
- — Sutter Lambda 10-2 and 10-3 filter wheels and shutters
- — Sutter Lambda DG-4 high speed filter changer
- — Vincent Associates shutters
- — Vincent D3 shutter controller
- — Zeiss Axioplan2 microscope
- — Zeiss Axioimager 2 microscope
- — Zeiss Axiovert microscope
- — Zeiss MCU-27, MCU-28, and MCX-2 stage controllers.

Other types of microscope equipment may be added to this list in the future. Equipment that is not currently supported or is not motorized can be represented by a "manual" component.

Before Installation

Before you begin installing, you ***must already*** have the following items correctly installed, configured, and functioning:

- *Image-Pro Plus* version 7.0 or higher
- *Capture* version 7.0 or higher
- The appropriate *Windows* system drivers required by your hardware.

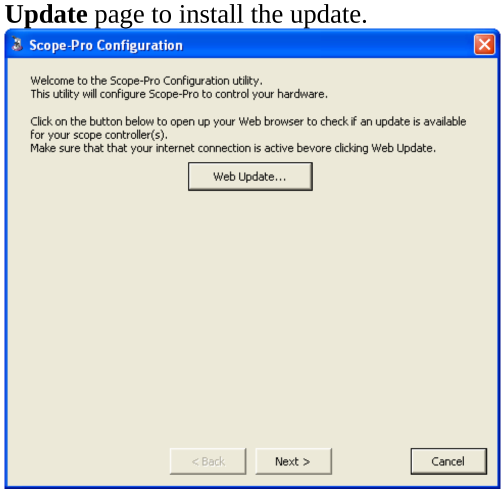
Make sure that your microscope is working properly beforehand!! We also recommend that you create some reference calibrations for each of your objectives.

Scope-Pro Configuration Utility

The *Scope-Pro* configuration set-up continues with these steps:

1. You will see the *Welcome* dialog. Click **Next** to continue.

2. *Scope-Pro* will give you the opportunity to use your web browser to access the *Media Cybernetics* web site to check for an update to your microscope controller. Click the **Web Update** button, and follow the instructions on the **Update** page to install the update.



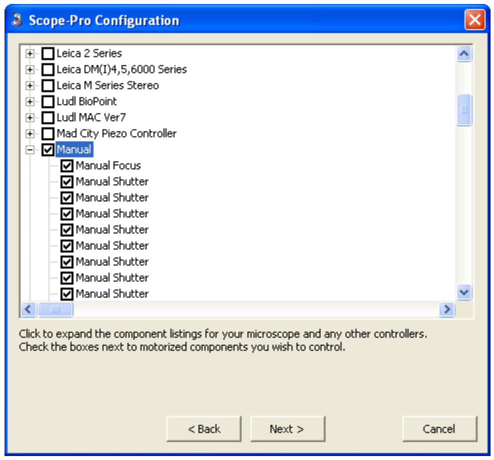
3. Click **Next** to continue.

4. Click **Web Update** to search our web site for updated drivers.

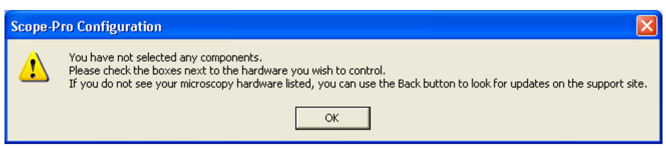
5. Select the hardware controllers and components that you have available.

6. Click in the small box that appears before (to the left of) the name of each stage and/or scope hardware controller or component that is part of your microscopy system. Note that there is a scroll bar to allow you to see the rest of the list of components.

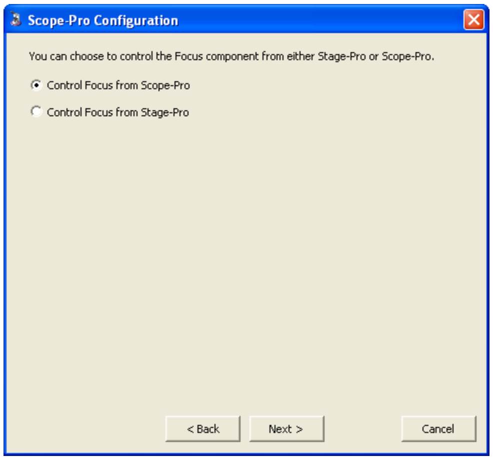
As you highlight each controller, its description will be shown in the **Description** area, but the controller component is not selected for installation until there is a **check mark** in the box next to it:



7. Click **Next.**
If you have forgotten to select a controller or component, you will be informed and given the option to go back and complete the selection. ***Setup*** cannot continue without at least one controller selected. Press **OK** to return to **Step 17**.



8. Select the focus component's location.
When the configuration includes an X/Y stage, Z focus, and one or more *Scope-Pro* components such as shutters or filter wheels, the Z **Focus Selection** page is displayed next:

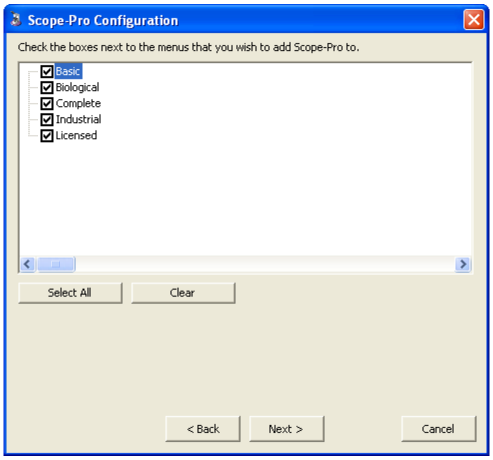


This page is skipped if it is not needed. If you have both a X/Y stage and one or more Scope components, other than the Z Focus component, you can choose to control the Z Focus component from within either the *Scope-Pro* or *Stage-Pro* user interfaces.

The *Stage-Pro* interface is the default location for the Z Focus component, and has a much richer set of controls for the Z Focus. If you do not have an X/Y stage, you can install the dummy X/Y stage, which will then give you the option of putting the Z Focus in the *Stage-Pro* interface.

9. Click **Next** to continue.

10. Select the appropriate menu.
When *Scope-Pro* has been installed to a product supporting multiple menus, the **Menu Selection** page is displayed next:

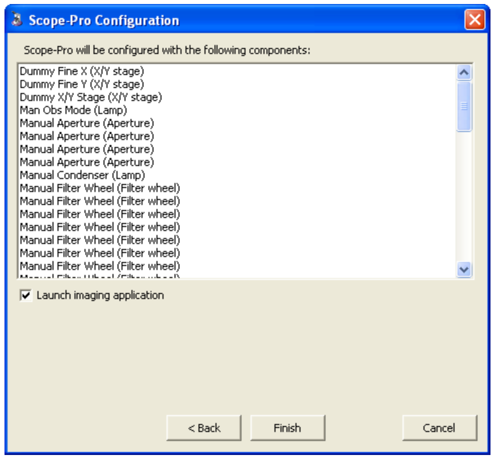


This page is skipped if it is not needed, e.g. if there is only a single, fixed menu similar to earlier *Image-Pro* versions. You may choose which menus to modify to add the *Scope-Pro* menu items. Menus that already have the *Scope-Pro* menu items installed will be checked automatically so that the menu selection need only be made once. When you click the **Next** button, you will be warned if no menus have been selected.

11. Click **Next** to continue.

12. Review your selections.

A **Review** page appears, summarizing the selected configuration prior to making any of the changes:



The top section of the review page lists the selected components, sorted alphabetically, followed by a listing of the menus that have been selected for modification. Note that the **Next** button is replaced by **Finish**, and that it is still possible to **Cancel**. Note also the option to run *Scope-Pro* automatically in the imaging application.

13. Click **Finish**.
The *Stage-Pro* and/or *Scope-Pro* components will be installed to your *Image-Pro* directory, *Stage-Pro* and/or *Scope-Pro* configured for the components installed, and menu items added to *Image-Pro*. The "*Stage-Pro*" and/or "*Scope-Pro*" menu items will be found in *Image-Pro's* ***Acquire*** menu.

14. *Setup* completed.
You can now run *Image-Pro* from the previously installed icon or **Start** menu item, and access *Stage-Pro* and/or *Scope-Pro* from the ***Acquire*** menu items mentioned in **Step 19**.

Working with *Scope-Pro*

This section describes the procedures for starting the microscope controller module, *Scope-Pro,* for the ***first*** time.

Before you select the "*Scope-Pro*" menu item, be sure that all of the configured controllers have been properly connected to the PC, turned on, and have been setup (as necessary). For setup procedures, consult the manufacturer's manual. Once all of the hardware is connected and configured, start *Image-Pro Plus*, and go the ***Acquire*** menu.

The first time the "*Scope-Pro*" command is invoked, the *Scope-Pro* components are not yet configured. You will see an error message stating that you need to configure *Scope-Pro*; it will offer you a choice between configuring *Scope-Pro* manually and using the ***Configuration Assistant****.* You will then be presented with the ***Configure*** tab page. The Configure program lets you determine which hardware components you have in your system, but you will still need to supply important about each component.

Information that you may need to complete this configuration includes:

- The PC serial port to which each controller is connected. You may also need to know how the communications parameters must be set for each controller, if any of the parameters are changeable via switch settings, etc. or a
- USB port
- Other controller-specific settings, such as card slot or address, type of component (i.e., type of filter wheel, or number of filter wheel positions), etc.

The first part of this section describes the process of completing the configuration of your *Scope-Pro* system, and common controls or groups of controls that may be used to configure each of the different types of components.

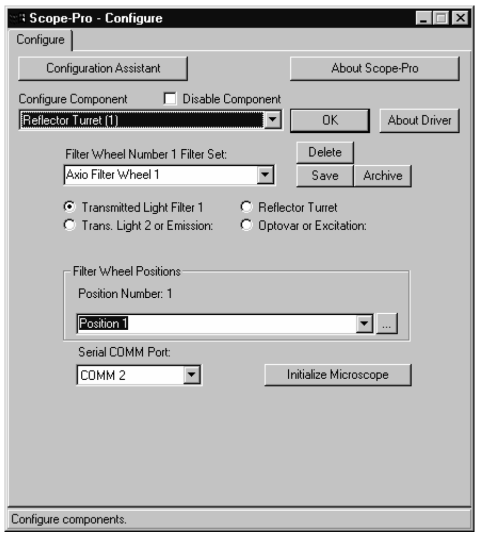
The most crucial items at this point relate to communications port configuration. Each separate controller component will need a separate port, and all will default to the **COMM 1** serial port. Check your cabling to determine the correct port for each controller. You should also check your hardware documentation for other serial port settings if applicable.

Many of the newer devices supported by *Scope-Pro* require installation of Windows system drivers that *Scope-Pro* needs in order to communicate with the device.

Configuring *Scope-Pro*

To begin setting up the *Scope-Pro* module, follow the steps below:

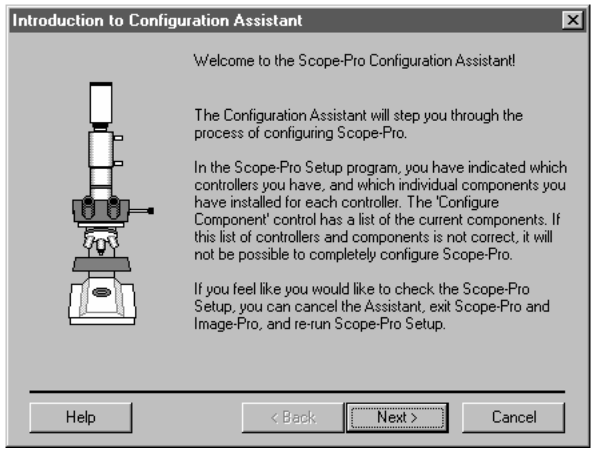
1. Select the “*Scope-Pro*” command in the *Acquire* menu.
   Scope-Pro will detect that the newly installed components have not been configured.



15. Configure each component.
    As described in the following section, each installed component will have its own configuration controls, which are displayed on the configuration page. The Configuration Assistant is designed to help you select each component from the Configure Component drop-down list, and verify that each is set correctly (or at least adequately).

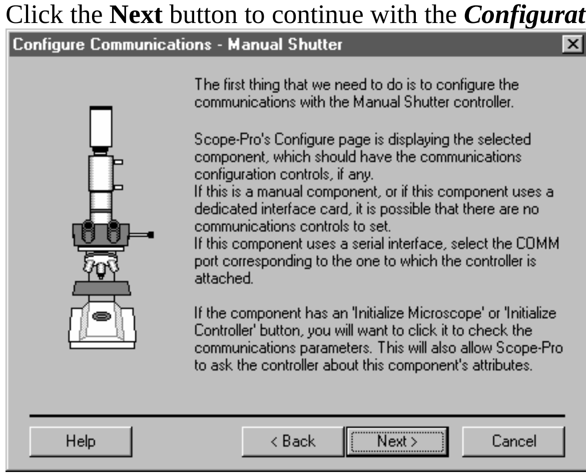
Note: *The remainder of this section describes the configuration process using the* ***Configuration Assistant****. If you want to configure Scope-Pro manually, please go to the section titled* ***Selecting Components*** *.*

Clicking the **Configuration Assistant** button invokes the following series of screens:



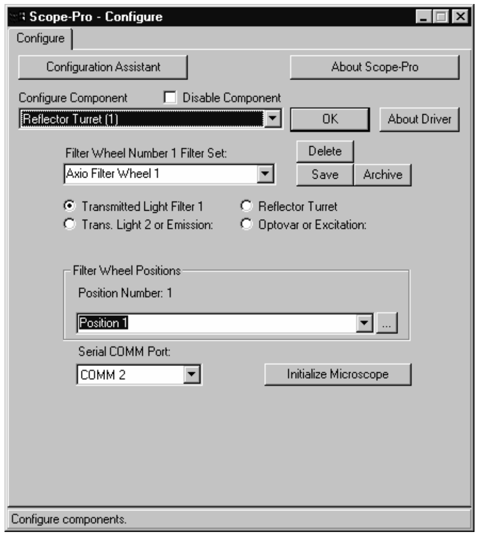
The ***Configuration Assistant*** is designed to help you set up the controllers that you selected when you installed *Scope-Pro*, and initialize the various components of each controller.

16. Click the **Next** button to continue with the ***Configuration Assistant***.



The first step of configuration establishes communication with each of the controllers. The communications configuration for each controller will be displayed on the ***Configure*** tab page for the first component in the **Configure Component** list for each controller. The ***Configuration Assistant*** will display a **Configure Communication** page for each controller that you installed when you set up *Scope-Pro*. It will also display the *Scope-Pro* ***Configure*** tab page with the appropriate component selected:

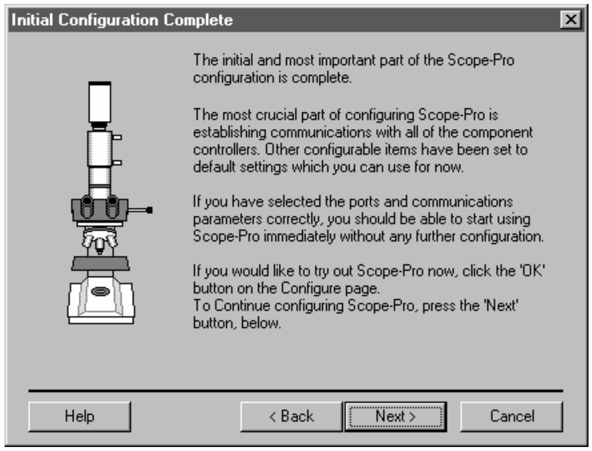
17. Click the **Next** button to continue with the ***Configuration Assistant***.



18. Select the appropriate component settings, and click Next on the Configuration Assistant page. ***Configuration Assistant*** will automatically select the next item to be configured.

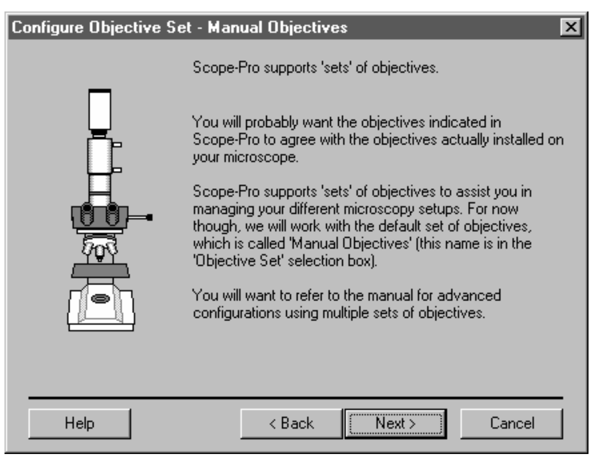
Note: *The illustration above is a representation of the types of controls for a filter wheel component that may be present on the* ***Configure*** *tab page. The next section discusses the different types of controls that may be present for each type of component.*

After configuring the communication with each controller, you have the option to try out *Scope-Pro* now; using each component's default configuration, or going on to refine the configuration, as indicated here:



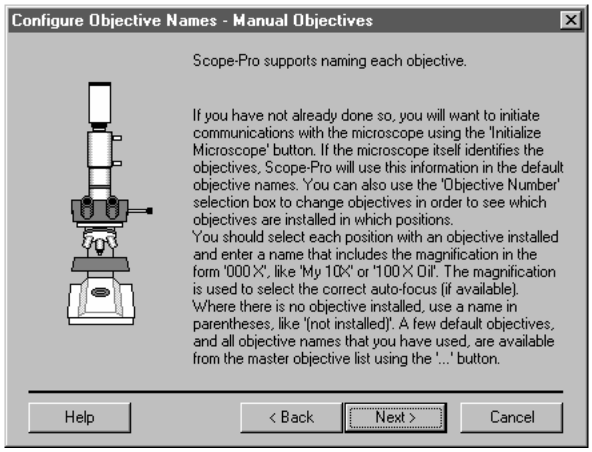
19. If you decided to try out *Scope-Pro* using the default configuration settings, click **OK** on the ***Configure*** tab page. The message, "Configuring *Scope-Pro*" will appear.
When finished, *Scope-Pro* will check for any errors. If any errors are found, *Scope-Pro* will display an error message, and indicate which component is reporting the error on the ***Configure*** tab page. If the ***Configuration Assistant*** is still running, it will display a page indicating possible causes for the error.

20. Click **Cancel** to stop the ***Configuration Assistant***, or **Next** to configure other components.
After the communications, the ***Configuration Assistant*** chooses to configure the objectives, if installed:

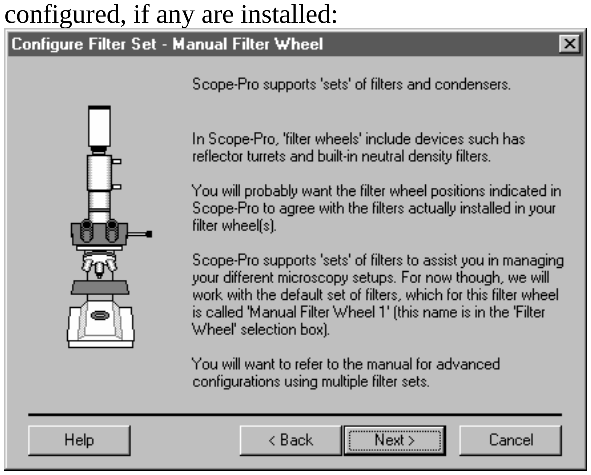


Again, the appropriate part of the ***Configure*** tab page will be displayed.

The next step is to name the objectives:

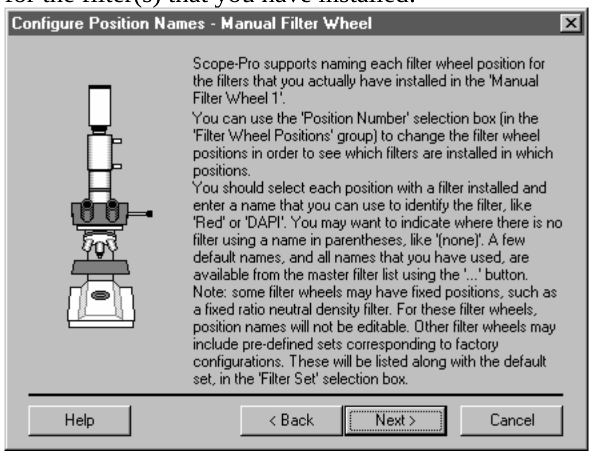


21. Click **Next** to continue. The filter wheels are the next component to be configured, if any are installed:

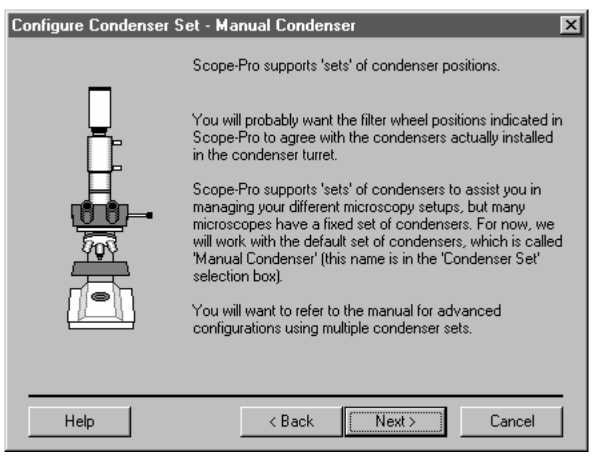


The ***Configure*** tab page displays the next filter wheel component that you chose during the setup process.

22. Name the filter set (as indicated below) and each position of the filter wheel for the filter(s) that you have installed:

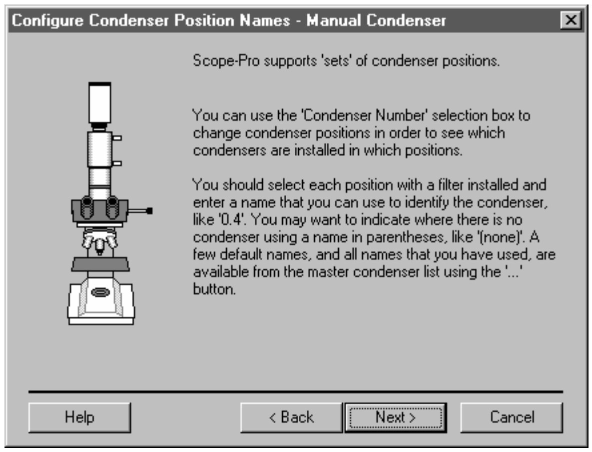


23. Click **Next** to continue.
The ***Configuration Assistant*** will also help you set up a condenser, if one is installed:

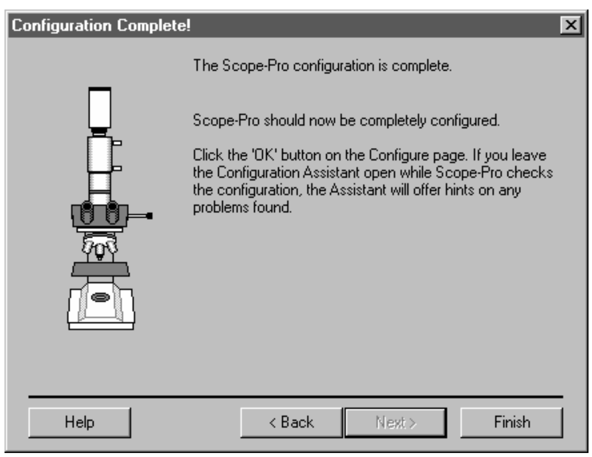


The condenser component is selected on the ***Configure*** tab page.

24. Click **Next** on the ***Configuration Assistant*** page to name and save your condenser settings.
You will probably want to save your condenser settings according to the number of positions available.



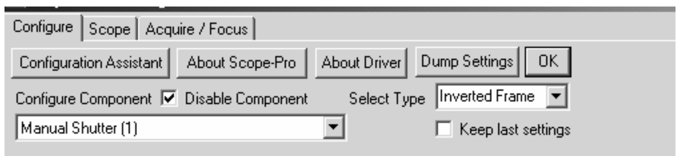
25. Click **Next** on the ***Configuration Assistant*** page to continue.
The ***Configuration Assistant*** indicates that the configuration process is complete:



26. Click the *OK* button on the *Configure* tab page.
Scope-Pro will attempt to initialize each of the controller components. When all of the controller components initialize successfully, the ***Scope*** tab page will be enabled, and you can proceed to use *Scope-Pro*.

Selecting Components

The *Scope-Pro* ***Configure*** tab page and the ***Configuration Assistant*** work together to support configuring each of the components that you have installed separately. The top portion of the ***Configure*** tab page is fixed, and lets you select which component to configure. One component at a time is configured, and will display whatever controls are appropriate for configuring that component on the rest of the ***Configure*** tab page. The fixed portion of the ***Configure*** tab page is illustrated below:



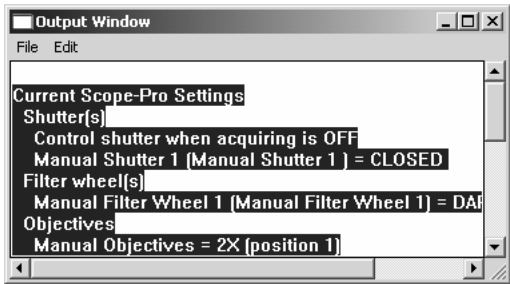
The installed components are listed in the **Configure Component** drop-down list, by groups according to the type of component. The name of each individual component will include manufacturer information to help identify the controller for the component. When a component is selected from the list, the component-supplied configuration controls will be displayed in the lower portion of the ***Configure*** tab page. Examples are provided in sections discussing each type of component, below.

The ***Configuration Assistant*** button launches that feature, which is described in the preceding pages.

The **OK** button is used to inform *Scope-Pro* that all of the components have been configured, and that *Scope-Pro* should try to initialize (details below).

The **About Driver** button is used to display a dialog containing information about the installed driver for the currently selected component.

Dump Settings: Click this button to send a text listing of the current *Scope-Pro* settings to the **Output Window**, as shown here:



Disable Component: Click this button to disable the component selected in the **Configure Component** list box. You may use this option to selectively disable components that will not configure because the corresponding hardware is either not present or not responding.

Keep last settings: Check this box to retain the hardware settings from your last *Scope-Pro* session.

Select Type: Choose a microscope type from the drop-down list box to use the *Graphical User Interface* (GUI) to set up your components. You will need to restart *Scope-Pro* after selecting an item from the list. The configuration GUI is described in the section *Using the Configuration GUI* at the end of this chapter.

Configure Component: The **Configure Component** control will list all of the components that you installed in the *Scope-Pro* ***Setup*** program. Select the specific component from the list. The lower portion of the ***Configure*** tab page will display whatever settings are required to configure the selected component

The Configuration Process

Whenever *Scope-Pro* is invoked, the software will attempt to initialize all of the configured components. If the initialization fails for any reason, the ***Configure*** tab page will be displayed and the ***Scope*** and ***Acquire/Focus*** tab pages will be not be available.

Initially, none of the installed components will have been configured, and *Scope-Pro* will display a message to this effect. Each component will need to be configured, but *Scope-Pro* and the individual components have been designed to make that as easy to complete as possible.

Whenever any component from a specific controller is configured for the first time, all of the configurable parameters for **ALL** of that controller's components will be defaulted for you. In most cases, this will mean that to start working quickly, you will only have to set or verify the communications parameters for each controller (see Serial Port Configuration below).

However, while these default configurations will get you working quickly, they cannot in most cases accurately reflect your actual configuration, and a message to that effect will be displayed whenever a controller's configuration has been defaulted. You will want to refer to the specific component section later in this section to see how you will want to refine the configuration, and for tips managing different configurations or working sets as you attack different types of experiments in the lab.

To fully default your **entire** configuration, you will need to select at least one individual component from each of the controllers in your setup. For instance, if you have an automated microscope and a separate shutter or filter wheel, you will want to select the first component listed for each controller, which will default all of the other components' parameters. Selecting the first component listed for each controller also allows you to check the communications parameters for each controller. Since there are two controllers, and they will both be defaulting to COMM1, at least one COMM port setting will need to be changed.

When you believe that all of the components have been configured correctly, click the **OK** button. *Scope-Pro* will then attempt to initialize all of the installed components as before. If any of the components fail to initialize after the initial configuration is complete, and if the component can be disabled without affecting other components, you will be allowed to continue working while that component is disabled. However, if the component cannot be successfully disabled, or the failure is a communications error, you will be returned to the ***Configure*** tab page, and the ***Scope*** and ***Acquire/Focus*** tab pages will remain disabled (hidden)

When you have successfully configured and initialized all of the installed components, the ***Scope*** and ***Acquire*** tab pages (described in the next section) will be displayed. You will probably still have occasion to return to the ***Configure***, whenever you make changes to your configuration, such as adding, removing, or changing objectives or filters, etc.

User-Defined Names

Some of the information maintained by the ***Configure*** tab page includes user-friendly names for the components and/or their positions or settings. For instance, you can give a user-friendly name for each filter wheel to help identify it by its position in the light path. You can name each position of that filter wheel by the particular filter that is installed, and each objective by its type and magnification.

For some components, their sub-components (i.e., the particular filters installed in a filter wheel, or the particular objectives in an objective turret) can change from configuration to configuration along with the requirements of different experiments. For these components, you will be able to save different sets of filter or objective names corresponding to the different configurations under different filter wheel or objective set names. All of the configurations saved in this manner will be available for easy recall later. In addition, *Scope-Pro* will be accumulating a “master list” of the filters and objectives used in your filter or objective sets to make it easy to select any of these later.

The Initialize Microscope Button

Many components will have an **Initialize Microscope** or **Initialize Controller** button on their ***Configure*** tab page. This button is supplied when the controller allows you to verify which accessories are actually installed, and/or their characteristics.

When this button is present, you will want to configure that component in several stages:

- On the ***Configure*** tab page for the controller's first component, set all of the serial communications parameters according to which port is in use and the controller's switch settings (if any).
- Make sure that all accessories are free to move and not locked down or physically blocked, and then click **Initialize Microscope** (or **Initialize Controller**).
- If the initialization fails, check cabling and communications parameters.
- Select each of the remaining components managed by this controller and verify the component-specific parameters. Whatever can be determined from the controller will be used to default the settings.
- Click **OK** when done.

Manual Control

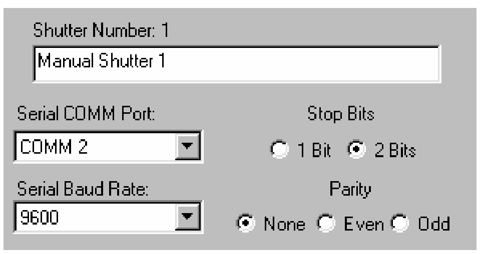
In many cases, your microscope configuration will have one or more accessories that are not motorized and must be manually controlled. *Scope-Pro* provides a special component to support this type of configuration, and the configuration for this component is featured in many of the illustrations in this section. When you install this component to support the manual accessories, you will be prompted to position the accessory whenever a change in position is necessary. This component can be used to assure that the manual portions of a microscope configuration is returned to a known state, i.e., for the setup of a particular experiment or procedure. However, this component is inherently not conducive to automation, so manual components are not recommended for the components that will change with every exposure, such as shutters and/or filter wheels.

Serial Port Configuration

The communications port configuration will be maintained by the first of the components listed for each controller (as they are listed in the **Configure Component** drop-down). The controls necessary to configure the communications will be added to the bottom of the set of controls normally required for the component in question.

You will need to specify the computer's COMM port used to communicate with the controller, at the very least. Note that in many cases, some or all of the serial communications parameters shown above are fixed by the controller. In such cases, you may only have to specify the communications port.

The following illustrates a typical configuration dialog for a shutter controller with serial port controls added:

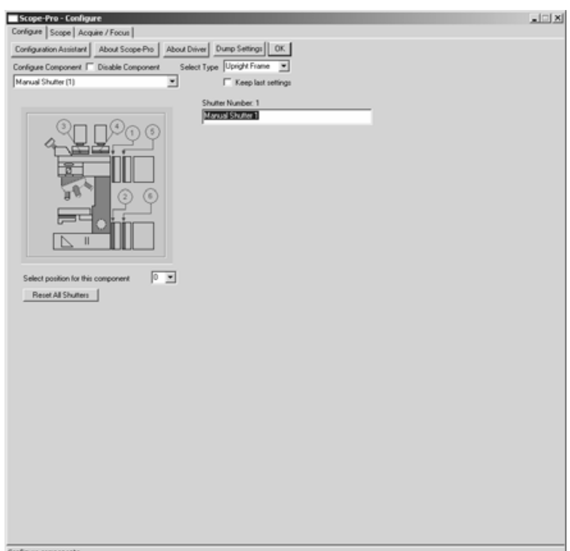


Using the Configuration GUI

Scope-Pro 7.0 includes a configuration GUI that enables you to see how your hardware components are set up in your microscopy system. To use the configuration GUI, select one of the microscope types from the drop-down list on the ***Configure*** tab page:

You will see a message asking you to restart *Scope-Pro*. You do **not** have to restart *Image-Pro Plus.*

After you have restarted *Scope-Pro*, the ***Configure*** tab page will display a diagram, something like this:



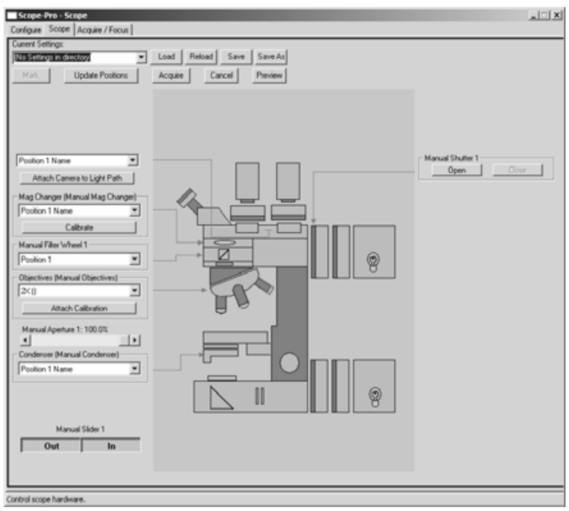
When you select a specific component from the **Configure Component** drop-down list box, the possible positions for that component are displayed on the diagram. In the example above, the manual shutter has 6 possible positions on the microscope.

Select position for this component: Choose one of the numbered positions for the component (the manual shutter in this example) from the drop-down list box.

Reset all shutters: Clicking this button will reset the location of the manual shutter back to 0.

Once you have chosen the positions for all of your components, you can switch to the ***Scope*** tab.

Scope-Pro will display the ***Scope*** tab dialog, with the diagram indicating the position of your components. The manual shutter location appears on the right side of the diagram, as shown here:



Other selected components are also displayed on the ***Scope*** tab page diagram. To change the position of any of them, return to the ***Configure*** tab page, and choose a component from the **Configure Component** drop-down list box. Then you can select a location for that component as you did for the manual shutter.

Note: The first time that you switch to the new GUI, all components that can be assigned to multiple positions will be set to 0 (not used). They will not appear on the **Scope** tab page. To display these components on the **Scope** tab *page*, switch to the ***Configure*** *tab page* and assign each component to a real position.

Working with Stage-Pro

Introduction to the *Stage-Pro* Module

Stage-Pro is a tool that can be used to automate sampling across a given area of the sample under examination. Usually you will want to traverse a much larger area than can be encompassed within a single field of view at the working magnification of the microscope. *Stage-Pro* uses the ability of the motorized stage to move to precise and repeatable locations to help you traverse these larger areas, locate features of interest, and return to them at a later time.

The key to successful application of *Stage-Pro* in your procedures and experiments is proper setup. Setup of *Stage-Pro* includes (at a minimum) component setup, configuration, stage squaring, lens calibration, and setting the scan area. Each of these topics will be discussed in following sections, in the order in which you will encounter them.

Setup

To begin setting up the *Stage-Pro* module of *Scope-Pro*, follow the steps below.

Stage-Pro can be configured with the **Setup** program to have an X/Y stage component – and optionally, a Z focus component. These components are often, but not always, driven by the same controller. Each controller accepts commands from the computer and drives the axis motor(s).

In the *Stage-Pro* **Setup** program, you must select the correct controller for the X/Y stage controller that you have installed. If you have Z focus control and it uses a separate controller, you must select the correct Z focus controller as well. In subsequent pages of the **Setup**, you must then select the corresponding components.

For example, if you have an Olympus AX-80 Provis microscope with a Prior X/Y stage, you will need to select:

- *both* the Olympus Provis and Prior controllers;
- check Z Focus component on the Provis components page;
- check the X/Y stage on the Prior components page.

Configuration

Once *Stage-Pro* has been set up, communications and other configuration parameters need to be set up. The ***Configure Tab*** page of *Stage-Pro* can be used to set these parameters. For most controllers, the communications parameters will default correctly for the usual factory settings. However, you will need to specify the step size for the specific X/Y stage that you have installed, and ptionally, the step size for any Z focus component.

The **Step Calib** button on the ***Configure Tab*** page of *Stage-Pro* allows you to refine the hardware calibration provided by the manufacturer of your equipment.

Clicking this button brings up the following dialog:

The step mod indicates how many “pulses” the stage will require to move one calibrated unit. If you wish to adjust the calibration provided with your equipment, you can calibrate the step size using the Step Calibration routine.

The **X/Y step size** is critical for correct operation of *Stage-Pro* with your stage, and is usually specified by the stage documentation. The Step size specifies for a single increment of the move command how far the stage will move along the associated axis. For example, a stage may move .05 or 4 microns per single step. If the wrong step size is specified, all subsequent movements, lens calibration, etc. will be off by a corresponding magnitude (i.e., the 80X difference between the actual step size of .05 microns and the specified step size of 4 microns).

The **Z step** is often less critical, and with clamp-on Z focus components can only be approximated in any case. *Stage-Pro* includes a Z calibration capability that can precisely calibrate your stage’s Z travel in absolute units if you have a precision standard of known thickness.

TIP: **Step calibration is absolutely critical to achieving maximum accuracy in stage movement**. This will only become apparent when you attempt to capture tiled images, and will appear as an overlap or gap between the tiles, even though you may have double-checked the accuracy of your spatial calibration.

Stage Squaring

Stage squaring is the process of aligning the camera with the axes of travel of the stage. For many of the *Stage-Pro* operations to succeed, the X-axis of the stage must be aligned well with the horizontal edge of the image acquired by your capture device. If your optics and capture device are properly aligned (so that the focal plane of the camera is perpendicular to the light path), this will also guarantee that the
Y-axis of the stage aligns with the vertical edge of the capture image.

Directional Note: When Stage-Pro/Scope-Pro refers to the upper left corner of an area, the stage/scope will move in a negative direction. When it refers to the lower right corner, the stage/scope will move in a positive direction.

Lens Calibration

Many of the *Stage-Pro* operations are based on the size of a captured frame – essentially the physical size of the camera's field of view. **Lens calibration** is the process of determining the size the captured frame (and by extension, of a single captured pixel). This allows *Stage-Pro* to step precisely from one field of view to an adjacent field of view, and also to provide to *Image-Pro Plus* a precise spatial calibration that can be used in subsequent operations to measure feature sizes and/or distances.

Step-by-step instructions for the lens calibration procedure that *Stage-Pro* provides are included in the initial setup, and the procedure described in detail in the ***Lens/Mag*** tab page discussion.

A dummy calibration is included in *Stage-Pro* and *Scope-Pro*. The dummy calibration settings are not saved when you end your *Stage-Pro* session, so you will need to reload the dummy calibration each time you want to use it.

Scan Area

Once the size of a captured frame is known, you can specify the area that you want to traverse during sampling. This area is referred to as the **Scan Area**. You can specify the scan area by its physical dimensions, by the number of frames in each direction, or by using the stage joystick to traverse the desired area.

If you will be separately analyzing several similar areas on your sample (i.e., separate tissue cells or individual well-plate wells), you may want to specify a small area, and then set the origin (top-left corner) of the area to the various desired locations on your sample. Conversely, if your sample should be analyzed as a whole, you can specify a larger area covering the entire significant portion of the sample.

For some applications, you may want to scan the area with **contiguous** or adjacent frames, so that all of the specified area is acquired (especially when tiling to a single image). In other applications, you may want to sample at intervals across the total area without necessarily acquiring 100% of the area (**non-contiguous** sampling).

Stage-Pro has two pages that are used to set up the ***Scan Area*** and the ***Scan Pattern*** that is used to traverse the Scan Area. *Stage-Pro* also provides a wizard that can be used to fully configure a **Scan Area** from start to finish

The **Scan Area** is dependent on the size of the captured frame, so you will probably want to have scan areas defined for each lens that you will be using regularly. For example, the frame area captured using a 4X lens will be just about ½ the width and height of the area captured using a 2X lens. So to cover the same area of your sample, the scan area should be set to have about twice as many frames in each direction.

If you are setting your scan areas to cover a consistent area that you will be examining regularly (e.g., to cover one well of a multiwell plate, or an area specified by your protocol), you will want to give each scan area a name. *Stage-Pro* maintains a list of named scan areas so that any of them can be selected from the list during a subsequent session.

Scan Area controls on ***The Stage Tab*** page allow you to step from field to field within the current scan area, and *Stage-Pro* acquisitions will acquire all of the fields in the current scan area.

Z Focus

If your microscopy hardware includes **Z focus control**, *Stage-Pro* provides a variety of functionality that you can use to assure that your sample is in focus and/or to analyze your sample at multiple depths (Z planes). *Stage-Pro* will support your auto-focus hardware (if you have that option) and provides software equivalents to hardware auto-focus as well.

If your hardware supports auto-focus, you may want to use **hardware auto-focus** while acquiring. This will be most effective if your sample is flat or sloping slightly such that a single frame is all or mostly in focus, but may require re-focusing when you move to another location on the sample. Keep in mind that hardware auto-focus circuitry will require a live video feed from your capture device (and so will only work with video capture devices). Most will sample a region near the center of the video when determining whether the sample is in-focus or not, and some support different ranges of travel depending on the lens magnification.

Stage-Pro provides a number of features that work with a **stack or set of images** collected at different Z focus positions. All of these features require you to specify:

- the number of Z planes and
- the Z travel limits over which the set should be collected (include the highest and lowest positions of interest across your entire sample, not just at the current X/Y position).

These limits are relative to the current Z origin, so you will want to set the Z origin to a position most nearly in focus, and then set the travel limits.

One of these Z-stack features is a **software auto-focus** that analyzes a stack of images and selects the image with the most in-focus material. Another related feature is the **multi-plane focus**, which makes a composite image from the set by selecting the most in-focus areas. For instance, if your sample slopes with the left side higher than the right, the multi-plane focus feature will select areas from the higher Z plane images on the left side of the composite image and areas from the lower Z plane images on the right side.

Both the software auto-focus and multi-plane focus features give you control over the algorithm used to determine what is “in-focus”. For most **bright-field work**, the maximum contrast option will work the best (this is the algorithm used by most hardware auto-focus circuitry). For **fluorescence work**, either minimum or maximum intensity may give better results, depending on the operations to follow.

You can also simply collect the stack of images for further depth analysis and processing in subsequent operations.

Z plane controls on ***The Stage Tab*** page allow you to step from plane to plane within the current Z travel limits, in addition to the acquisition options mentioned above.

Sample Patterns

Stage-Pro provides a further aid to **automating repetitive tasks** that is useful if your sample is organized into any repetitive grid (e.g., multiwell plates) or your stage supports multiple slides. **Sample patterns** specify the characteristics of the overall grid, and at each point of the grid the current scan area will be sampled during acquisition. In other words, sample patterns act as an automation of repetitively moving your origin and sampling the relocated scan area.

A **sample pattern** is defined by the number of rows and/or columns (it is perfectly reasonable to have a single row or a single column), and the corner-to-corner spacing between the rows and columns. For example, in a typical 96-well multiwell plate, there are 8 rows of 12 columns, which are spaced about 1 centimeter apart (corner-to-corner).

Use ***The Sample Pattern Tab*** page in *Stage-Pro* to define new sample patterns or select from existing ones. There is also a wizard you can use to fully configure a sample pattern from start to finish.

Groups

When a protocol specifies a repetitive arrangement of samples where sample patterns are useful, it may often specify a **logical grouping of those samples**. For instance, some wells or one slide may be a control or reference for the rest of the samples. *Stage-Pro* supports defining named groups that represent rectangular sub-areas of the grid as a whole. You can then choose to select a single group for acquisition or acquire from all groups in ***The Sample Pattern Tab*** page.

Starting Stage-Pro

This section describes the procedures for starting the *Stage-Pro* module for the ***first*** time. If you have already opened or used *Stage-Pro* before, the screens will appear differently than presented here.

> ***Important:*** Remember, if you configured the X/Y stage component **or** the X/Y and Z stage components, then ***Stage-Pro*** was installed. If you configured a single Z-focus component, then you installed *Scope-Pro* and need to refer to *Section 3 – Working with Scope-Pro*.

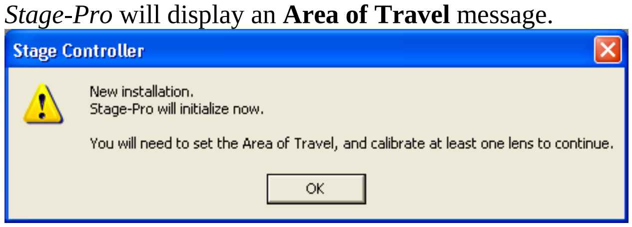
Before you select the ***Stage-Pro*** command, be sure that your motorized stage controller has been properly connected to your PC. For set-up procedures, consult the manufacturer's operating manual. Once the stage is connected, start *Image-Pro Plus* and go to the *Acquire* menu.

Note: *Your stage controller may have other configurable options not presented here. Please refer to the release notes, and your hardware guides.*

Also, if you have any Scope-Pro components (shutters, filters, objectives, etc.) installed, configuring Stage-Pro for the first time will be much simpler if you have configured Scope-Pro first.

To begin using the *Stage-Pro* module for the ***first*** time, follow the steps below:

1. Select the "*Stage-Pro*" command in the *Acquire* menu.
2. *Stage-Pro* will display an **Area of Travel** message.

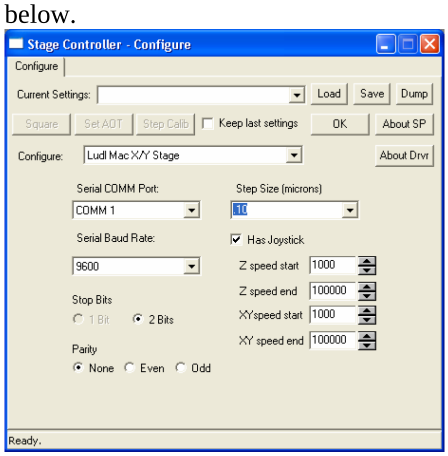


27. Click **OK**.
If *Stage-Pro* is ***not*** communicating properly, you will receive a warning similar to the one below.

This warning may appear if:

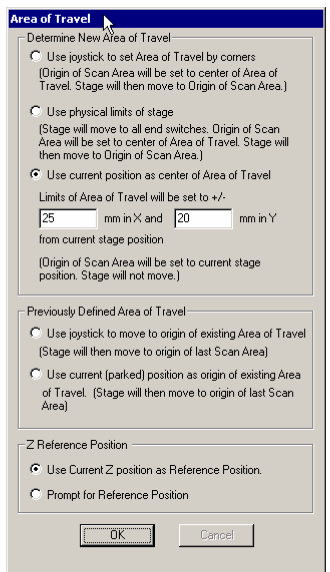
- *Stage-Pro* is not configured properly;
- The wrong COMM port is selected (note that the default is 1);
- Your equipment (i.e., microscope) is not turned **on**; or
- You are trying to install a component that is not present.

28. If *Stage-Pro* ***is*** properly communicating with the stage, you will see the ***Configure*** tab page. Make any necessary changes to the dialog box shown below.



29. Select the settings for each component as appropriate for your stage controller.
Make sure that the serial port settings on the X/Y Stage are correct, and that the Step Size is set correctly for each component. If you have a Z focus, you will need to configure that as well.

You will see the **Select Area of Travel** dialog box (you can also access this dialog box by pressing on the Set area of travel button on the *Configure* tab page):



30. Select the appropriate radio button for the *Area of Travel* option and the Z Reference position, and click **OK.**
If you have not set the area of travel, please do so now.

31. Click **OK.**

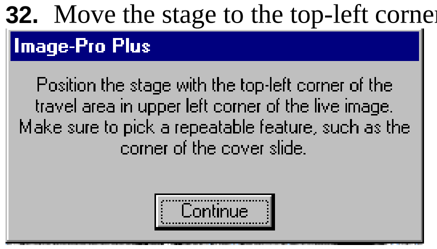
The following warning dialog box appears:

Important: At this point, you ***must*** have the stage positioned so that **the lenses, condenser, and the focus motor are *clear* of the path of stage movement**. The stepper motors are strong enough to break lenses if they are driven into an obstruction.

How you will proceed depends on which method you choose to set the Area of Travel.

If you are setting the area of travel **by corners**, proceed with **Step 32**If you are setting the area of **maximum travel**, skip to **Step 36** If you have set the current position as the **center** of the Area of Travel, skip to **Step 14.**

32. Move the stage to the top-left corner of the desired area of travel:



Directional Note: When *Stage-Pro/Scope-Pro* refers to the upper left corner of an area, the stage/scope will move in a negative direction. When it refers to the lower right corner, the stage/scope will move in a positive direction.

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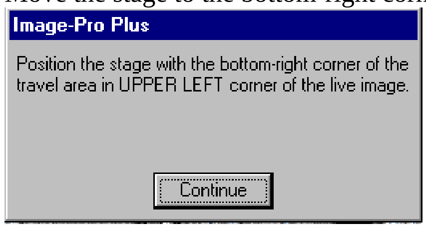
33. Position the stage using the joystick and then click *Continue*.

34. Move the stage to the bottom-right corner of the desired area of travel:



35. Position the stage, using the joystick, so that the bottom-left corner of the area of travel is in the *top-left corner* of the live preview. Then click *Continue*.
Proceed by skipping to Step 12.

36. If you are setting the area of maximum travel, you will see a stage movement warning.

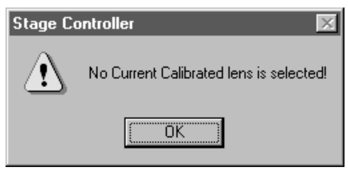
Important: At this point the stage is driven to its end limit switches (as far as it will go) in the X and Y directions to determine the travel limits. You must have the stage positioned so that the lenses, condenser, and other equipment are clear of the path of stage movement. The stepper motors are strong enough to break lenses if they are driven into something.

37. Click *OK*. You will see the *Halt* dialog box.

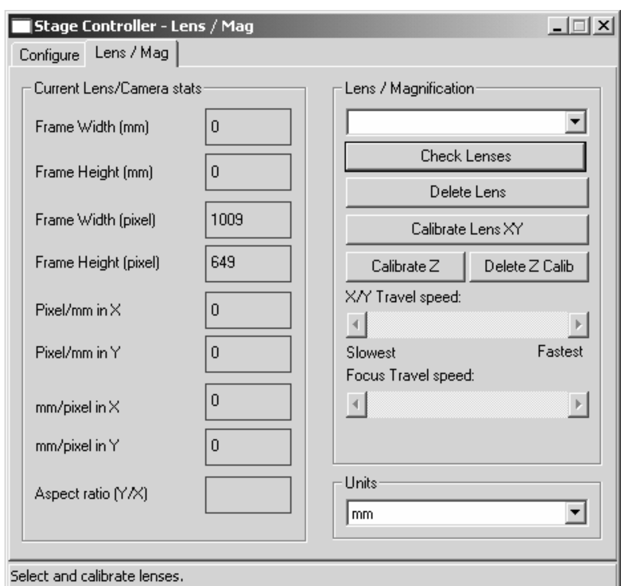
The stage will start to move. If the stage does not start moving or it does move and is about to hit something, click the **Halt Stage** button. You will get another dialog box with **OK** and **Cancel** buttons.

OK starts the limit finding process again (i.e., if the stage was about to hit a lens, you halt it, move the lens out of the way, and want to continue). **Cancel** is for when the stage is not responding (i.e., you gave the incorrect port information). **Cancel** will take you back to the ***Configure*** tab page.

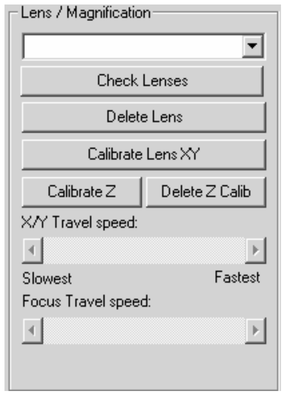
38. Finish finding the limits.
Once the limits have been determined and stage motion has stopped, you will get a message box telling you that no current calibrated lens is selected.



39. Click *OK*.
You will see the ***Lens/Mag*** tab page.



Note: If *Scope-Pro* is controlling the objective, this part of the tab will look different.



In this case, if you have already configured the Objective component in Scope-Pro, and attached appropriate calibrations to the lenses, clicking the Check lenses button will import Scope-Pro's list of lenses and calibrations. If you have not yet configured Scope-Pro, and you do have a motorized objective, you may want to pause in configuring Stage-Pro and configure Scope-Pro.

Once the Objective component of Scope-Pro is configured, click the Check Lenses button on the dialog shown above, and you have finished configuring Stage-Pro. Please continue with Chapter 7 to learn about the Stage-Pro tabbed dialogs.

If you are not going to use Scope-Pro to control your objectives, please continue with the next step.

40. Click on the *Lens/Magnification* list box and pick the lens that you want to calibrate.

The first time that you select a lens in *Stage-Pro*, you will get a message box telling you that the lens is not calibrated and asking if you want to calibrate it now. At least one lens must be calibrated. The dialog pictured below will come up when ever you select an un-calibrated lens or press the **Calibrate Lens XY** button. It will default to the second choice, *Import Calibration.*

- **Selecting Calibrate by stage movement** uses the method described later in this chapter.

- **Selecting Import Calibration** displays a drop-down list box. You can select a current reference calibration to load as the current *Stage-Pro* calibration.

To use the default selection, **Import calibration** option, you must have a good *Image-Pro* reference calibration from an image captured through the objective that you want to create a *Stage-Pro* calibration for, and from the camera that you plan to use with *Stage-Pro.* There are a number of ways to get that *Image-Pro* calibration, but the easiest way is to use the *Calibration Wizard* in *Image-Pro*.

If you do not see your lens, use the **New** button to create a new lens setting. Refer to ***The Lens/Mag Tab*** page for more details.

41. Click *OK*.

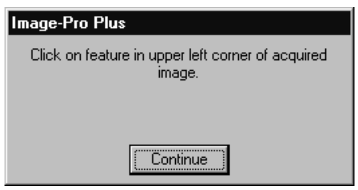
Important: *Stage-Pro* assumes that you have already installed your capture board and camera, and that you can acquire an image.

If you are importing a calibration, you will see the **Calibration Confirmation** dialog now. Click **Yes** to accept the calibration, or **No** to reject it, and leave the lens uncalibrated.

If you have chosen to calibrate by stage movement, *Stage-Pro* brings up *Image-Pro's* live video window and a message box telling you to move the stage, so that some small identifiable feature in the image is in the upper left corner of the live video window.

It helps to minimize or move the **Capture** dialog box out of the way, and to also resize the **Preview** window.

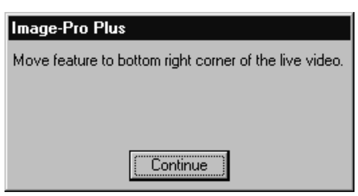
42. After you have moved the feature to the top left of the *Preview* window, press *Continue* in the message box.
Stage-Pro will capture the frame, maximize it, and place it in a workspace. Another message box now tells you to move your cursor to the feature in the acquired image (not the preview), and click on it.



The **live preview** will be turned *off*, so that you can easily see the captured image.

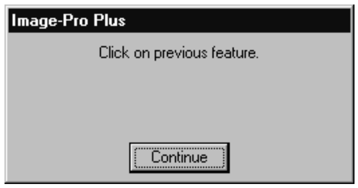
If the object you selected was close enough to the top left corner of the preview window, it should be visible in the image workspace. If not, use the scroll bars to bring it into view.

43. Click on the feature in the upper left corner of the acquired image.
Click on the acquired image ***workspace***, not the preview. The previous message box is replaced with one instructing you to move the stage so that the ***same*** feature is in the lower right corner of the live video window, and **live preview** is restarted.

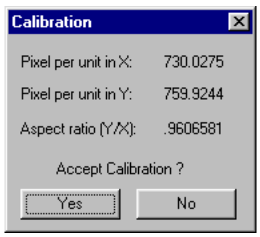


Tip: It is important to use a small enough object that you can identify and click on exactly the same point of the object in both calibration images, in order to get the most accurate spatial calibration possible.

44. Move the object (feature) to the bottom right of the *Preview* window and click *Continue* in the message box.
Stage-Pro captures the frame, maximizes it, and places it in the workspace. The last instruction dialog box then appears.



45. Click on the feature.
Now the live video window and the workspace are closed. You will see the **Accept Calibration** dialog box.



Note: Most capture devices, when set up correctly, should display an aspect ratio very near 1.0. The inability to calibrate your lenses to a 1.0 aspect ratio may indicate that the camera needs to be aligned with the axis of the stage movement. See the **Stage Square** button on T***he Configure Tab*** page.

46. Click *Yes* to calculate all the frame and camera information and send it to the *Lens/Mag* tab page, or click *No* to redo the calibration.
Once you have calibrated one lens, you have access to all the other tab pages (***Lens/Mag***, ***Stage***, ***Scan Area***, ***Scan Pattern***, ***Sample Pattern***, and ***Acquire***). At this point you can calibrate your other lenses, or go on with the capture process.

You must have at least one lens calibrated to do anything, and you must calibrate each lens before you can use that lens.

Use with Macros

This section describes how to combine your motorized stage with the power of *Image-Pro Plus'* macro facility. Your *Stage-Pro* module includes a set of *Auto-Pro* functions, which can be used to develop macros for controlling the movement of your stage automatically. For example, you might create a macro that automatically samples a specimen in a prescribed pattern.

Note: The material presented in this section assumes familiarity with the *Auto-Pro* facility. If you are not acquainted with *Auto-Pro*, you may want to review Section 1 in your *Auto-Pro Referenc*e first. Please refer to that manual, for complete descriptions of the *Scope-Pro* and *Stage-Pro* macros.

Moving the Stage

When you are manually controlling the stage, you can move the stage to a specified position or you can move it in single-step increments relative to its current position. The same methods of movement can be performed from a macro using the following basic *Auto-Pro* functions:

FUNCTION	DESCRIPTION
IpStageXY	Moves the stage to a specific position on the XY plane. When recording a macro, this statement is created by clicking the **Record XY** button in the ***Stage-Pro*** page.
IpStageZ	Moves the focus to a specific position on the Z-axis. When recording a macro, this statement is created by clicking the **Record Z** button in the ***Stage-Pro*** page.
IpStageStepXY	Moves the stage one logical step left, right, up or down on the XY plane. When recording a macro, this statement is created using the buttons in the **Stage (XY)** group box in the ***Stage-Pro*** page.
IpStageStepZ	Moves the stage one logical step up or down on the Z plane. **Stage (XY)** group box in the ***Stage-Pro*** page.

Each of these functions, and their parameters, are fully described in the *Auto-Pro Reference Guide.*

Moving to a Specific Position

The **IpStageXY** function is used to move the stage to a specific horizontal position. The following example shows how it could be used to acquire samples from five different positions on a slide.

```
 1.   Sub Sample5( )
 2.   ‘ Acquire 5 samples
 3.   ret = IpStageControl(SETORIGIN,STG_VAL) ‘Start at top
 4.   ret = IpStageVal= STG_UPLEFT
 5.   ret = IpStageXY(8,5)         ‘Move to position 8mm/5mm
 6.   ret = IpAcqSnap(ACQ_NEW)   ‘Acquire Image
 7.   ret = IpStageXY(10,5)      ‘Move to position 10mm/5mm
 8.   ret = IpAcqSnap(ACQ_NEW)   ‘Acquire Image
 9.   ret = IpStageXY(9,6)       ‘Move to position 9mm/6mm
10.   ret = IpAcqSnap(ACQ_NEW)   ‘Acquire Image
11.   ret = IpStageXY(8,7)       ‘Move to position 8mm/7mm
12.   ret = IpAcqSnap(ACQ_NEW)   ‘Acquire Image
13.   ret = IpStageXY(10,7)      ‘Move to position 10mm/7mm
14.   ret = IpAcqSnap(ACQ_NEW)   ‘Acquire Image
15.   End Sub
```

When using this approach, keep in mind that the specified positions are measured from the current origin. To be sure that the stage is positioned at exactly the same physical location during subsequent executions of the macro, you must make sure that the same origin is used each time. In the macro above, this was done by initializing the origin at the upper-left corner of the stage (statement 3 in the example above).

Recording a Specific-Position Movement

To create a macro like the one above, you can manually type the statements into a script file, or you can record the steps using the following procedure:

47. Select the *Stage-Pro* command from the Acquire menu. This will display the *Stage-Pro* dialog.

48. Select the *Record Macro* command from the Macro menu. This will begin macro recording.

49. Set the appropriate origin in the *Set Origin To* group box in the *Stage-Pro* dialog. This action will write an IpStageControl statement to your macro.

50. Move the stage to the first position. You may use either the controller’s joystick or the XY fields in the *Stage-Pro* dialog’s Stage and focus positions group box to do this. When the stage is properly positioned, go to the next step.

51. Click the *Record XY* button in the *Stage-Pro* dialog. This action will write an IpStageXY statement into your macro, which will cause the stage to be moved to this point when the macro is executed.

52. Click the *Snap* button in the *Acquire* window. This will capture the image at that particular position.

Note: If you want to do additional processing on the acquired image (e.g., histogram analysis, filtering, saving to disk), perform those commands in this step too.

Repeat steps 5 and 6 for each position that you want to record.

Moving Stepwise

The IpStageStepXY function is used to move the stage in single steps. In the example below, the stage is advanced in 2mm steps.

```
1.    Sub Sample2mm( )
2.    ‘ Acquire samples every 2mm

3.    ret = IpStgVal=2.0                'Set X step size
4.    ret = IpStageControl(SETSTEPX,IpStgVal)’Set Y step size
5.    ret = IpStageControl(SETSTEPY,IpStgVal)’Set Y step size

6.    ret = IpAcqSnap(ACQ_NEW)          ‘Acquire Image
7.    ret = IpStageStepXY(STG_LEFT)     ‘Step Left 1
8.    ret = IpAcqSnap(ACQ_NEW)          ‘Acquire Image
9.    ret = IpStageStepXY(STG_UP)       ‘Step Up 1
10.   ret = IpAcqSnap(ACQ_NEW)          ‘Acquire Image
11.   ret = IpStageStepXY(STG_RIGHT)    ‘Step Right 1
12.   ret = IpAcqSnap(ACQ_NEW)          ‘Acquire Image
13.   End Sub
```

When moving the stage in steps, remember to set the step size. In the macro above, this was done with the **IpStageControl** statements that appear on lines 3 and 4.

Recording a Stepwise Movement

To create a macro like the one above, you can manually type the statements into a script file, or you can record the steps using the following procedure:

1. **Select the *Acqurie:Stage-Pro* command.** This will display the ***Stage-Pro*** dialog. Position the stage as necessary.

2. Select the *Macro:Record Macro* command. This will begin macro recording.

3. **Set the X and Y step sizes in the *Stage-Pro*'s Stage and focus positions group box.** This action will write the appropriate **IpStageControl** statement(s) to your macro.

4. **Click the *Snap* button in the *Acquire* window.** This will capture the image at that first point.

Note: If you want to perform additional processing on the acquired image (e.g., histogram analysis, filtering, saving to disk), perform those commands in this step, too.

5. Advance the stage one step by clicking the *Up*, *Down*, *Left*, or *Right* button in the *Stage-Pro*'s *Stage XY* group box. This action will write the appropriate IpStageStepXY statement to your macro.

6. Repeat steps 3, 4, and 5 for each point that you want to process.

Moving the Focus

The focus-oriented functions, **IpStageZ** or **IpStageStepZ**, can be used to move the stage along its Z-axis. The stage can be positioned at a specific point (using **IpStageZ**) or moved up or down one step at a time (using **IpStageStepZ**). If your stage-controller hardware is equipped with an auto-focus feature, the **IpStageStepZ** function can be used to automatically position the stage at the proper focus point. Do not use this in a macro unless you have the appropriate hardware installed.

The example below illustrates how this might be used in a macro. In this example, statement 3 performs the auto-focus.

```
1.  Sub CenterSample( )
2.  ret = IpStageControl(SETORIGIN,STG_CENTER)’ Go To Center
3.  ret = IpStageStepZ(STG_AUTO)            ‘ Adjust Focus
4.  ret = IpAcqSnap(ACQ_NEW)                ‘ Acquire Image
5.  End Sub
```

Recording the Focus Position

The method used to record a focus movement in a macro is similar to the method used to record a movement of the XY plane. If you want to program the focus so that, during playback, it is positioned at a specific vertical point, follow the steps below.

1. **Select the *Acquire*:*Stage-Pro* command.** This will display the *Stage-Pro* dialog.

2. Select the *Macro:Record Macro* command. This will begin macro recording.

3. **Move the focus.** You may do this using either the hardware’s manual control or the “Z” field in the *Stage-Pro* dialog’s Stage and focus positions group box. When the focus is properly positioned, go to the next step.

4. **Click *Record Z* in the *Stage-Pro* dialog.** This action will write an IpStageZ statement into your macro, which will cause the focus to be moved to this point when the macro is executed.

Remember that when the macro is played back, the focus is positioned with respect to the current Z-axis origin. If you want to ensure that the stage is at the same vertical position each time the macro is executed, be sure your macro initializes the origin, using the appropriate **IpStageControl** statement, before moving the focus with **IpStageZ**.

Recording a Focus Step

If you want to program the focus so that, during playback, its position is moved in steps, follow the steps below.

1. Select the *Acquire:Stage-Pro* command. This will display the *Stage-Pro* dialog.

2. Select the *Macro:Record Macro* command. This will begin macro recording.

3. Set the Z step size in the *Stage-Pro*'s *Stage and focus positions* group box. This action will write an IpStageControl statement to your macro.

4. Move the focus up or down by one step by clicking *Up* or *Down* in the *Focus (Z)* group box. This action will write the appropriate IpStageStepZ statement to your macro.

Getting the Current Stage Position

Many times it is useful to know the stage's current position. You might use it to determine the size of a specimen or to return the stage to that position at a later step in the macro. With *Auto-Pro*, stage position can be queried using the **GETX**, **GETY**, or **GETZ** options with the **IpStageControl** function.

In the example below, the X and Y coordinates are obtained (in statements 5 and 6) and stored in variables X1 and Y1. These variables are then used in statement 10 to reposition the stage back to that point.

```
1.  Sub 4ImageSample( )
2.  Dim X1 as Single, Y1 as Single

3.  ret = IpStageControl(SETSTEPX,2.0)    'Set X step size
4.  ret = IpStageControl(SETSTEPY,2.0)    'Set Y step size

5.  ret = IpStageControl(GETX,X1)         'Get X Position
6.  ret = IpStageControl(GETY,Y1)         'Get Y Position

7.  ret = IpAcqSnap(ACQ_NEW)              'Acquire Image
8.  ret = IpStageStepXY(STG_LEFT)         'Move 1 Step Over
9.  ret = IpAcqSnap(ACQ_NEW)              'Acquire Image
10. ret = IpStageXY(X1, Y1)               'Move back to Start
11. ret = IpStageStepXY(STG_UP)           'Move 1 Step Up
12. ret = IpAcqSnap(ACQ_NEW)              'Acquire Image
13. ret = IpStageStepXY(STG_LEFT)         'Move 1 Step Over
14. ret = IpAcqSnap(ACQ_NEW)              'Acquire Image
```

```
End Sub
```

Selecting and Executing a Pattern

It is also possible to use the *Stage-Pro* macros to automate processes using the scan area and patterns. The following example assumes that you have set a scan area and pattern, and have saved the settings to the file **`“sampptn.stg”`**. Below is a simple example of how you can use these macros:

```
Sub PatternSample( )
Dim Frame as Integer, NFrames as Integer
ret = IpStageSettings(“SAMPPTN.STG”, STG_LOAD) ‘Load area and pattern
                                               settings
ret = IpStageGet (STG_NUM_FIELDS, 0, NFrame)   ‘Find out how many frames
for Frame=1 TO NFrames-1                       ‘Loop through pattern
ret = IpStageField(Frame)                      ‘Move to Next Position
ret = IpAcqSnap(ACQ_NEW)                       ‘Acquire Image
Next Frame
```

Note: The Stage-Pro demo script, `STGDEMO.SCR`, includes a macro, `DemonstrateWellPatterns`, which illustrates automating a process with both scan areas and sample patterns.

Appendix A: Glossary of Imaging Terms

Active Window

The image window that is currently selected. If only one image window is open, it will always be the active window. If more than one image window is open, the active window is the frontmost window. Its title bar is highlighted.

Angle dot screen

A technique for representing gray tones through dots of varying sizes placed at regular intervals on a grid. The distance between the dots on the grid is the resolution in dots per inch (DPI). The positions of the dots on the grid fall on two sets of imaginary lines at 90 degrees to one another. The angle of these imaginary lines to the horizontal is 45 degrees, which is the conventional angle in printing processes using the screen technique.

AOI or
Area of Interest

Area of Interest. A contiguous subset of pixels defined within an image, which may be arranged in any polygonal shape. They are used to isolate the subset from the rest of the image.

API

Application Program Interface

BCG

Brightness, Contrast, and Gamma

Bilevel

The simplest way to represent an image in digital terms, with each pixel represented by a single bit that is either on or off. Also referred to as “Black and White”, “Line art” or “Halftone”.

Bilinear Scaling

A scaling technique that interpolates a pixels rescaled value based upon its relationship to surrounding pixels. This technique creates smoother scaled output than traditional “factor” or “pixel replication” scaling methods.

Bit

The smallest unit of information recognized by a computer. A *pixel* is represented by one or more computer bits. The number of bits per pixel directly determines the number of colors or gray shades that can be represented. See *Bit Depth*.

Bit Depth
The number of pixels used to represent one pixel value. Also referred to as *Pixel Depth* and *bits-per-pixel* (BPP).

Bitmap
A two-dimensional array used to represent an image. Each cell in the array contains a value which describes a sample of the image in terms of its color.

Contrast this with Vector graphics, where an image is created by describing its elements as geometric objects.

BMP
Image bit-mapped format used by Windows and OS/2.

BPP
Bits Per Pixel. Describes the "depth" of an image. Bilevel images are only 1 BPP, True Color images are 24 BPP.

Brightness
The amount of white in an image. The brighter the image, the more white it contains, and as brightness is increased, each color in the image is shifted more toward white.

Calibration, Device
The process of setting *Color Map* attributes to compensate for the variations in quality of images due to differences in printers, scanners, and the materials associated with them.

You can calibrate your printer(s) or scanner(s), save the calibration(s), then load them each time you use a different scanner or printer, or as your printer toner or ribbon age.

Calibration, Intensity
The process of redefining the intensity scale associated with an image, to express intensity values in another unit of measure. For example, intensity values can calibrated to reflect density, or temperature.

Calibration, Spatial
The process of redefining the spatial scale in an image to express distance between pixels in a unit of measure other than pixels. For example, you might calibrate the spatial scale to measure microns or kilometers.

CCITT
Consultative Committee for International Telegraph and Telephone. The committee developed the CCITT compression methods for FAX data, which are used to compress Bilevel image files.

Channel
The luminance and color components associated with a color image. An RGB image contains 4 channels: Red, Green, Blue, and Luminance. The Luminance channel represents intensity without regard to color.

Chunk, RGB Chunk
The 3-byte (24-bit) group that represents the red, green and blue values for a pixel. A chunky image data line is made up of consecutive chunks, with no gaps or fillers between chunks.

Class
See *Image Class*.

CMY
Color model in which color is expressed in varying amounts of Cyan, Magenta, and Yellow (CMY). CMY is the standard color model in printing.

Color Correction
Making changes to brightness, contrast, color, highlight, shadows in an image to compensate for perceived deficiencies.

Color Model
A mathematical model describing color. There are several Color Models are used today. Image-Pro supports the popular models. See the *Color Model* discussion in *Section 1* of the *Image-Pro Reference Guide*, and CMY, HSI, HSV, RGB, and YIQ entries in this Glossary.

Color Plane
See *Plane*.

Color Reduction
Optimization or reduction of the number of colors in a color image. Accomplished using the “Convert To” menu's “Palette” command. Often performed to reduce the amount of space require to store a color image.

Compression
Mathematical technique that allows an image to be stored with less memory. Redundancies in the internal representation of the image are identified and given a code; the data in the redundancies is then replaced by the code.

Contrast
The sharpness of an image. The higher the contrast in an image the larger the difference between white and black in the image, or (in color images) the more spread out the color range is. As contrast is increased, all the colors or gray shades in the image spread apart.

DLL
Windows Dynamic Link Library

Document ID

A document ID (*DocId*) is assigned to an image window when it is opened. It retains this ID for the duration of its existence. IDs are assigned consecutively, in the order in which images are opened. The next higher ID number is used when a new window is created — e.g., if image 4 is already open, the next image is assigned an ID of 5.

Because of the dynamic nature of *DocId* (the mix and sequence of images on your desktop varies from session to session), macros involving multiple images should be recorded and played back from an empty imaging area (i.e., one in which there are no images open), or images should be selected relatively using the DOCSEL_NEXTID and DOCSEL_PREVID options described below. These measures will ensure that the recorded image numbers select the intended images on playback.

DPI

Dots Per Inch

Drag

To press and hold down a button on your pointing device while you move the cursor on the screen. You "drag" something when you want to move it to a new location on the screen, or when you want to resize it.

Error Diffusion

A halftoning technique where the difference between the image pixel value, and the targeted halftoned pixel is added to the value of the next image pixel.

Factor Scaling

A scaling technique that uses a zoom factor. The zoom factor allows scaling to be accomplished quickly.

File Format

The method with which an image is stored to disk; based on image class, compression type, and halftone pattern. TIFF and BMP are examples of file formats.

Gamma

A nonlinear logarithmic contrast correction factor which is used to adjust the contrast in dark areas of the image.

Gray Level

In gray scale images, the brightness value assigned to a pixel. In an 8-bit image, this value ranges from 0 to 255 (from black, through shades of gray, to white).

Halftoning

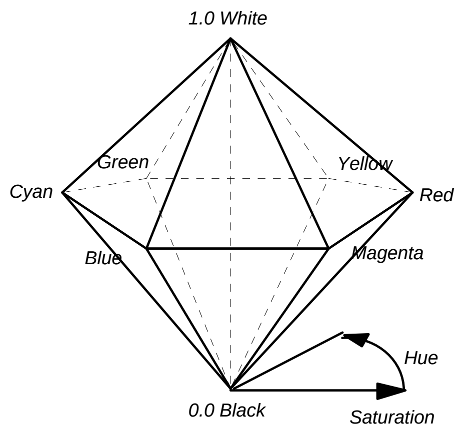
A method of simulating a gray scale image using patterns of very small black and white dots.

Horizontal Differencing

Technique used for enhancing LZW compression. Horizontal differencing takes advantage of the fact that, in many images, the difference between adjacent pixels if frequently 0 or a small number. Instead of compressing the actual value, LZW then compresses the differencing values of the adjacent pixels.

HSI

Color model in which color is expressed in varying amounts of Hue, Saturation and Intensity. The color space for the HSI model is normally represented by a double hexcone, with Intensity forming the primary axis as shown in the drawing below.



HSV

Color model in which color is expressed in varying amounts of Hue, Saturation and Value. HSV differs from HSI in the way Intensity is derived. See *HSI* above.

Huffman

See *Modified Huffman*.

Icon

A symbol that represents a tool or item. For example, the symbols on the Ribbon are icons of the available tools.

Image

The “document” that *Image-Pro* works upon. Also may refer to the original artwork, graphics, or photo that you scan or import.

Image Class

Image category, determined by **bit depth.** Image classes supported by *Image-Pro* include *Bilevel* (1-bit), *Palette* (8-bit), *Gray Scale* (8-bit), *Gray Scale 12* (12-bit) and *True Color* (24-bit).

Image Conversion
The process of changing an image from one class and/or format and/or compression type to another.

IMG
Image class developed by Digital Research, and used extensively by Ventura Publisher.

Instance, Image Instance
A rectangular view of a virtual image. An instance may encompass the entire virtual image, or define just part of it. Instances provide access to virtual image data since virtual images cannot be accessed directly.

Library
(*Imaging*) A collection of image information stored in a database. (*Bio*) A collection of clones which partially or completely represent the complexity of genomic DNA or cDNA from a defined biological source.

Lossless
Type of **compression** that allows full recovery of the original image and is fully reversible.

Lossy
Type of **compression** that degenerates an image during compression and is not fully reversible.

Luminance
Intensity irrespective of color. Usually derived from the mean of the RGB color values.

LUT
Lookup Table
A table in which BCG adjustments are maintained, and through which the image bitmap is rendered. This allows you to experiment with the BCG qualities without affecting your actual image data. Also lets you improve the image for viewing purposes without affecting the integrity of your data for analytical purposes.

MColor
A method of converting a 24 bit color image to a 8 bit palette image. The MColor technique, developed by Media Cybernetics, uses a fixed 256 color palette, representing a range of values across the color spectrum. During MColoring, the 24 bit image is reduced to 8 bits using a dithering technique to convert the pixel values into MColor palette indicies..

Modified Huffman
Compression type which is a variation on **RLE**. Modified Huffman uses a standard table of patterns that represent typical patterns in an image. These tables are usually tuned to the type of data they are used to compress.

MSP
Image file format developed by Microsoft Paint.

Normal Scaling

Scaling is accomplished using the following formula:
`Dest(x)=Source(x * SourceWidth/DestWidth)`

Normalized Image Data

A method of representing image data utilizing 1 byte per color channel, regardless of image class. For *Bilevel Gray Scale* and *Palette* images, normalized data is arranged as 1 byte per pixel. Normalized Bilevel pixel values are either 0 or 255. For RGB images, normalized data is arranged as 3 bytes per pixel, representing the 3 color channels.

Palette

For a *Palette* class image, the 256 element array used to specify the RGB values associated with that image. Each pixel in the image contains an index to the palette, which contains the RGB value for that pixel.

See *Appendix C* for additional discussion of palette images.

PColor

A method of converting a 24-bit color image to an 8-bit palette image using a palette defined by the user.

PCX

Image file format developed by ZSoft Inc.

Pixel

Picture element. The smallest element in a digitized image.

Pixel Depth

The number of pixels used to represent one pixel value. Also referred to as *Bit Depth* and *bits-per-pixel (BPP).*

Plane, Color Plane

An arrangement of RGB image data such that all Red values are located in one array, all Green values in a second array, and all Blue values in a third. Contrast with Chunk above.

Polyline

A series of lines connecting pairs of points (vertices).

Pseudo-color

The process of assigning RGB color values to replace gray levels for display purposes. Usually used to highlight certain intensity ranges in a *Gray Scale* image, with color.

Response Lookup Table

Three arrays of 256 lookup values for the Red, Green, and Blue channels. This table is produce by converging the BCGM controls into it.

Resolution

See *Spatial Resolution.*

RGB

Color model in which color is expressed in varying amounts of Red, Green, and Blue (RGB). RGB is used by most digital imaging devices.

RGB Chunk

See *Chunk.*

Ribbon
The graphic bar along the left side of the *Image-Pro* application window that displays icon controls for AOI creation, zooming, selected commands, and adjustments to brightness, contrast, and gamma.

RLE or Run Length Encoding
Run Length Encoding. A compression technique that encodes data "runs" (lengths of identical information).

Sharpen
A filtering process that intensifies edges and details in an image by increasing the difference between gray values of neighboring pixels. Sharpen produces a crisper image.

Smooth
Bilinear scaling option that may be used during printing and resizing. This is a form of resizing that takes into account adjacent pixel values to generate in-between values for the resized image, so that the new image does not look as if pixels were arbitrarily removed or added. When this option is not selected, scaling is done using pixel replication (bigger) and decimation (smaller).

Spatial Resolution
Image attribute defined by a two-dimensional (width and height) grid of pixels.

Threshold
A value used to separate gray values into 2 values. Gray values less than the threshold will be set to 0. Gray values greater or equal to the threshold will be set to 255. Typically the threshold is set to 128.

TIFF
Tagged Image File Format. A general-purpose file format recognized as a standard for image files.

Tile
One page panel in a multi-page poster-size print.

TGA
TARGA image file format developed by TrueVision Inc.

True Color
24-bit color. Image class in which each pixel contains 256 shades each of red, green, and blue, therefore the image can contain up to 16.7 million colors.

YIQ
A component analog color model wherein "Y" represents luminance value, and "I" and "Q" represent the color components. YIQ is the standard in broadcast television.

Virtual Image

The memory-based copy of an image. It contains the image data and the image attributes. Image attributes can be accessed using the HilImControl and HilImControlInst commands; Image data can only be accessed through an image instance.

Zoom Factor

A zoom factor is the factor used to rescale an image. Positive factors are used to "zoom in" (expand), and negative factors are used to "zoom out" (shrink).

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