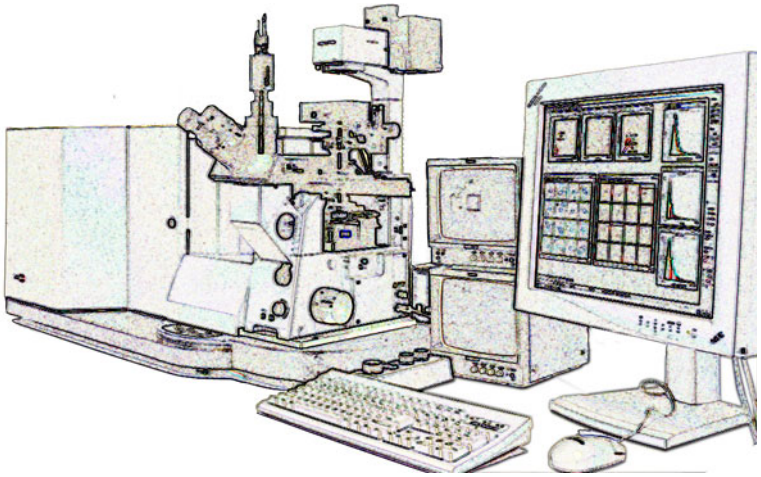
iCys™

RESEARCH IMAGING CYTOMETER



CompuCyte

User Guide

DOC190-0037-001 Revision B

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Chapter 1

Introduction

This manual provides information about using the iCys™ Research Imaging Cytometer and the iCys™ Cytometric Analysis Software. The iCys Research Imaging Cytometer is an instrument which performs rapid biochemical measurements and genetic analysis of cellular and tissue specimens. The iCys Cytometric Analysis Software allows you to configure the instrument, scan or view a plate, slide or other carrier, and analyze the resulting data.

This manual will familiarize you with the basic components and operations of the entire iCys system.

Included in this chapter:

- ❑ Manual Organization, page 1-2
- ❑ System Overview, page 1-3
- ❑ iCys Intended Use, page 1-4
- ❑ Technical Support, page 1-4

iCys™ RESEARCH IMAGING CYTOMETER

Manual Organization

Chapter 1, “Introduction”

Introduces you to the iCys hardware and software, and provides technical support information related to the iCys system.

Chapter 2, “iCys™ Research Imaging Cytometer Hardware Reference”

Provides information on the hardware components, safety requirements, system maintenance, and functional specifications for the iCys system.

Chapter 3, “iCys™ Cytometric Analysis Software Application Overview”

Provides detailed information about setting up and generating an iCys run.

Chapter 4, “Generating Analytical Data”

Provides detailed information about the analysis parameters for an iCys run.

Appendix A, “Installing iCys™ Research Imaging Cytometer”

Provides hardware installation information.

Appendix B, “Using the iNovator Toolkit”

Provides information about using the optional iNovator Toolkit to create macros that extend the capabilities of iCys.

Appendix C, “Administrator Tasks”

Provides information about performing iCys administrative tasks.

Appendix D, “Glossary”

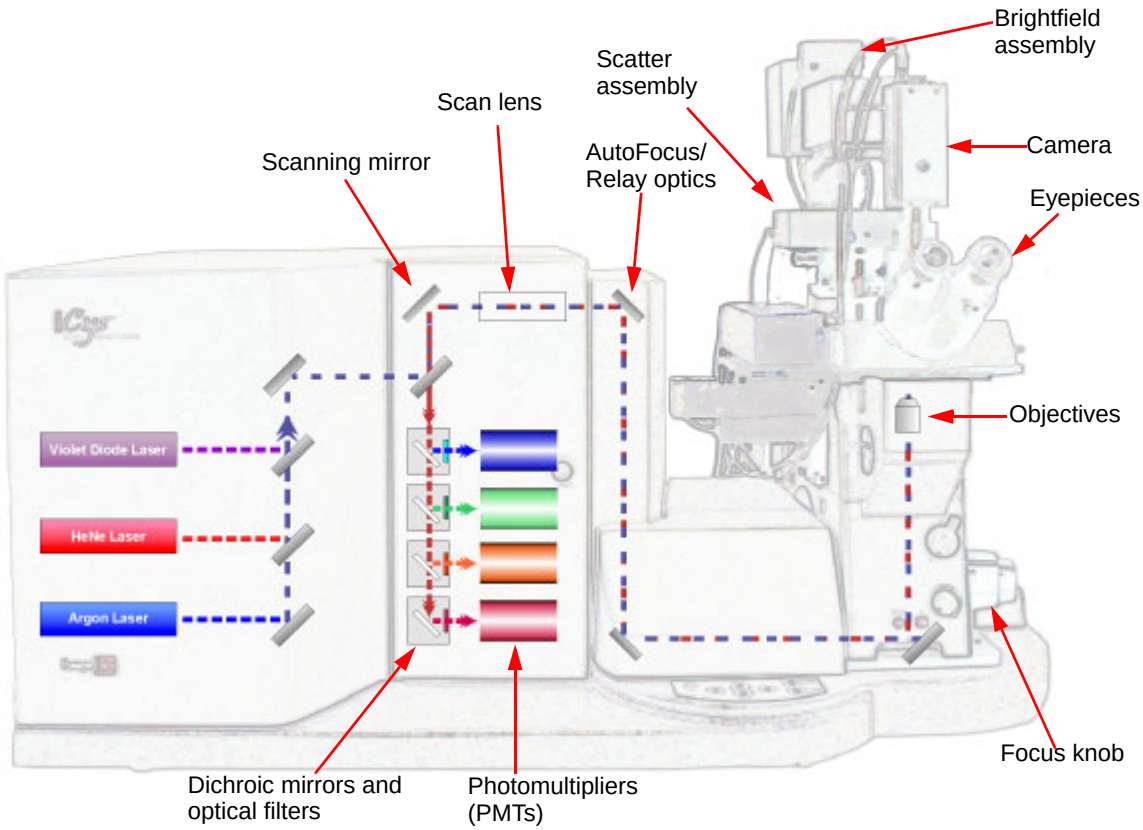
Contains a glossary of terms used within iCys.

System Overview

The iCys™ Research Imaging Cytometer is a microscope-based instrument that uses lasers and photomultiplier tube detectors to measure constituents and other properties of cells based on multicolor fluorescence detection. The iCys instrument scans microplates, slides, and other carriers, analyzing and recording data about each scanned sample.

Figure 1-1 illustrates the laser and data path of the iCys Research Imaging Cytometer. The iCys instrument, its components, and basic operation are described in detail in Chapter 2, "iCys™ Research Imaging Cytometer Hardware Reference."

Figure 1-1: iCys Laser and Data Path



iCys Hardware

The iCys Research Imaging Cytometer consists of the following major hardware components:

- The optics/electronics unit creates the laser scanning beam. It contains the fluorescence sensors and controls laser light shutters, the motorized stage, and other functions.
- The microscope unit is an Olympus IX-71 microscope with a motorized stage, brightfield lamp, and a forward scatter detector. Optionally available are the AutoFocus mechanism, EpiFluorescence assembly, and color camera.

- The computer runs on the Windows XP® operating system. It controls iCys operations, stores, and analyzes data using iCys software.

iCys Software

The iCys software includes:

- The iCys Cytometric Analysis Software is used to configure the iCys instrument and set up the desired parameters for viewing and analysis.
- The optional iNovator Toolkit allows you to generate more complex analyses during an iCys run. For more information, see Appendix B, "Using the iNovator Toolkit."
- Optional iBrowser Analysis Software allows you to analyze and display saved scan data from an iCys run. For information about iBrowser, see the *iBrowser User's Guide*.

iCys Intended Use

The iCys™ Research Imaging Cytometer is an instrument which performs rapid biochemical measurements and genetic analysis of cellular specimens and tissue specimens in microplates, slides, and other carriers. iCys visually displays samples under analysis without destroying them.

iCys is intended for research use only. iCys is not for use in diagnostic procedures.

iCys is manufactured by CompuCyte Corporation, 12 Emily Street, Cambridge, MA 02139-4507.

Technical Support

CompuCyte technical support is available from 8 A.M. to 5 P.M. (EST). You may contact us the following ways:

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Outside US & Canada: 617-492-1300 (prompt 2)

Fax: 617-577-4501

E-mail: techsupport@compucyte.com

Chapter 2

iCys™ Research Imaging Cytometer Hardware Reference

This chapter describes the iCys™ Research Imaging Cytometer hardware, along with maintenance, and safety issues.

Included in this chapter:

- About iCys, page 2-2
- Optics/Electronics Unit, page 2-4
- Argon Ion Laser Support Elements, page 2-6
- The Desktop Computer, page 2-7
- Microscope Unit, page 2-8
- The Delivery and Collection Optics, page 2-12
- iCys Hardware Setup, page 2-13
- iCys Specimen Carriers, page 2-17
- iCys Safety Requirements, page 2-24
- General Maintenance Information, page 2-30
- Functional Specifications, page 2-31
- Performance Specifications, page 2-33

iCys™ RESEARCH IMAGING CYTOMETER

About iCys

The iCys™ Research Imaging Cytometer has the following major hardware components:

- The optics/electronics unit creates the laser scanning beam. It contains the fluorescence sensors and controls the laser shutters, and other functions. This unit is described in greater detail in “Optics/Electronics Unit” on page 2-4.
- The microscope unit consists of an Olympus IX-71 microscope with a motorized stage, eyepieces, a CCD camera, brightfield source, optional EpiFluorescent mercury burner, and a forward scatter detector.This unit is described in greater detail in “Microscope Unit” on page 2-8.
- The Remote Control unit provides fine X and Y control, and if your iCys system uses the optional AutoFocus feature, coarse and fine AutoFocus control.
- The desktop computer runs on the Windows XP® operating system. It controls iCys hardware and software operations, collects scan data, and stores and analyzes the data.

Figure 2-1 shows some of the hardware components for the iCys system.

Figure 2-1: iCys Selected Hardware Components

Optics/Electronics | Microscope, brightfield source, EpiFluorescence, camera, and automated stage | Camera and focus monitors, Remote Control unit | Monitor

Not shown in the figure are the desktop computer, the argon ion laser cooling fan, and power supplies for the argon laser, brightfield lamp, and optional EpiFluorescent mercury burner.

Note: For information about installing iCys, see Appendix A, “Installing iCys™ Research Imaging Cytometer.”

Optics/Electronics Unit

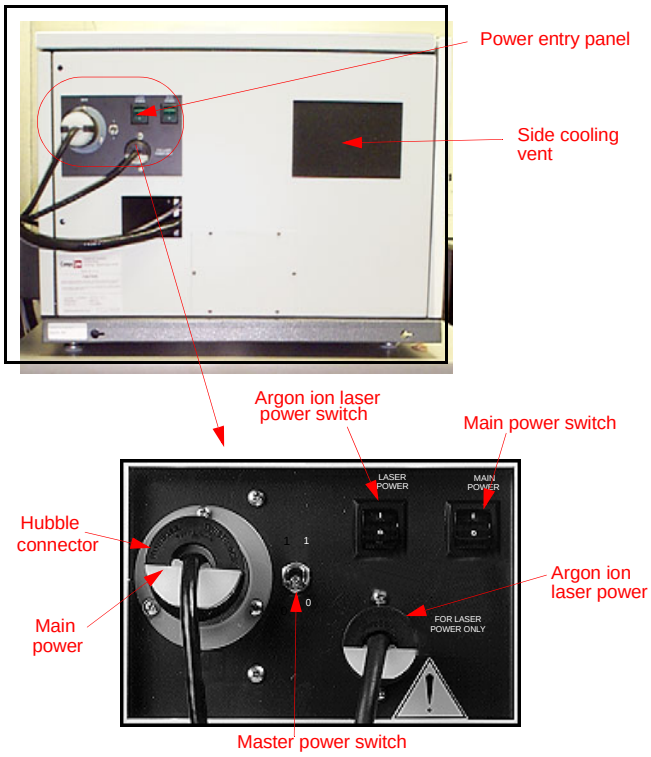
The optics/electronics unit includes the following components:

- **One to three lasers**—Argon ion (488nm) is always included, you can optionally add HeNe (633 nm), and Violet diode (405nm)
- **Laser beam steering optics**
- **Galvanometer**—Creates the laser scanning beam
- **Scan lens**—Introduces the scanning laser beam into the microscope
- **Electronics and power supplies**—Controls various functions of the instrument such as the motorized microscope stage, the galvanometer scanner, and the laser shutters
- **Fluorescent light collection optics**—Collects light from the specimen and transmits it through a series of optical filters to the photomultiplier tubes

Power Entry Panel

The power entry panel is on the left side of the optics/electronics unit, as shown in Figure 2-2.

Figure 2-2: iCys Power Entry Panel



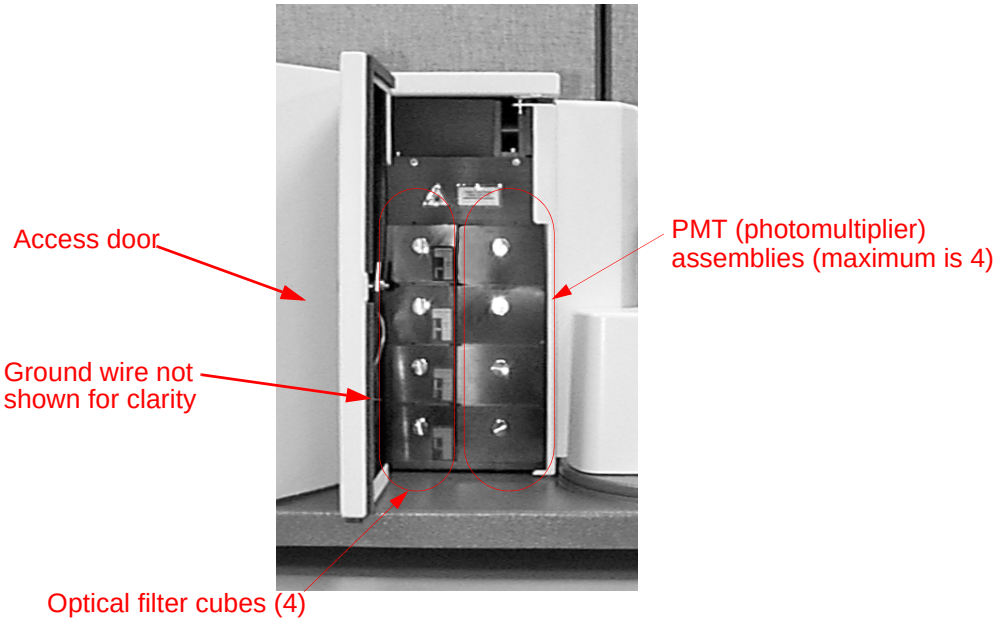
The instrument is connected to power mains through the special Hubble connector as shown above. The master power switch switches all power to the unit and must be in the “On” position for the unit to function. The breaker should be left in the “On” position unless the instrument is being serviced. The argon ion laser power supply is

connected to the outlet shown and switched on by the illuminated laser AC switch above the outlet. Power for the rest of the system, including the accessory outlets on the right of the optics/electronics unit, is controlled by the second illuminated main AC power switch.

PMT/Filter Access

Figure 2-3 is a close-up photo of the opened PMT/filter access door.

Figure 2-3: PMT and Filter Access



To gain access to the filter and PMT assemblies you must open the door on the front panel using a 5/32” Allen wrench, which is supplied. To replace the filter cubes, turn the knob clockwise until the cube is loose.

The PMT assemblies process the light selected by each filter and are located directly to the right of the associated filter cube.

Note: Do not replace the PMT assemblies or move the filters, unless under the advisement of service personnel.

Accessory Outlets

There are two accessory outlets located on the right side of the optics/electronics unit. The accessory outlets are controlled by the main power switch.

Argon Ion Laser Support Elements

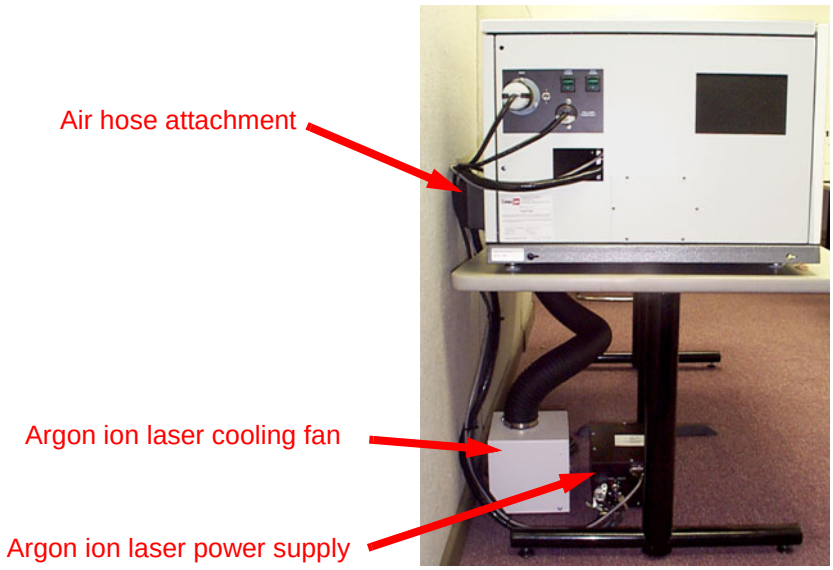
The argon ion laser support elements consist of the following:

- ❑ Argon ion laser cooling fan
- ❑ External power supply

Argon ion laser cooling fan

The laser cooling fan is attached through an air hose either at the rear or the side of the optics/electronics unit as shown in Figure 2-4. Power for the fan is supplied by a cable through the cable port on the left-side panel.

Figure 2-4: Argon Ion Laser Cooling Fan Attachments

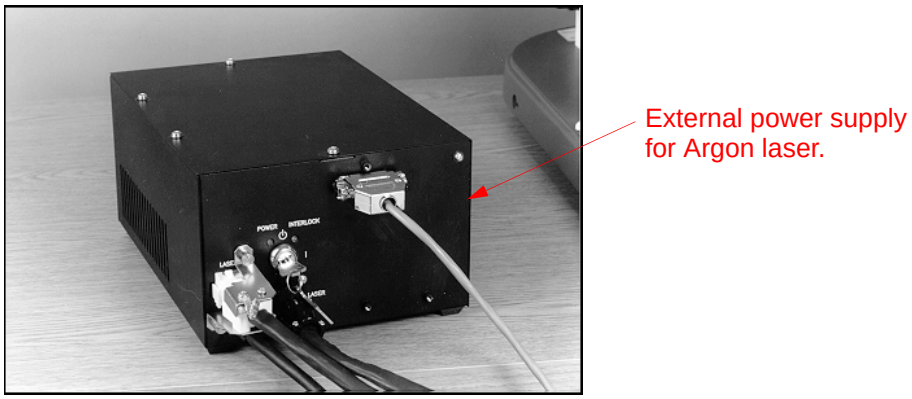


External power supply

The supply is connected to the optics/electronics unit via three cables, as shown in Figure 2-5:

- ❑ One cable is for AC power
- ❑ A second (large) cable is used to send power from the laser power supply to the laser
- ❑ A third (smaller) cable carries signals from the electronics to the laser power supply for controlling and monitoring the laser power and current

Figure 2-5: Argon Laser External Power Supply



Note: Do not place the laser external power supply or the external fan on the same table as the optics/electronics unit.

The Desktop Computer

The imaging cytometer is controlled, and data is obtained and analyzed using the iCys software. iCys operates on the supplied computer, which runs the Microsoft Windows XP operating system. Two proprietary circuit boards installed in the computer convert analog signals from the cytometer into digitized data, and write it to system RAM for processing and storage. The computer is equipped with a monitor, mouse, and keyboard. In addition to the iCys software and circuit boards, the computer also contains an EPIX frame grabber card, and is supplied with EPIX XCAP-Lite image viewing and analysis software.

Microscope Unit

The microscope unit consists of the following:

- A standard Olympus IX-71 microscope.
- WH10X eyepieces.
- An adapted motorized stage – the manual stage positioning controls, located in front of the microscope, allow for coarse or fine operator control of the motorized stage positioning.
- A brightfield illumination column and lamp housing and TH4-100 power supply for brightfield lamp.
- An optional EpiFluorescence (Reflected fluorescence) system, including a power supply for the 100W mercury burner.
- Sony CCD Video Camera.
- A forward scatter detector located above the microscope stage.
- The scanning laser beam is directed into the microscope through a side port on the microscope. This beam is directed to the microscope objective and focused on the sample located on the microscope stage. Fluorescent light, collected by the microscope objective, emerges through this port and is relayed to the photomultiplier tubes located in the optics/electronics unit.
- Other optional microscope accessories may also be included.

The Microscope

The microscope supplied with iCys is an Olympus IX-71 which comes with an Olympus objective turret containing 10X, 20X, and 40X Universal Plan Semi-Apochromatic objectives. It also contains a crosshair alignment tool.

Note: Do not remove, replace, or add objectives to the microscope unit.

iCys can be purchased with or without the AutoFocus feature. The microscope functions are directly affected by the presence of AutoFocus, as follows:

- If your iCys system does not have the AutoFocus feature, you will need to manually move the objective lens into the correct positions as discussed in “Using the Microscope without AutoFocus” on page 2-8.
- If your system does have AutoFocus, see “Objective Lens Automated Movement” on page 2-9.

Using the Microscope without AutoFocus

If your iCys system does not have the optional AutoFocus feature, then you will need to manually focus the microscope on the specimen in the area to be used during the run before starting a Test Scan or Scan & Save run.

You can manually focus by either:

- ❑ Turning on the brightfield source and viewing the specimen area directly through the eyepiece.
- ❑ Using the CCD camera with either the stand alone monitor or the computer monitor.

➢ In either case, to manually focus the microscope:

1. Lower the objective lens turret to the full downward position.
2. In the Scan Area Setup window, described in "Setting up a Microplate Scan" on page 3-17 and "Setting up a Freeform Carrier Scan" on page 3-22, select Move stage to active well ( ), then click on the region of interest to move the stage into position.
3. Select **Instrument** ⇒ **Brightfield**, and switch the microscope Light Path Selector lever to Observation ( ).
4. If necessary, adjust the brightfield lamp power.
5. From the microscope, rotate the coarse or fine focus knob until the specimen appears to be in focus on the eyepieces, the camera monitor or the computer monitor
6. Adjust the focus by the system offset for the objective lens used in the run.
7. The system is now ready to scan the well or scan area.

Objective Lens Automated Movement

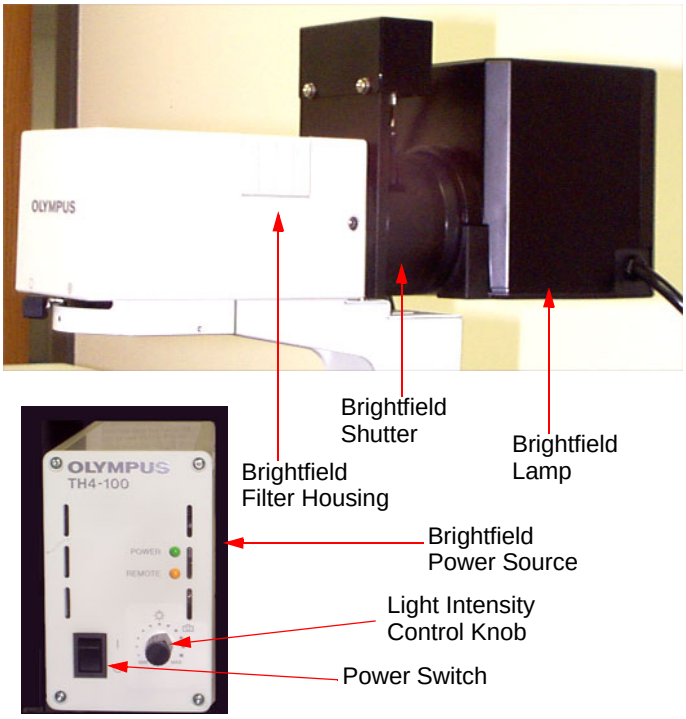
When scanning, the microscope automatically moves the objective lens into position for scanning. When not scanning, there are three possible locations for the objective lens:

- ❑ **Objective Home**—The home base for the lens. The lens is entirely retracted. The objective moves to the home position when the objective lens is explicitly returned home. After the objective lens is homed, it is driven to the Clear Position.
- ❑ **Clear position**—The lens is retracted enough to be clear of any carriers, but is not retracted to the Home position. The objective moves to Clear when a Scan & Save run is completed, is manually stopped, or when the stage is moved to the Load position.
- ❑ **Retraction offset**—A carrier-specific retraction that allows the stage to move without bumping into the objective. The objective moves to the retraction offset when:
  - ❍ A Test Scan completes or is stopped.
  - ❍ The stage is moved using the Move to Active Well command between wells or rooms in the Scan Area Setup window.
  - ❍ You view events in the Field Image window as described in "Locating and Viewing Events in the Field Image Window" on page 4-45.

Brightfield Lamp

The brightfield illumination column and lamp allow you to view your samples directly from the microscope stage. Brightfield is only useful when using direct observation.

Figure 2-6: Brightfield Illumination Assembly



When using the brightfield lamp, you also need the TH4-100 power supply that powers the lamp.

For information about using the brightfield lamp, see “Observing Samples with the Brightfield Source” on page 2-14.

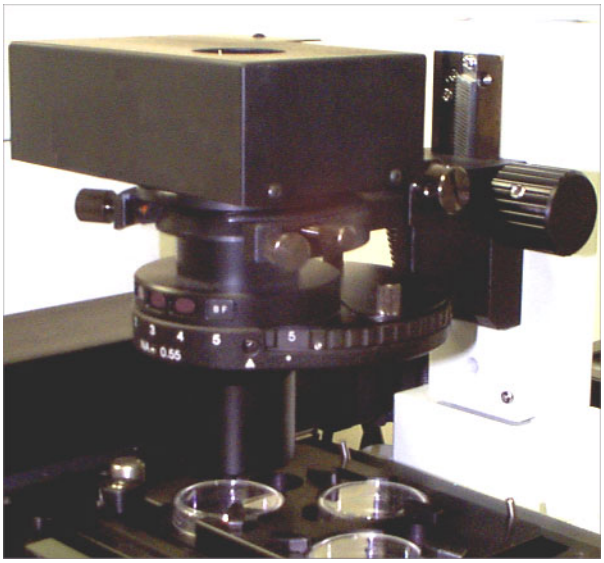
For detailed information about the IX71 microscope and brightfield illumination, see the Olympus *Instructions: IX71/IX51 Inverted Research Microscope/Inverted Basic Microscope.*

Forward Scatter Detector

The Forward Scatter detector allows you to generate scatter images in iCys. You can use scatter detection to show relief details in an otherwise flat image. iCys provides two types of scatter imaging: Light Loss and Shaded Relief. Scatter detection is only available when the Scatter channel is selected in the Instrument Setup window.

For information about using the Scatter detector, see “Selecting the Forward Scatter Method” on page 3-48”.

Figure 2-7: Forward Scatter Assembly



EpiFluorescence System

Note: The iCys EpiFluorescence system is an optional feature of the Olympus IX71 microscope that allows you to view samples directly using reflected fluorescent light to highlight particular features.

For information about using the EpiFluorescent system, see “Observing Samples using EpiFluorescence” on page 2-15.

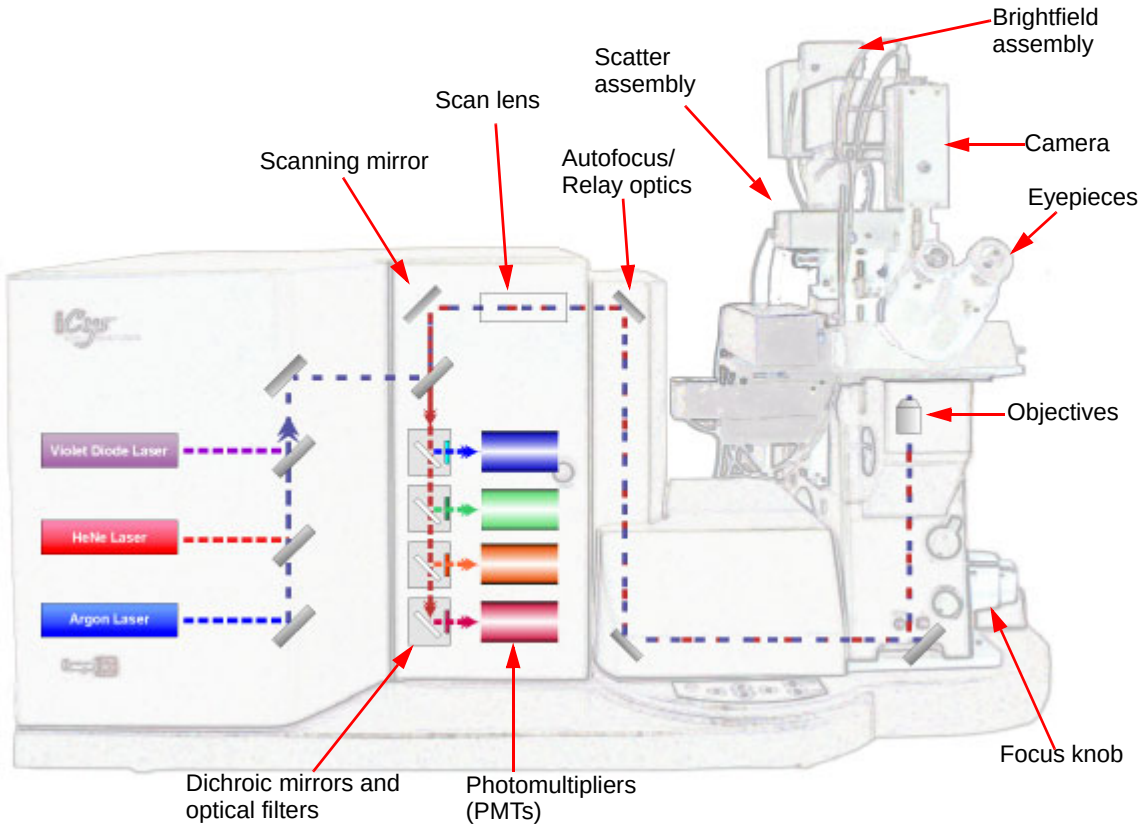
For information about using the EpiFluorescent system hardware, see “Optional EpiFluorescence Illumination Assembly” on page 2-31.

For detailed information about the EpiFluorescence system, see the Olympus *Instructions: Manual/Motorized Reflected Fluorescence System*.

The Delivery and Collection Optics

Figure 2-8 illustrates the delivery and collection optics for the iCys system:

Figure 2-8: iCys Delivery and Collection Optics



Delivery Optics

The beams from all lasers are directed concentrically to the main beam splitter through laser beam steering elements. The mirror reflects the laser beams upward to the scanning mirror, which is driven by a galvanometer, creating a beam sweeping across the scan lens. The scan lens creates a scan line which is reflected at 90 degrees by a fully reflective mirror. Light reflected from the mirror passes through a pair of relay lenses, is reflected off a 90 degree full reflective mirror towards the microscope, and is then directed upwards through the objective lens to illuminate the specimen.

Collection Optics

The same optical configuration collects fluorescent light from the specimen, directing emitted light up to the main beam splitter. Fluorescent light passes through the main beam splitter to the dichroic mirrors, optical filters, and photomultiplier tubes.

iCys Hardware Setup

iCys allows you to observe or scan samples using a number of different methods. The setup requirements for each method are described in this section.

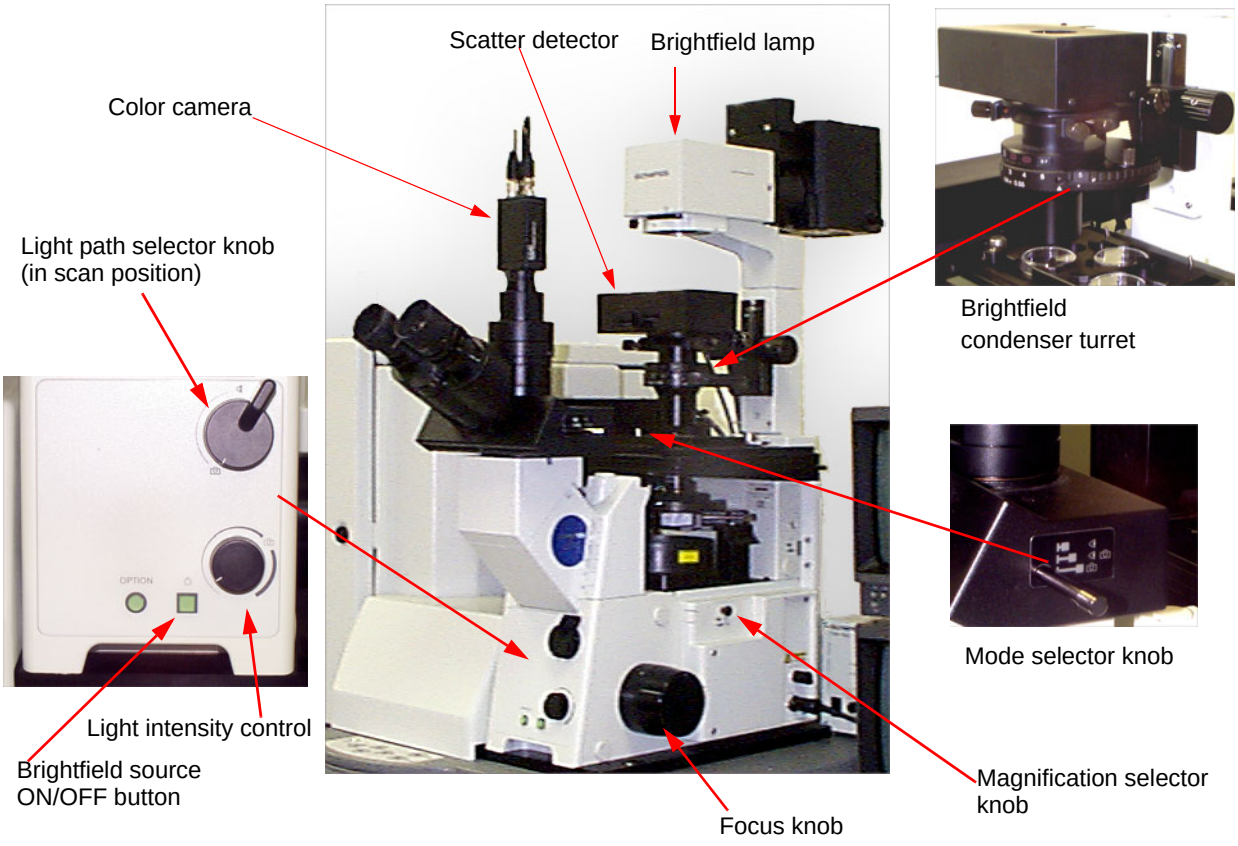
This section contains the following information:

- ❑ Scanning Samples with iCys, page 2-14
- ❑ Observing Samples With the Video Camera, page 2-16
- ❑ Observing Samples with the Brightfield Source, page 2-14
- ❑ Observing Samples using EpiFluorescence, page 2-15

iCys Microscope Components

iCys uses an Olympus IX71 microscope with a number of options for different scanning and viewing methods.This section shows the components you need to use to set up the iCys hardware for the different methods of scanning and observation.

Figure 2-9: iCys Microscope Selected Components



Scanning Samples with iCys

➢ To scan samples with iCys, you need to make sure that the microscope is set as follows:

1. Set the light path selector lever to side port ( ).

Note: When set to observation ( ), scanning will not take place.

2. Set the magnification selector knob to 1X magnification (the pushed in position).
3. If your system has the optional EpiFluorescence feature, set the EpiFluorescence cassette to position 1.
4. Set the condenser filter turret to position 5.

Note: Position 5 of the condenser filter turret is used for brightfield and Forward Scatter and must be empty.

5. Make sure the condenser is rotated in the down position (not rotated upwards).
6. After the hardware setup is complete, you can use the iCys application to set up and scan as described in Chapter 3, "iCys™ Cytometric Analysis Software Application Overview."

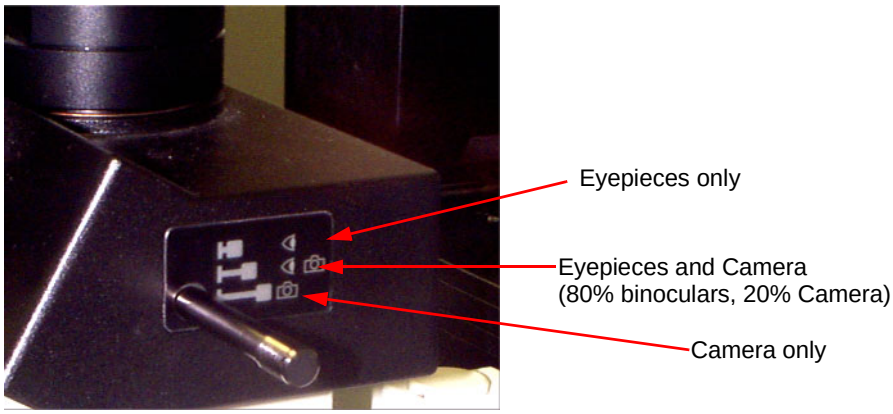
Observing Samples with the Brightfield Source

➢ To observe samples using the brightfield source:

1. Set the light path selector lever to observation ( ).
2. Turn on the following:
   - The TH4-100 Power Supply (shown in Figure 2-6, "Brightfield Illumination Assembly," on page 2-10).
   - The brightfield ON/OFF switch (shown in Figure 2-9, "iCys Microscope Selected Components," on page 2-13).

   You may want to set the Light Control Intensity knob on the TH4-100 to "Max" and use the Light Intensity Control knob on the main instrument to control the brightfield illumination.
3. Select Brightfield from the iCys Instrument menu to open the brightfield shutter. For more information, see "Instrument Menu" on page 3-6.
4. If your system has the optional EpiFluoresence feature, set the EpiFluorescence cassette to position 1.
5. For viewing, if the camera is on (as described in "Observing Samples With the Video Camera" on page 2-16) you can select the viewing mode by sliding the Light Path Selector knob as shown in Figure 2-10:

Figure 2-10: Light Path Selector Knob



6. Focus the sample using the microscope focus knob or, if available, the rocker switch and the fine focus knob on the Remote Control unit.

7. After the hardware setup is complete, you can use the iCys application to view the samples on the microscope stage.

Observing Samples using EpiFluorescence

➢ If your iCys system includes the optional EpiFluorescence feature, you can use it to observe samples as follows:

1. Set the light path selector lever to observation ( ).

2. If necessary, deselect Brightfield from the iCys Instrument menu.

3. Turn on the Power Supply Unit for the mercury burner.

4. Set the EpiFluorescence cassette to the desired filter position. The following filters are supplied with iCys:

Table 2-1: EpiFluorescence Filters

Turret Position	Filter Name	Filter Short Name	Excitation Light Color	Emission Color
2	U-MNU2	NU	Violet	Blue
3	UMWIBA2	WIBA	Blue	Green
4	U-MWIG2	WIG	Green	Red

5. Make sure the shutter on the EpiFluorescence cassette is open:

6. For viewing, if the camera is on (as described in "Observing Samples With the Video Camera" on page 2-16) you can select the viewing mode by sliding the Light Path Selector knob as shown in Figure 2-10, "Light Path Selector Knob," on page 2-15.
7. Focus the sample using the microscope focus knob or, if available, the rocker switch and the fine focus knob on the Remote Control unit.
8. After the hardware setup is complete, you can use the iCys application to view the samples on the microscope stage.

Note: When using EpiFluorescence, you may cause photobleaching in the exposed samples. Photobleaching is caused by exposing fluorophores to light which, over time, destroys the fluorophores irreversibly and results in loss of fluorescence. The degree of photobleaching increases with the time and intensity of light exposure.

When using the EpiFluoresence feature prior to scanning, minimize sample exposure and when possible scan in areas that have not been exposed to EpiFluorescence illumination.

For detailed information about the EpiFluorescence system, see the Olympus *Instructions: Manual/Motorized Reflected Fluorescence System*.

Observing Samples With the Video Camera

iCys comes with a 3CCD camera module, EPIX PIXCI® Imaging Board, and XCAP-Lite image viewing software. You can use the camera while observing to magnify and save images from the computer monitor.

➢ To use the video camera:

1. Turn on the color camera monitor.
2. From your PC, click on XCAP for Windows to open the EPIX XCAP-Lite application.
3. When iCys scans a sample or is moved from one event to another, the visual output is displayed on the camera monitor, if on, and on your PC, if XCAP-Lite is open.

From XCAP-Lite, you can capture and save screenshots of desired images. See the online help for XCAP-Lite for more information.

iCys Specimen Carriers

The first step in running a scan is to load your specimens. After the specimens are loaded, you can set up scan parameters as described in Chapter 3, “iCys™ Cytometric Analysis Software Application Overview.”

iCys provides a number of options for specimen carriers:

- Microtiter plates (also referred to as “microplates” or “plates”) with 6, 12, 24, 48, 96 or 384 wells.
- Single standard slide.
- Four slide carriers.
- 3-petri dish holder.
- Chamber slide.
- Clatch slide—a type of chamber slide used for immunophenotyping studies.

Additionally, you can define custom carriers for iCys, as described in Appendix C, “Administrator Tasks.”

About Specimen Carriers

All specimen carriers fit onto the plate holder on the iCys stage. iCys accepts the following types of carriers:

- Microtiter plates. In general, microplates contain cellular or DNA samples. CompuCyte recommends the following types of microplates:
  - 96-well, plastic (such as Whatman cat# 7716-2380)
  - 96-well, glass, skirtless (such as Whatman cat# 7716-2370)
  - 96-well, glass, skirted (such as Whatman cat# 7716-2375)
  - 48-well, tissue culture-treated (such as BD Falcon #353078)
  - 24-well, tissue culture-treated (such as BD Falcon #353047)
  - 12-well, tissue culture-treated (such as BD Falcon #353043)
  - 6-well, tissue culture-treated (such as BD Falcon #353046)
  - 384-well, optical quality plastic (such as Whatman #7706-2103)
  - 384-well, optical quality plastic (such as Greiner #781096)
- Standard slide carriers and “4-slide carriers.” Slides are used for both tissue and cellular samples.

  Slide carriers can be ordered from Compucyte.
- Petri dish carriers. Petri dish carriers can hold up to three 35mm x 10mm culture dishes (such as Corning #430165).

  Petri dish carriers can be ordered from Compucyte.
- Chamber slide carriers. Chamber slide carriers are designed to hold the Lab-Tek II 2-chamber slides (Nalge Nunc #155379).

Chamber slide carriers can be ordered from CompuCyte.

- ❑ Clatch slide carriers.
- ❑ 4-slide carriers.

Loading Specimen Carriers

This section describes loading the various types of specimen carriers supported by iCys.

Note: If your iCys system does not use the optional AutoFocus feature, make sure that the objective turret is in the full downward position before loading a carrier (that is, selecting Load or Instrument ⇒ Load Plate) onto the microscope stage.

Loading a Microtiter Plate

➢ To load a microplate on the microscope stage.

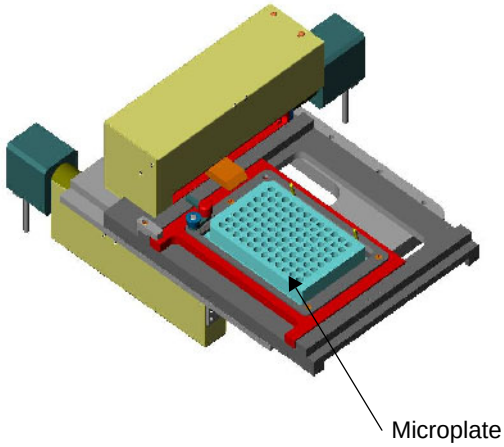
1. Invoke the load operation by doing either of the following:
   - ❍ Select **Load** from the taskbar
   - ❍ Select **Instrument** ⇒ **Load Carrier** from the menu bar

   The Load operation causes the stage to go to the load position and the plate release bar to be retracted.
2. Place the plate onto the stage. The beveled corners should be to your left and the column and row labels readable from your point of view.

Note: If your iCys system uses the optional AutoFocus feature, make sure that microplate wells to be scanned are at least 1/3 full to allow AutoFocus to correctly focus.

Do not touch the bottom of the plate. Any contamination can adversely affect your data.

Figure 2-11: Microscope Stage with 96-Well Plate



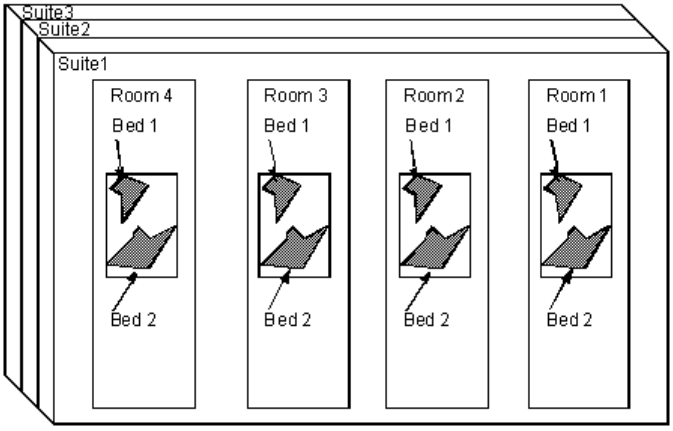
Loading Other Carrier Types

Loading Slide Carriers

iCys supports the use of two types of slide carriers:

- A standard slide carrier, which holds a single slide. The standard slide carrier is shipped as part of the standard iCys installation.
- A “4-slide carrier” which holds four slides and can be ordered from CompuCyte.

Figure 2-12: Sample 4-Slide Carrier Layout

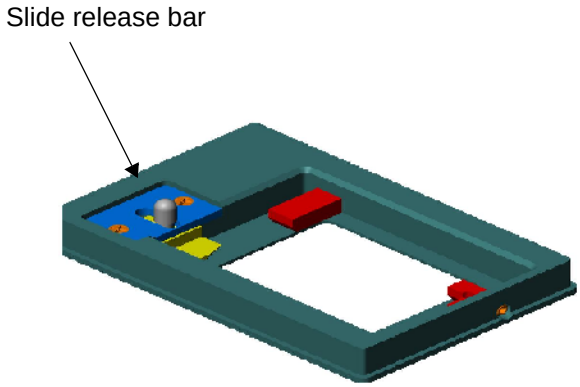


Note: Slides within a 4-slide carrier are loaded upside down. Therefore, they are numbered from right to left.

Loading a Standard Slide

Use the provided slide holder to analyze specimens on a microscope slide. The standard slide holder fits into the same space on a stage as a microtiter plate.

Figure 2-13: Standard Slide Carrier



➢ To load a standard slide tray:

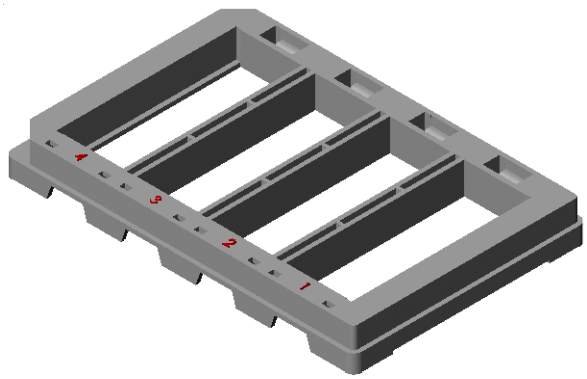
1. Holding the slide release bar to the left, insert the slide into the slide holder, coverslip down.
2. Slowly release the slide release bar. Ensure that the slide is secure.
3. Invoke the load operation; click **Load** on the taskbar or select **Instrument** ⇒ **Load Plate** from the menu bar.
4. Place the slide holder on the stage with the slide release lever at the lower left. Ensure that the slide holder is secure.

Note: Do not touch the coverslip. Any contamination can adversely affect your data.

Loading a 4-Slide Carrier

You can order 4-slide carriers from CompuCyte. These carriers allow you to scan multiple samples with less human intervention. The 4-slide carrier fits into the same space on a stage as a microtiter plate.

Figure 2-14: 4-Slide Carrier



➢ To load a 4-slide carrier:

1. Snap the slides into their respective slide holders on the underside of the carriers, coverslip down.

Note: Since 4-slide carriers are loaded upside down, they are numbered from right to left.

2. Ensure that the slides are secure.

Tip: For slide carriers, make sure that the slides themselves are loaded into the tray with the cover side on the bottom (that is, upside down). Also, make sure that each slide is loaded into its holder correctly. The slides must be loaded flush with the mounting surface on the bottom of the carrier.

3. Invoke the load operation; click **Load** on the taskbar or select **Instrument** ⇒ **Load Plate** from the menu bar.
4. Place the slide holder on the stage with the bevel at the lower left. Ensure that the slide holder is secure.

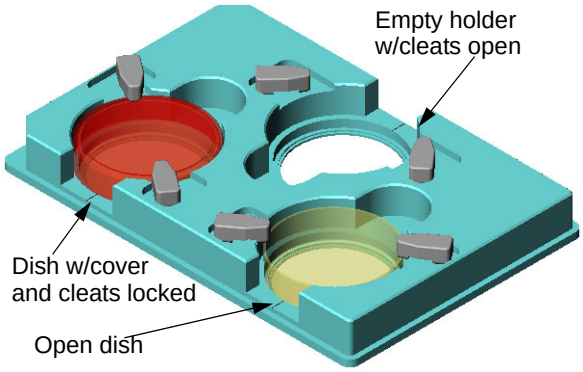
Note: Do not touch the coverslip. Any contamination can adversely affect your data.

Loading a 3-Petri Dish Holder

You can order 3-petri dish holders from CompuCyte. These holders allow you to scan multiple samples with less human intervention. The 3-petri dish holder fits into the same space on a stage as a microtiter plate.

Note: If your iCys system does not use the optional AutoFocus feature, make sure that you only scan one petri dish at a time. Between petri dishes in the same carrier, you must lower the objective turret before moving the stage.

Figure 2-15: 3-Petri Dish Holder



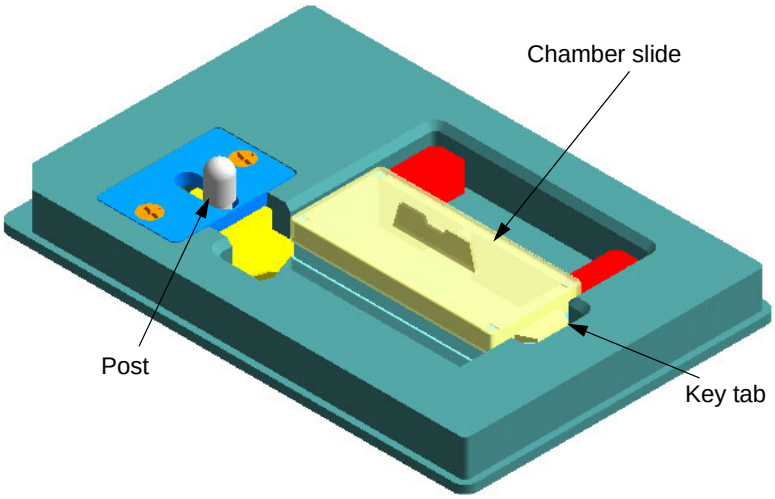
➢ To load a 3-petri dish holder:

1. Place the petri dishes into their holders. Petri dishes can be scanned with or without their covers. When using Forward Scatter, you will obtain better images scanning without covers.
2. Move the cleats to lock the petri dish into place.
3. Invoke the load operation; click **Load** on the taskbar or select **Instrument** ⇒ **Load Plate** from the menu bar.
4. Place the petri dish holder on the stage with the single petri dish holder facing the rear of the instrument.
5. Ensure that the holder is secure.

Loading a Chamber Slide

You can order a chamber slide holder from CompuCyte. The chamber slide holder allows you to use a 2-chamber Lab-Tek II slide with iCys.

Figure 2-16: Chamber Slide Carrier



➢ To load a chamber slide in the chamber slide holder:

1. Hold the chamber slide by the long sides.
2. Pull the spring-loaded post back (towards left), and carefully place the slide into holder, with the key tab engaged into the matching opening on the holder.
3. Position the slide firmly against the 2 rear stops and fully to the right, towards the key tab cutout. Slowly release the post.
4. Make sure that the slide is flat against the rear stop and lies flat on the bottom of the holder.
5. Chamber slides can be scanned with or without their covers. When using Forward Scatter, you will obtain better images scanning without covers.

iCys Safety Requirements

iCys has been designed for safe and reliable use for laser cytometry of microplates, slides, and other carriers. The iiCys Research Imaging Cytometer's optics/electronics unit contains up to three Class 3b lasers inside and is itself a Class 3R laser product.

Warning: If the iCys system is used in a manner not specified by CompuCyte Corporation, the protection provided by the equipment may be impaired.

All lasers are intense sources of coherent light and may cause injury if used improperly. In addition, high voltages are present inside the iCys Research Imaging Cytometer. Neither are accessible to the operator, unless the door and/or covers are opened and the lasers are energized. Be aware of these potential dangers and take the proper precautions.

Note: All service functions must be performed by a trained authorized service person.

Laser Safety Labels

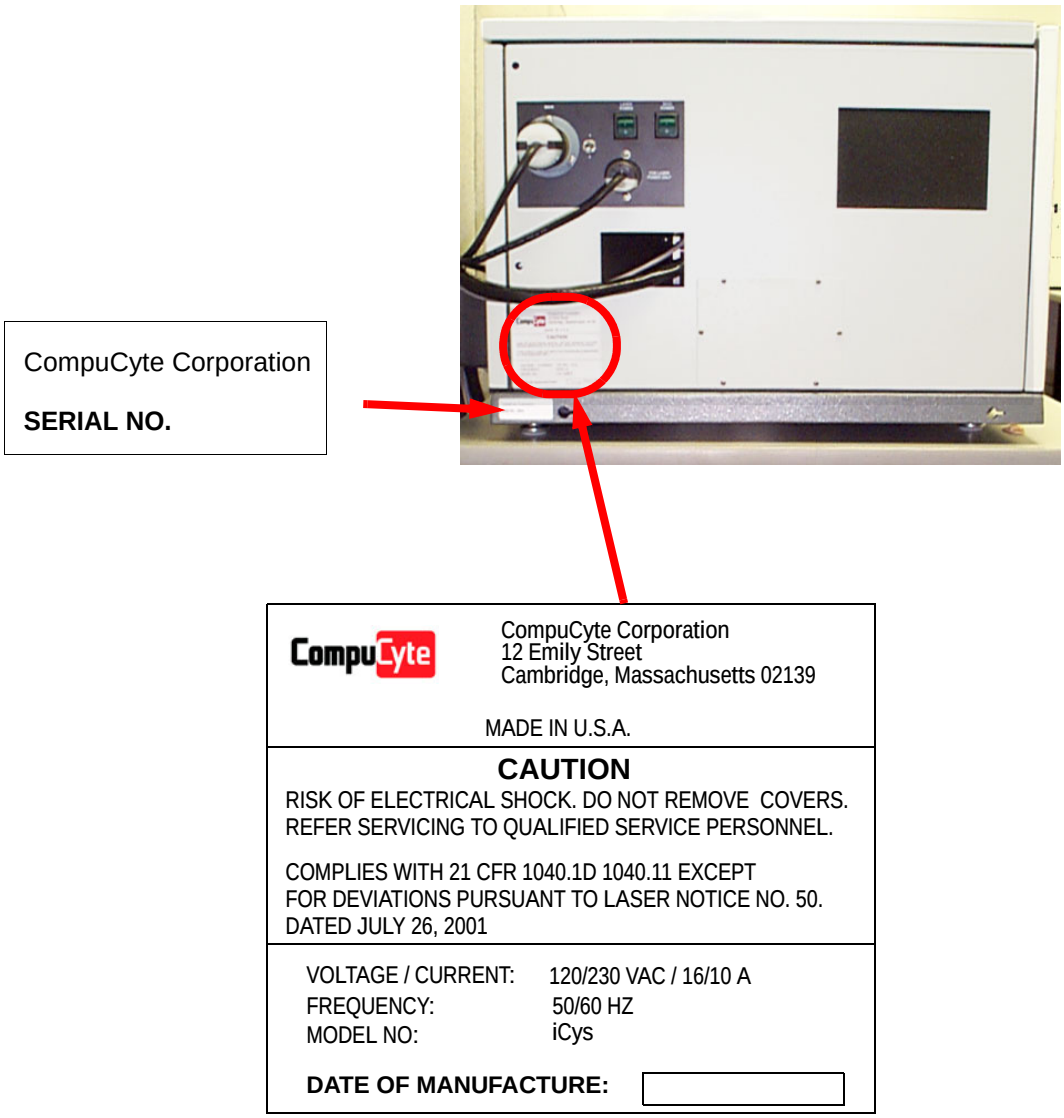
The iCys system comes equipped with the following safety labels:

- ❑ Identification and Laser Certification Label
- ❑ Classification label
- ❑ Access Panel labels

Identification and Laser Certification Label

Figure 2-17 shows the location of the identification labels.

Figure 2-17: iCys Labels



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Classification Label

The classification label is located on the left side of the main instrument by the Identification and Laser Certification labels.

Access Panel Labels

The access panel label is for the main instrument housing lasers. This label is located in three places:

- ❑ Inside access panel
- ❑ PCB mounting panel
- ❑ Rear cover

Caution - Class 3B laser radiation when open
Avoid direct eye exposure

Aperture Labels

This symbol indicates the location of the system laser aperture (the objective lens). The label is located on the right face of the black housing directly below the objective lens turret.

Laser Aperture

Safety Symbols

The iCys system comes equipped with the following safety symbols:

- ❑ Protective earth 5019
- ❑ On (power) 5007
- ❑ Off (power) 5008
- ❑ Pinch Point (hand in rollers) 8011H-ISO

Protective earth 5019

Protective earth
This symbol indicates the location of the protective earth ground for the main instrument.

It is located on the inside left wall of the main instrument enclosure.

On (power) 5007

On (power)
This symbol indicates the ON position of the main power switches and the laser power switch.

It is located near or on these switches on the left side of the main instrument housing.

Off (power) 5008

Off (power)
This symbol indicates the OFF position of the main power switches and the laser power switch.

It is located near or on these switches on the left side of the main instrument housing.

Pinch Point (hand in rollers) 8011H-ISO

Pinch Point
This symbol indicates possible pinch points between the stage and the instrument cover to the left of the stage and between various components of the stage.

It is located on the instrument cover directly to the left of the stage.

Safety Feature: Key Locked Door

The front door panel can only be opened with a special hex key. Other panels require tools to open and should be accessed only by authorized service personnel.

Safe Operating Guidelines

Warning: Use of controls or adjustments or performance of procedures other than those specified herein may result in hazardous radiation exposure.

Laser Safety

The following safety recommendations apply:

- ❑ Do not attempt to service the laser system.
- ❑ For all service functions call a trained CompuCyte authorized service person.
- ❑ Do not remove the internal safety shields

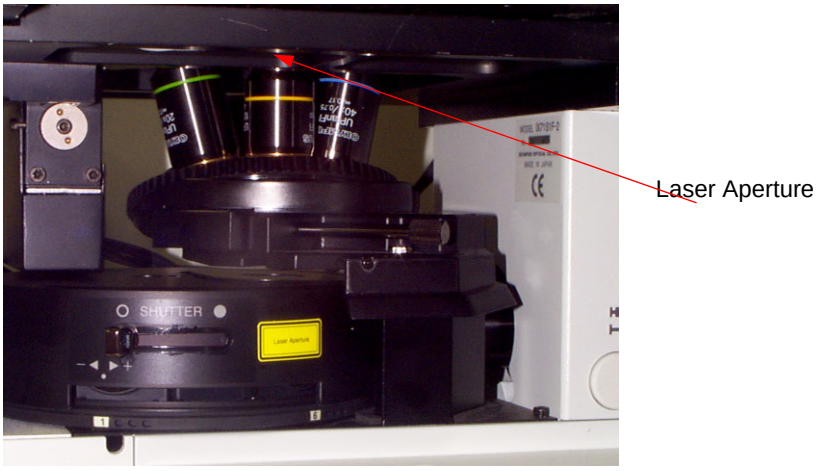
Warning: Viewing the laser output with certain optical instruments (for example, eye loupes, magnifiers, and microscopes) within a distance of 100 mm may pose an eye hazard.

Laser Aperture at Objective Lens

Laser light is emitted from the objective lens that is currently selected for use by rotating the objective lens turret.

Warning: Do not remove objective lenses or objective lens port caps.

Figure 2-18: iCys Laser Aperture



Electrical Safety

The following safety recommendations apply:

- ❑ Keep all panels and covers in place when operating the system to prevent the chance of electrical shock.
- ❑ Refer all servicing of high voltage or line voltage to an authorized CompuCyte service representative.
- ❑ The CompuCyte iCys Research Imaging Cytometer requires surge protected mains or other external surge protection to meet the surge requirements of EN61326. Surge protection capable of eliminating surges up to 2kV line to ground, and 1kV line to line is required.

- CompuCyte highly recommends the use of a surge protector for the computer and all peripheral devices, such as your monitor and printer.

Mechanical Safety

The following safety recommendations apply:

- Do not place your fingers between the moving stage and the iCys housing
- Do not place fingers in specimen area within the stage

Laboratory Safety

The following safety recommendations apply:

- Follow established safety procedures for handling specimens.
- Avoid direct contact of skin and mucous membranes with dyes and other chemicals that may cause injury.

General Maintenance Information

iCys Maintenance

The general maintenance for the components of the iCys are listed below:

- ❑ Microscope
- ❑ Computer
- ❑ Optics and Electronics Unit

Microscope

Clean the lenses by blowing air across the lens surface using a filtered air source and/or wiping lightly with gauze. Avoid abrasives or solvents as they can permanently damage the finish. Do not disassemble any part of the microscope.

Computer

When your computer has gathered too much dust turn it off and disconnect all power cords. You can then wipe it with a damp piece of cloth. To clean the monitor screen, spray window cleaner on a soft cloth and gently wipe the screen.

Do not unplug the power cord while the system is on. After you have switched the power off, wait ten seconds before switching the power on again.

CompuCyte highly recommends the use of a surge protector for the computer and all peripheral devices, such as your monitor and printer.

Note: Any additions and deletions to hardware or software must be done with prior approval of CompuCyte Corporation.

Optics and Electronics Unit

The optics and electronics unit contains no user-serviceable parts.

Do not block the output of the laser cooling fan.

Functional Specifications

The following specifications apply to the iCys system.

Microscope

Table 2-2 describes the specifications for the microscope used with iCys.

Table 2-2: iCys Microscope Specifications:

Olympus IX-71 microscope base	
Objectives	❑ 10x magnification — 0.3 N.A. ❑ 20x magnification — 0.5 N.A. ❑ 40x magnification — 0.75 N.A
	Crosshair alignment
Eye Viewing	WH10x eyepieces, F/22
Video Viewing	Sony CCD Video Camera, with EPIX frame grabber card
Brightfield Illumination Assembly	❑ 100W precentered Halogen bulb ❑ Brightfield power source, TH4-100 ❑ NA 0.55, 27mm Working Distance condenser with 5 position manual turret ❑ Illumination Column allows 30 degree tilting
Optional EpiFluorescence Illumination Assembly	❑ 100W Mercury Burner ❑ Mercury Burner power source ❑ Filter Slider with three filter pockets ❑ WIG, WIBA, and NV filter cubes installed in Fluorescence Mirror Unit Cassette

Motorized stage

The range and resolution of the motorized stage is:

- ❑ **Travel range**:
  - ❍ X axis = 119.3mm
  - ❍ Y axis 116.3mm
- ❑ **Resolution**—0.5 microns

Lasers

Embedded lasers

Table 2-3 describes the embedded lasers available for use with iCys. iCys comes with the Argon laser; the HeNe and Violet lasers are available as additional features.

Table 2-3: iCys Lasers

	Wavelength (nm)	Rated output (mW)	Beam divergence (mRad)
Argon ion	488	20	0.95
Helium Neon	633	5	1.0
Violet diode	405	60	0.2 x 0.4 (elliptical)

All lasers are operated in constant wave mode (CW), that is, not pulsed.

Measurements +/-5% tolerance

Output drift:

- ❑ 405nm +/-2.5%
- ❑ 488nm +/-1%
- ❑ 633nm +/-2.5%

Measurements at objective

Table 2-4 describes the microscope measurements at the objective.

Table 2-4: Microscope Measurements at Objective

	10x		20x		40x	
	Power (mW)	Beam Divergence (mRad)	Power (mW)	Beam Divergence (mRad)	Power (mW)	Beam Divergence (mRad)
Argon ion (20mW setting)	3.50	75	3.40	142	3.09	309
Helium Neon	1.13	99.0	1.09	122	0.92	207
Violet diode	0.80	74	0.80	144	0.74	283

Forward Scatter Detector

Scatter detection is described in detail in “Selecting the Forward Scatter Method” on page 3-48.

- ❑ Light loss detection
- ❑ Shaded relief detection

Performance Specifications

Precision

Commercially available fluorescent beads are scanned on a microscope slide with the argon ion laser and measured in the red fluorescence. For 2500 beads, the CV of the red value is less than 3%, calculated by Full Width Half Max (FWHM).

Linearity

Commercially available fluorescent beads are scanned with the argon ion laser. Clusters for singlets and doublets are defined and measured for mean red value. The ratio of mean red value of doublets to singlets is in the range of 1.9 to 2.1.

Relocation Precision

Relocation of cells is within ± 6 microns on carriers which have not been removed from the stage. This has been verified by running commercially available fluorescent beads which are 6 microns in diameter.

Chapter 3

iCys™ Cytometric Analysis Software Application Overview

This chapter discusses how to use the iCys™ Cytometric Analysis Software to set up an experiment, scan, and save data.The iCys software is designed to facilitate automated data acquisition and analysis. Once you have set up the instrument for a particular type of experiment, you can save parameters such as instrument settings, the type of data you want to acquire and display, and the statistics to be collected in a Workspace file. Whenever you run a similar experiment these variables can easily be recalled and updated as needed.

For information about setting analysis parameters for iCys, see Chapter 4, “Generating Analytical Data.”

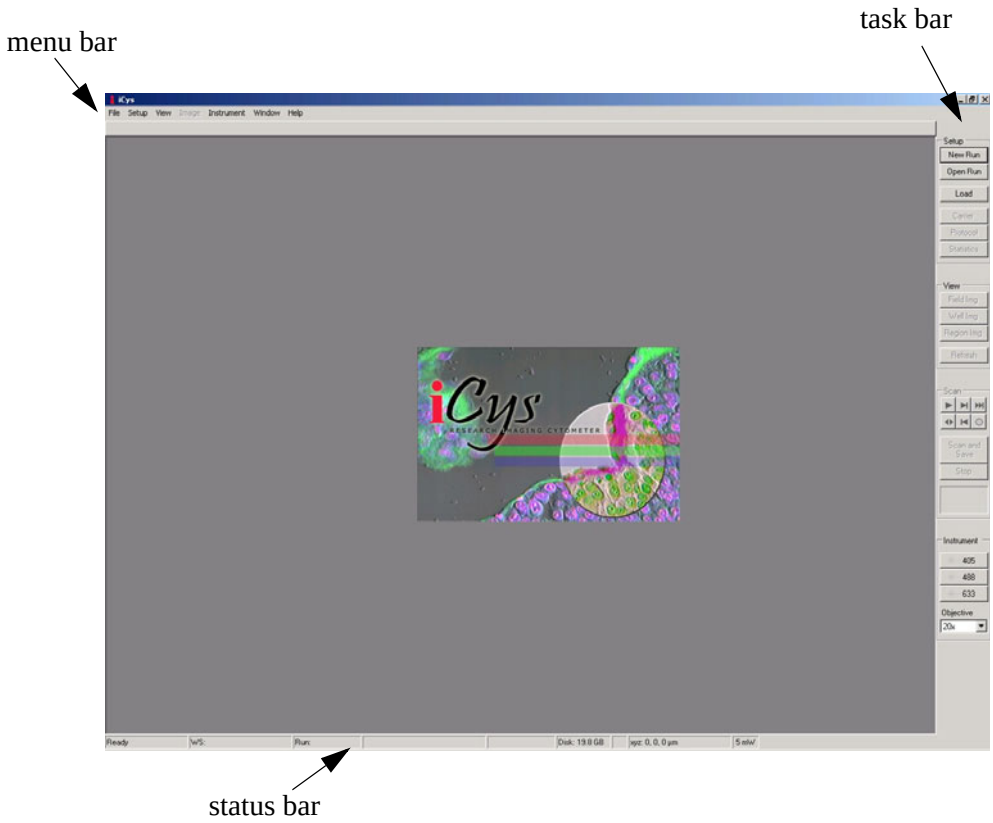
Included in this chapter:

- iCys Software Overview, 3-2
- Menu Bar Overview, 3-3
- iCys Status Bar, 3-8
- iCys Task Bar, 3-10
- Data Structure, 3-86
- Specialized Features, 3-90

iCys™ RESEARCH IMAGING CYTOMETER

iCys Software Overview

When the iCys Software is launched, it opens the startup screen pictured below.



The pulldown menus in the **menu bar** allow you to set the parameters for acquisition and analysis of data.

The **task bar** is designed to lead you sequentially through the workflow involved in setting up the instrument for an experiment. The task bar contains many of the features found in the pulldown menus.

The **status bar** provides information about the current status of the iCys system.

Each of these features is discussed in detail in this chapter.

Menu Bar Overview

This section describes the menu options available from the iCys menu bar.

- File Menu, page 3-3
- Setup Menu, page 3-5
- View Menu, page 3-5
- Instrument Menu, page 3-6
- Window Menu, page 3-7
- Help Menu, page 3-7

File Menu

The options in the File menu allow you to open, close, save, and print files and Workspaces.

When you select any option from this menu that opens a Workspace, the *Save Workspace*, or *Save Workspace As* window displays, allowing you to save the previous Workspace, as discussed in “Saving Workspaces” on page 3-87.

For more information about Workspaces, see “Workspaces and Workspace Files” on page 3-86.

The File menu options are as follows:

- **New Run**—Allows you to set up a new Run. You can specify a workspace to use. For more information on Run files, see page 3-10. The New Run command is also available from the Setup section of the task bar.
- **Open Run**—Opens a Run and its Workspace. If a previous Run is open, that Run and its associated Workspace are closed. Additional information on iCys file types can be found in “Data Structure” on page 3-86.

  Depending on certain file parameters and other information, you can use the Run file to either:

  - Reanalyze raw data or
  - Analyze event data.

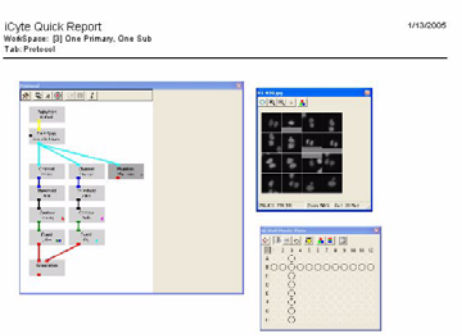
  The Open Run command is also available from the Setup section of the task bar, (see page 3-15.)
- **Close**—Closes the currently open Run file.

  When you close a Run, the Workspace is also closed. You will be prompted to save any changes you have made to the Workspace. For additional information about Run files, see “Data Structure” on page 3-86.
- **Open Workspace—**Opens an existing Workspace, closing the current Workspace. You are prompted to save changes to the current Workspace.
- **Save Workspace—**Saves the current Workspace in the WorkFiles folder. You cannot overwrite the template workspaces provided with iCys or a workspace

associated with a run. To save changes to these read-only Workspaces you must use the Save Workspace As command.

- **Save Workspace As—**Saves the current Workspace under another name.
- **Open Image**—Allows you to open an image file (that is, a file in *.BMP, *.FLF, *.JPG, or *.TIF format) using the standard *Microsoft Windows Open* dialog. The image is opened as a window in the iCys Workspace. Once opened, you can manipulate the image in the same way you do in other iCys image windows.
- **Import Protocol**—Imports the data acquisition protocol settings from an existing workspace. For information about protocols, see “Workspaces and Workspace Files” on page 3-86).
- **Export Events**—Saves an Event file under another name or as a text file.
- **Merge Runs**—Allows you to merge the data from two similar runs.

  Use the Merge Run feature to create a virtual run that contains specific data from two or more existing runs. For more information on merging runs see “Merging Runs” on page 3-91.
- **Preview Report**—Generates a snapshot of the current iCys window including the Scan View window, histograms, scattergrams, and gallery views. For example:



Note: iCys currently only provides reports in a single page format.

- **Print Report**—Sends the report to the default printer. If you have Adobe Acrobat installed you can also generate a PDF file using the Adobe PDF printer driver. (A copy of Adobe Acrobat is included with the iBrowser.)
- **Save Report**—Allows you to save the report as a JPEG (.JPG), TIFF (.TIF), or bitmap (.BMP) file in a location you specify.

Note: For all reports, iCys only includes those windows that are entirely inside the Workspace display area.

- **Exit**—Exits iCys. You will be prompted to save the current Workspace.

Setup Menu

- **Carrier Type**—This allows you to specify the type of carrier you will be using for the current run. Additional information on carrier types is found “iCys Specimen Carriers” on page 2-17.
- **Carrier**—Displays a graphic of the carrier and opens the Scan Area Setup window for this run. Additional information on Scan Area Setup is found in“Defining the Scan Area for Wells” on page 3-20 and “Defining the Scan Area for Freeform Carriers” on page 3-26.
- **Protocol**—Opens the Protocol Setup window. This window allows you to specify the details of how you want data collected and processed for the current run. This feature is covered in detail in “Setup Section” on page 3-10.
- **Statistics**—Opens the Setup Statistics window, from which you can define the Run Statistics for this run, as described in “Statistics” on page 3-60.
- **Protocol Summary**—Displays a textual description of the current protocol window. Buttons allow you to save the summary to disk, or to copy it to the clipboard, from which it can be pasted into other applications.
- **New Tab**—New Tab allows you to create new pages to better organize the display of your data. To create an element on a page, activate the page by selecting its Tab, then create the element.You can create and populate as many pages as you wish. To view a particular page, select its tab from the top of the iCys window.
- **Rename Tab**—Renames the selected Tab.
- **Delete Tab**—Deletes the selected Tab.

Tip: Right-clicking on an existing tab allows you to create a new tab, rename the current tab, or delete the current tab.

- **Show Administration Menu**—Displays an Enter Password dialog. When you enter the correct password, the Administration menu is added to the menu bar. See Appendix A, “Installing iCys™ Research Imaging Cytometer,” for information about the Administration menu.

  If the Administration menu is displayed, you can hide it by deselecting Show Administration Menu from the Setup menu.

View Menu

The options on the View menu allow you to configure the following iCys features:

- **New Scattergram**—Creates a new two-parameter scattergram. For information about scattergrams, see “Creating and Changing Scattergrams” on page 4-22..
- **New Histogram**—Creates a new one-parameter histogram. For information about histograms, see “Creating and Changing Histograms” on page 4-38.
- **New Gallery**—Creates a new gallery of images from a specified region. For information about galleries, see “Defining and Creating Galleries” on page 4-20.
- **Region Statistics**—Displays detailed statistics about each defined region. For more information, see “Displaying Region Statistics” on page 4-18.
- **Run Statistics**—Displays detailed statistics about each scan area. For more information, see “Displaying Run Statistics” on page 4-16.
- **Field Image**—Opens the Scan Field Image window (formerly Scan Data display), which allows you to control and monitor the scanning of your specimen. When no other active document is open, this window is titled Scan Field Image.
- **Well Image**—Opens the Well Image window. A Well image is a mosaic of all scan fields for a given scan area.
- **Region Image**—Opens an image of the scan fields contained in a specified region. (This feature is available only with the iNovator toolkit.)
- **Refresh**—Repaints all of the open windows on the iCys Workspace. This can be useful when you are adjusting various iCys parameters. If Auto Refresh is checked, Refresh is not available.
- **Auto Refresh**—Automatically repaints open windows in the iCys Workspace when adjustments are made to iCys parameters.

Image Menu

This feature is available only with the iNovator toolkit.

Instrument Menu

- **Home Stage**—Moves the microscope stage to its home position.
- **Home Objective**—Moves the microscope objective to its home position.
- **Load Carrier**—Moves the microscope stage to the appropriate position for loading a carrier.
- **Brightfield**—Clears the path from the brightfield lamp to the condenser, allowing the brightfield source to illuminate the sample.

Note: When Brightfield is selected, the Scatter sensor is moved out of the way, and is unavailable.

- **Oscilloscope**—Opens the Oscilloscope window. This allows you to view a representation of the scan outputs. The oscilloscope feature is described in detail in “Using the Oscilloscope Feature” on page 3-90.

Window Menu

- This menu lists the histogram and scattergram plots in the current workspace. Selecting a plot will bring it to the front of the window. If the plot is on a Tab that is not active, selecting that plot will activate the appropriate Tab.

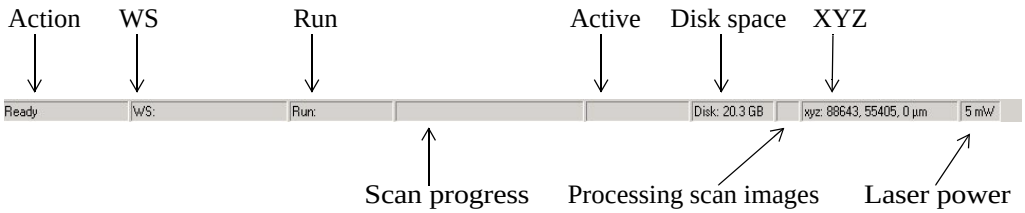
Help Menu

- iCys **User Guide**—This opens the iCys User Guide in PDF format.

Note: Adobe® Acrobat Reader® is required to read PDF files. If your system does not have a recent version of Acrobat Reader (version 4 or later), you can download it at no cost from the Adobe website.

- **CompuCyte.com**—If your computer is connected to the internet, this will point your browser to the CompuCyte website.
- **About iCys**—This displays information about the version of iCys you are running, including the software revision, serial number, and hardware configuration.

iCys Status Bar



The iCys status bar, at the bottom of the iCys window, displays the following information:

- **Action**—The current action, if any, being performed. These may include:
  - Ready
  - Test
  - Scan & Save Data
  - If a run is paused, the action field will be followed by the word *Paused*.
- **WS**—The name of the currently open Workspace.
- **Run**—The Run number and name of the Run being saved.
- **Scan Progress**—The first section of Scan Progress shows the field being scanned, in the format *xx* of *yyy* where:
  - **xx**— Number of fields scanned.
  - **yyy**—The total number of fields in this scan.

  The second part of **Scan Progress** in brackets reports:
  - If a microtiter plate is being used, the information in brackets indicates the identity of the well being scanned and the field being scanned in that well, in the format [XY, FZ], where XY indicate the well being scanned, and FZ indicate the field.
  - If a freeform carrier such as a slide is being used, the bracketed information indicates the room number of the slide and the field number being scanned. For information on freeform carriers see page 2-12 of this manual.
- **Active**—Indicates the currently active scan area whose data are displayed in the graphs and image windows.
- **Scan**—For repetitive scans only, described in “Setting Up a Repetitive Scan” on page 3-32, displays a scan count in the format *x* of *yy* where:
  - **x**— the number of scans performed. Except in the case of repetitive scanning, this number will be 1.
  - **yy**—the total number of scans requested.

- **Disk space—**The amount of disk space currently available on your iCys PC. If there is insufficient disk space, it could slow down or even stop iCys. Be sure to offload your data on a regular basis.
- **Processing Scan Images**—An hourglass appears while scanning is in progress.
- **XYZ**—When a scan is running, displays the current location of the carrier on the microscope bed.
- **Laser Power**—The laser power in mW.

iCys Task Bar

The iCys task bar is designed to guide you through the tasks necessary to set up an experiment and collect data.

Tip: The task bar can be docked on either side of the screen. To change its docking location, right-click anywhere on the task bar and choose the appropriate option.

The task bar consists of three main sections: the Setup area, the View area, and the Scan area. Each of these will be described in the following section.

Setup Section

Using the Setup section of the task bar you can:

- ❑ Select a Protocol Template
- ❑ Create a new Workspace Protocol
- ❑ Select a previously defined Workspace Protocol
- ❑ Select a Saved Run for viewing or re-analysis

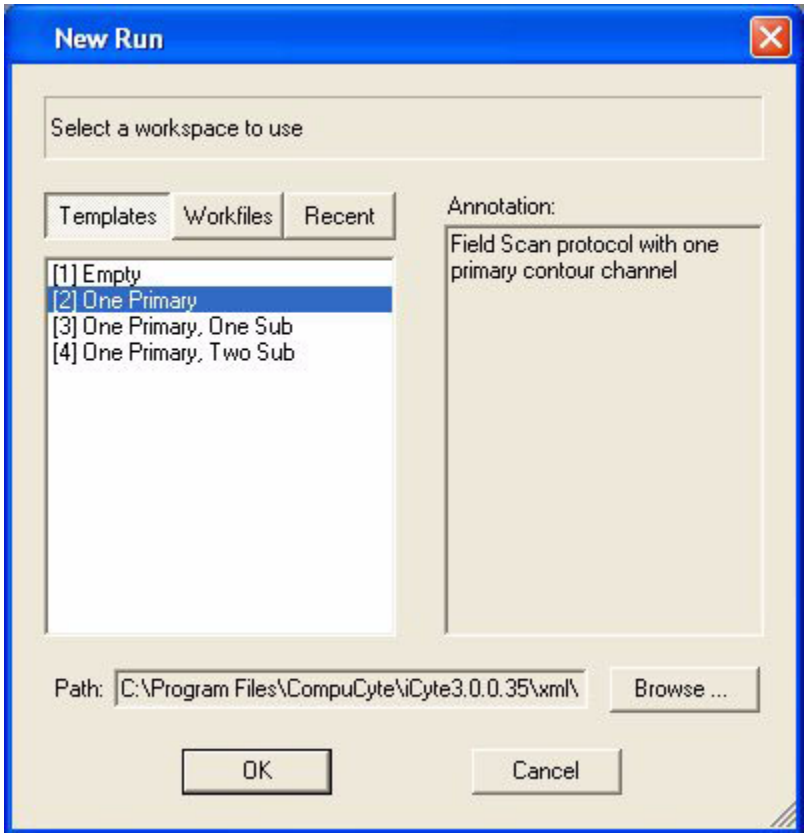
The tools in Setup allow you to define the settings specific to your analysis. In Setup you may define an analysis protocol or select a pre-defined protocol template.

Within the protocol you may:

- ❍ Select lasers and PMT detectors for the experiment
- ❍ Select primary and secondary channels
- ❍ Enable phantom contouring
- ❍ Define or select virtual channels
- ❍ Set up repetitive scans
- ❍ Select the general instrument settings (such as carrier type, etc.)

New Run

iCys allows you to choose among several run templates. These templates are provided with iCys and are designed to address some of the more common types of experiments. You may use these templates as they are, or alter them to suit your needs.



Highlighting a template displays any comments that were saved with it in the Annotation field.

If a workspace is already open when you choose New Run, you will be given the option of saving changes to it before it is closed.

Using the tabs you can choose from the templates provided with iCys, previously saved workspaces, or recently accessed workspaces. You can also browse for workfiles stored elsewhere.

iCys Templates

The following templates are included with the basic iCys software.

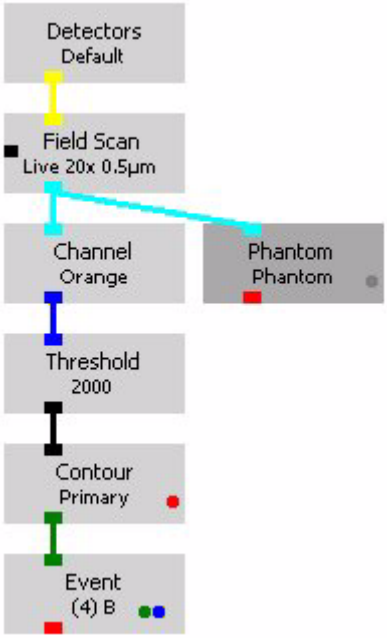
[1] Empty

The Empty template contains a 96 well plate carrier diagram and a blank protocol sheet. The Empty template can be used as a starting point in developing your own customized workspace.

[2] One Primary

The One Primary template has two tabs, Protocol and Data. The Data tab is an empty window provided for the display of histograms, scattergrams, etc.

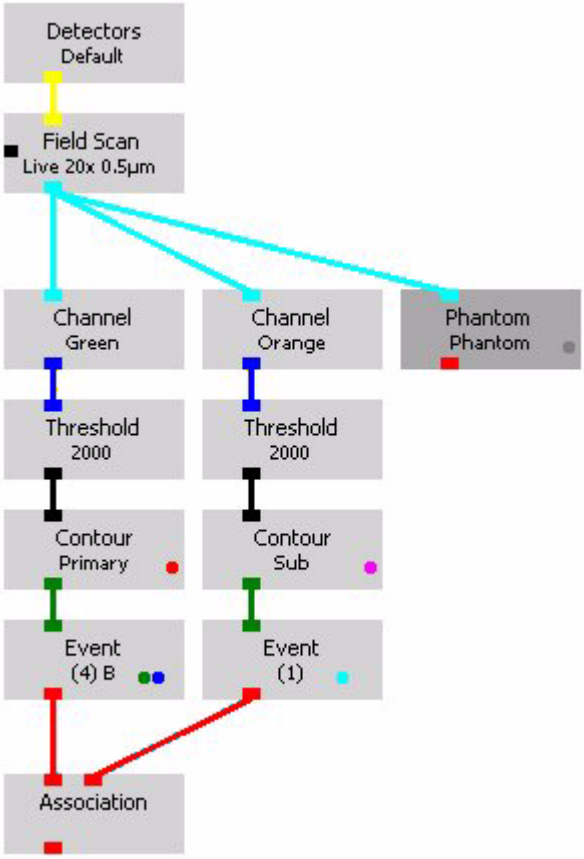
The Protocol tab contains a 96 well plate diagram, a Field Image window, and the protocol pictured below.



The protocol is designed to analyze data using a single contour, and includes a Phantom contour module.

[3] One Primary, One Sub

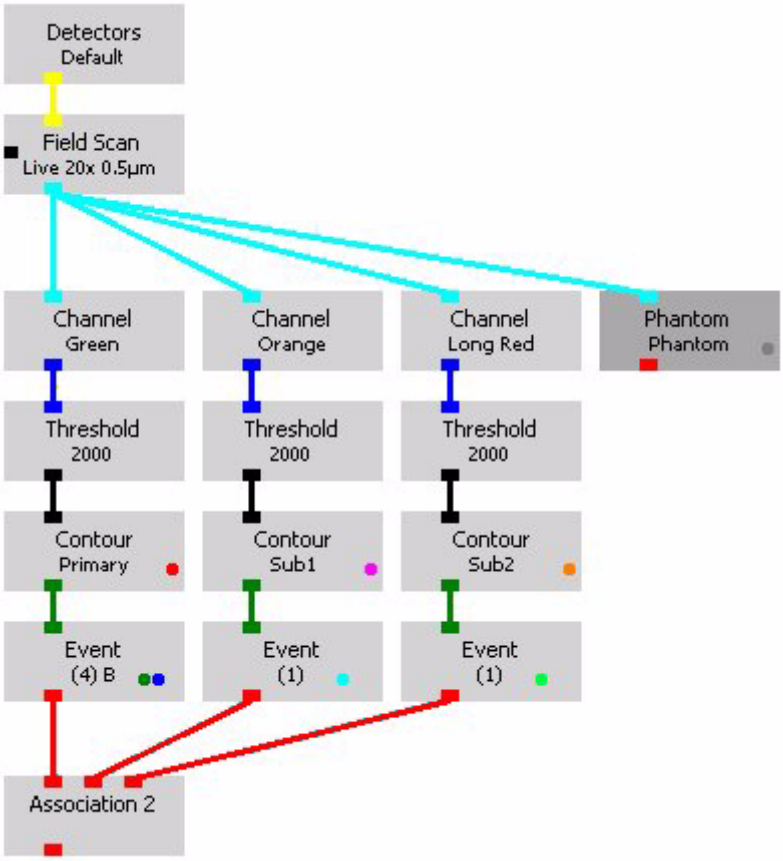
Like the One Primary, the One Primary, One Sub template has two tabs; a blank Data tab, and a Protocol tab containing a 96 well plate diagram, a blank Field Image window, and the following protocol.



This protocol analyzes data using two contours. These are combined in the Association module. As with the Primary Contour protocol, this protocol provides a Phantom contour module.

[4] One Primary, Two Sub

This template provides the same features as the previous two, but with the following protocol.



This protocol analyzes for a primary contour as well as two sub-contours, including an Association among the three. It also provides a Phantom contour module.

Note: Templates cannot be overwritten. If you wish to make changes to them, you must use the *Save as* option to create a new file.

Workfiles

Use this button to select a workfile from the Workfiles folder. Saved workfiles are stored in this folder.

Recent

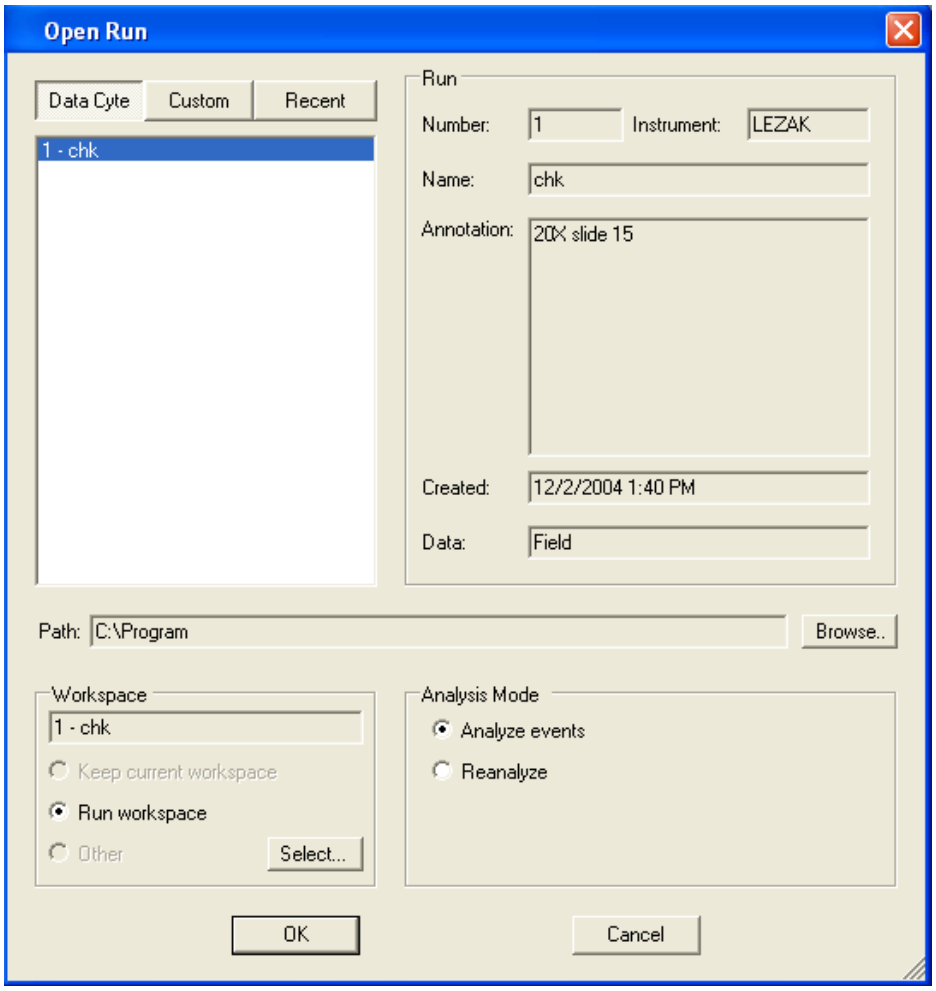
This button brings up a list of recently used workfiles.

Browse

Use this button to select a workfile not located in the Workfiles folder.

Open Run

Allows you to open a previously saved run, including workspace and data files. If a run is currently open, you will be prompted to save any changes.



On this page you can choose from runs saved in the DataCyte folder, custom runs, and recent runs. When a run is highlighted, information about that run is displayed in the Run field on the upper right of the window.

- ❑ **DataCyte**—This button points the system to the default data storage folder (DataCyte) on the iCys PC. Runs are stored here by default
- ❑ **Custom**—This button allows you to point the system to a specific alternate data location.

➢ To select the customized data location:

1. Click the Custom button.
2. Click the Change button in the Custom Location section.
3. Browse to an appropriate location and click OK. For example if you have moved runs from the iCys PC to a location on a remote server you might browse to a

folder named RemoteServer\iCysData to find the runs you want to reanalyze or the workspaces you want to use. Browse to the folder containing the run folder, not to the run folder itself.

- **Recent**—This button brings up a list of recently analyzed runs.
- **Browse**—Use to select a run stored in a location different from DataCyte or Custom folders.
- **Workspace**—In the Workspace area in the lower part of the window you can choose to analyze a run using:
  - The current (open) workspace
  - The workspace that was saved with the run you are opening
  - A different workspace that you may select

Note: In analyzing a run with a workspace other than the one saved with the run, you must ensure that the workspace and run are compatible.

- **Analysis Mode**—In the Analysis Mode area at the lower right of the window you can choose to analyze event data, or to analyze image data.

Load

Positions the stage for loading a carrier. See Chapter 2 of this manual for details on loading carriers.

- **Loading Specimen Carriers**—iCys supports two types of specimen carriers;
  - Microtiter plates (also referred to as “microplates” or “plates”) with 6, 12, 24, 48, 96, or 384 wells.
    For information about setting up microplate carriers, see page 3-17.
  - Freeform carriers, which encompasses all other carriers, including:
    - Single standard slide.
    - Four slide carriers.
    - 3 petri dish carriers.
    - 2-chamber slides.
    - Clatch slides.

    These carriers are considered “freeform” because the samples are not confined to a specific area as they are with microtiter plates. An important step in scanning samples on freeform carriers is defining the location of the samples before you begin.

Note: For information about setting up freeform carriers, see “Setting up a Freeform Carrier Scan” on page 3-22.

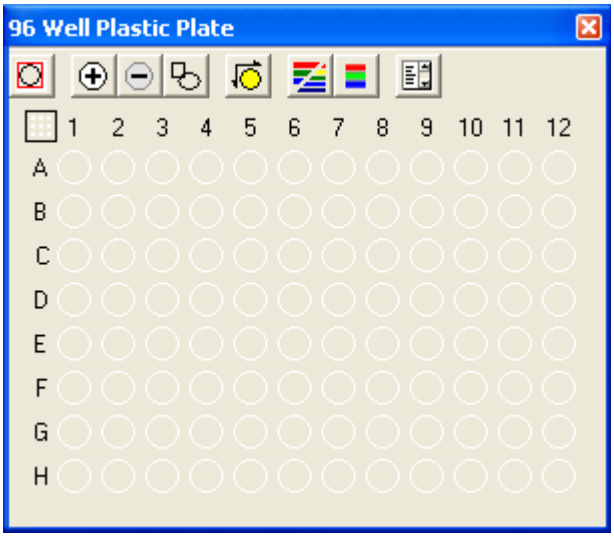
Note: For detailed information about the specimen carriers, see “About Specimen Carriers” on page 2-17.

Carrier

Clicking on this button opens a graphic of the carrier type currently selected. The menu at the top of the page allows you to perform such tasks as selecting scan areas, moving the iCys stage, and selecting a different carrier type. These are discussed below.

Setting up a Microplate Scan

For any microtiter plate, the Microplate Scan Area Setup window displays a graphic of the type of carrier in use (selected using the **S**elect Carrier Type button in the Carrier window). For example, for a 96-well plate a window similar to the following displays:



From this window, referred to as the Scan Area Setup window, you can:

- ❑ Select wells for scanning
- ❑ Select Active wells for data display or rescan
- ❑ Define the scan area for a well
- ❑ Display expression levels for wells

The following table summarizes the Setup window functions.

Table 3-1: Microplate Scan Area Setup Window

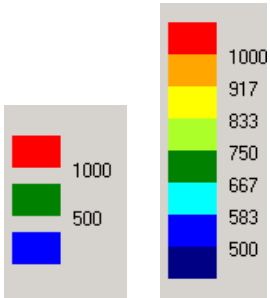
Option	Icon	Meaning
Select All Wells		Used in conjunction with Add to Scan Area and Remove from Scan Area, selects or clears all the wells in the plate.
Select Active Well		Select the wells you want to be active. Active wells can be used after a scan is complete to select specific wells for which to view data analyses or to rescan. For information about using Select Active well, see “Activating Wells or Scan Areas” on page 3-27.
Add to Scan Area		Designate the wells for scanning. Use with the Select all wells button to set up scanning for all the wells in the plate. Add entire rows and columns by clicking this button and then clicking on the row number or column letter.
Remove from Scan Area		Remove wells from scanning. Use with the Select all wells button to remove all the wells in the plate from the scan. Remove entire rows and columns by clicking this button and then clicking on the row number or column letter.
Define Scan Area		The scan area is the area of the well that is going to be scanned and analyzed. Defining the scan area is described in “Defining the Scan Area for Wells” on page 3-20.
Move stage to		Moves the stage to center the objective on the selected well.
Toggle shading mode		For expression level display—If Show Legend is selected, you can toggle between the following expression level legends:  For information about the expression level display, see “Showing Expression Levels” on page 3-20.
Show legend		For expression level display—The Show Legend button displays one of the two color map displays shown above. If the expression level display has not yet been used for this run, Show legend turns on the display. For information about the expression level display, see “Showing Expression Levels” on page 3-20.

Table 3-1: Microplate Scan Area Setup Window (Continued)

Option	Icon	Meaning
Select Carrier Type		Allows you to change the type of carrier.
Pull down menu	(None)	For expression level display—Select the run statistic for which you want to show the expression level in the well map. The run statistic dropdown displays all of the available run statistics for this scan. This menu is only displayed when Show legend has been chosen

Choosing the Wells to Scan

➢ To select all the wells in the plate:

1. Click the Add to Scan Area icon ( )
2. Click the Select All Wells icon ( ). All wells are now selected.

   All the wells will appear as black circles.

➢ To select individuals wells:

1. Click the Add to Scan Area icon ( ).
2. Click on the individual wells that you want to scan.
   - ✧ The wells you select will appear as black circles.
   - ✧ The wells that are not selected appear as white circles.

➢ To select rows or columns of wells:

1. Click the Add to Scan Area icon ( ).
2. Click on a row letter or column number.
   - ✧ The entire row or column you selected will appear as black circles.
   - ✧ The wells that are not selected appear as white circles.

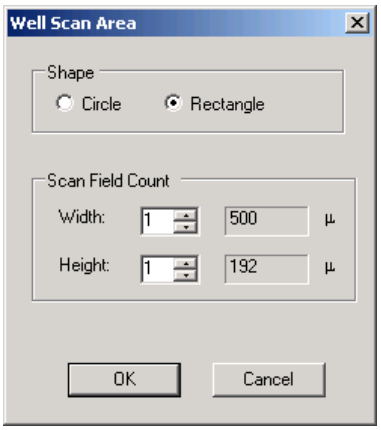
➢ To clear wells:

1. Click on the Remove from Scan Area icon ( ).
2. To remove individual wells, click on the wells you do not want to scan.
3. To remove a column or row of cells, click on the row letter or column number.
4. To remove all wells (and start over), click on the Select All Wells icon.

Defining the Scan Area for Wells

➢ To define the scan area:

1. Click the Define Scan Area icon. The Well Scan Area dialog displays:



2. Select whether you want the scan area to be a rectangle or a circle.
   - ✧ If rectangular, you need to set the **Scan field count**, which defines the number of scan fields in the scan area and the size of each scan area in microns.
   - ✧ If circular, you first need to select **Circle** from the Well Scan Area dialog, and then define the **Radius** of each scan area in microns.
3. Click OK. When you perform either a Test Scan or Scan & Save, iCys will generate the scan areas you set.

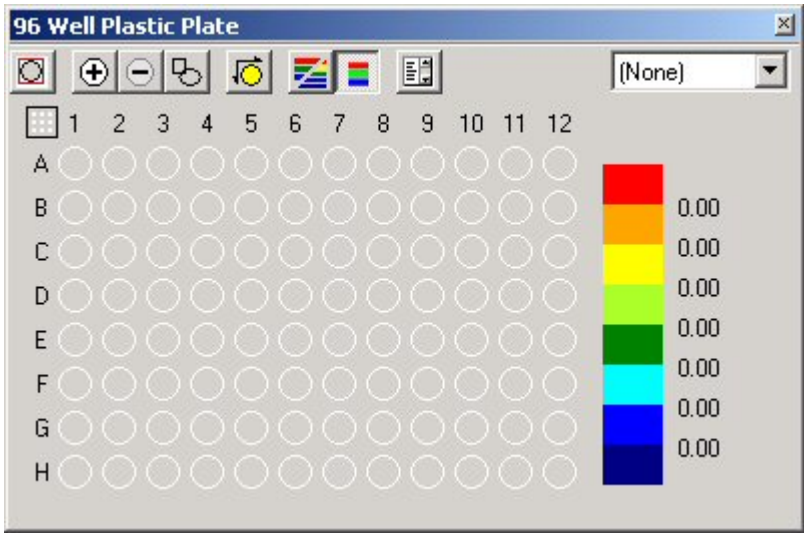
Showing Expression Levels

When scanning microplates, you can show the expression levels for a specific run statistic.You can use this information to help determine if your samples are generating sufficient data.

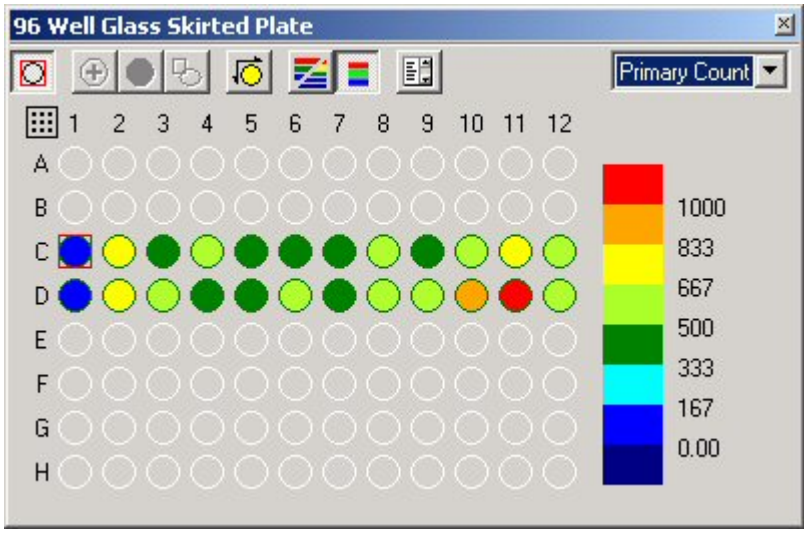
Use the Toggle shading mode icon, Show legend icon, and the run statistic dropdown to display expression levels in the well map during a scan.

➢ To display the expression level for a run statistic:

1. Begin a Test Scan or Scan & Save run.
2. Click the Show legend ( ) icon. The color legend displays to the right of the well map:

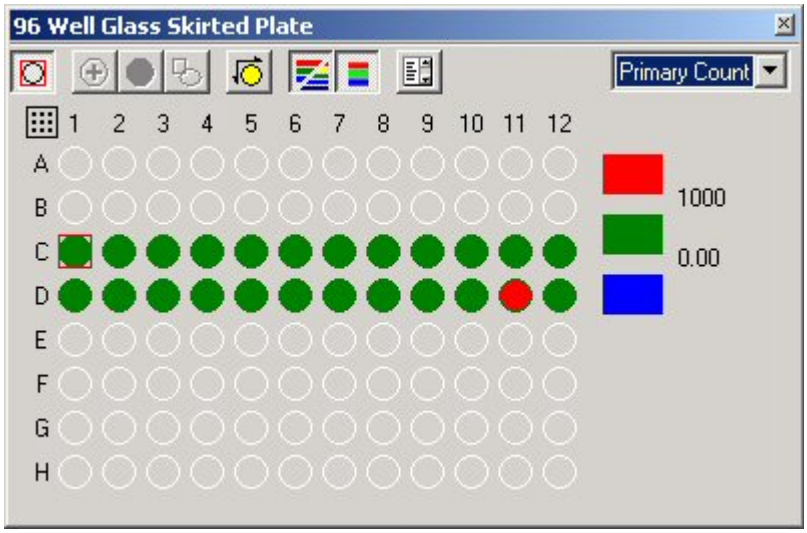


3. Select a run statistic from the run statistic dropdown box. For any wells that have been scanned, the well color will change to show the expression level for that feature. For example:



4. To change the expression level for a run statistic, click the Toggle shading mode ( ) icon. The eight-level color scale will be replaced by a three level scale, as shown below.

5.



Note: You can set the minimum (Min) and maximum (Max) boundaries for the expression display for each run statistic in the Run Statistics menu. If used, these boundaries are reflected in the temperature-style scale.

6. After the expression display is set up, you can:
   - ✧ Switch between to the two expression level displays using the Toggle shading mode icon
   - ✧ Select a different run statistic from the run statistic drop down

7. To turn off the expression display, select None from the run statistic dropdown and click Show legend again.

Setting up a Freeform Carrier Scan

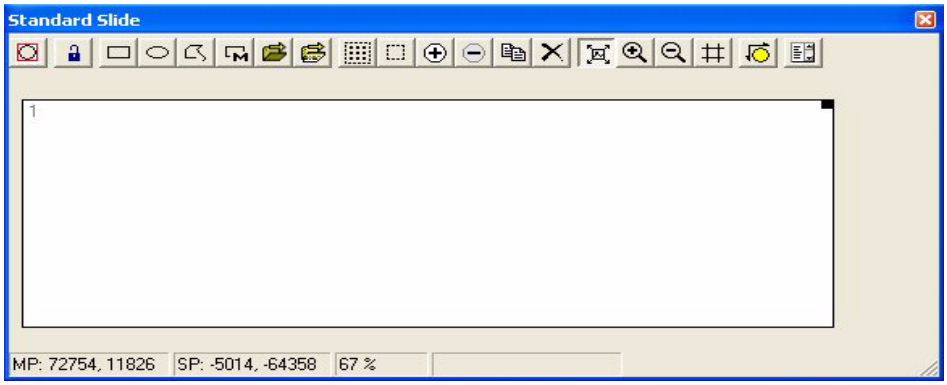
The Scan Area dialog box for freeform carriers, described in “Loading Specimen Carriers” on page 3-16, is analogous to the Scan Area dialog box for plates. It allows you to set the scan areas and other parameters for all non-plate carriers. The Scan Area Setup window allows you to:

- ❑ Define the location and size of scan areas
- ❑ Select scan areas for scanning, data display or data storage (the “active” scan area)

Note: If your system does not have the optional AutoFocus feature, **do not** select scan areas in multiple petri dishes. When scanning a petri dish carrier without AutoFocus, you must move the objective turret to the full downward position when moving between petri dishes (or the objective may hit the side of a petri dish).

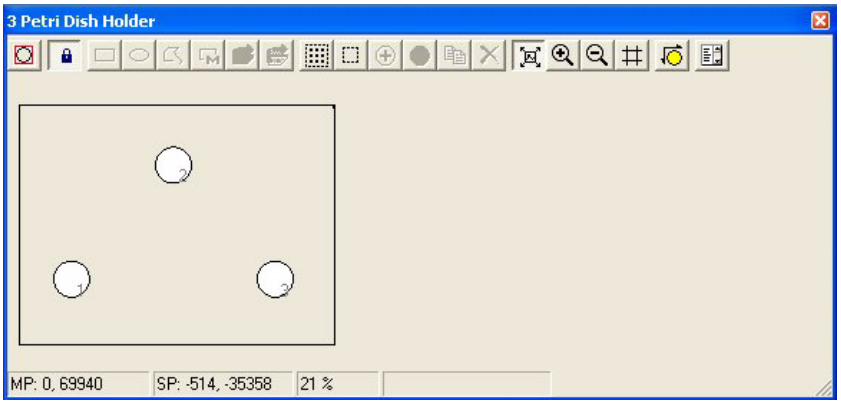
The Scan Area Setup window for freeform (that is, non-microplate) carriers displays a picture of the carrier.

For example, if the carrier is a standard, single slide, a window similar to the following displays when you click the Carrier button:



Note: To make changes to the Scan Area Setup window, you will need to click the Lock icon to unlock the editing area.

For a 4 slide carrier, petri dish, or other freeform carrier, a window that shows the outline of the selected carrier displays when you click the Carrier button from the task bar. For example, the Scan Area Setup window for a 3 petri dish holder appears below.



For more information about the available specimen carriers, see “About Specimen Carriers” on page 2-17.

Table 3-2 describes the options in the Freeform Scan Area Setup window.

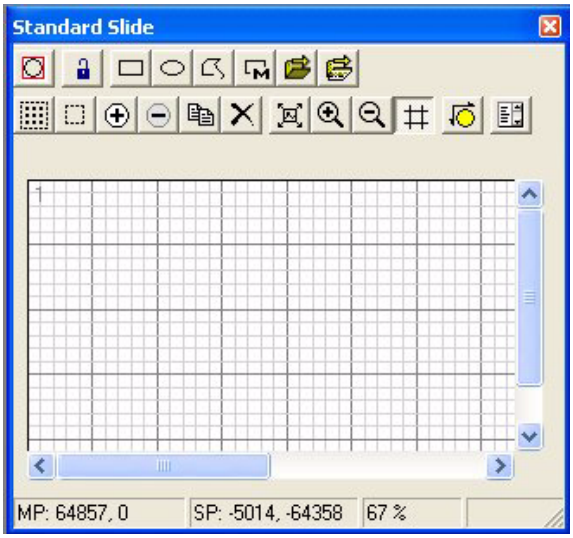
Table 3-2: Freeform Scan Area Setup Window

Option	Icon	Meaning
Select Active Well		Select the scan areas you want to be active. Active scan areas can be used after a scan is complete to select specific scan areas for which to view data analyses or to rescan. For information about using Select Active well, see “Activating Wells or Scan Areas” on page 3-27.
Lock Wells from Editing	,	Locks or unlocks the editing area. There are three lock states: **Locked**—In this mode, you cannot create or change scan areas. Only the Zoom In, Zoom Out, Grid, Label, Select Active Well, Select All, and Select Scan Areas are available. The Scan Area Setup window is in this state when: ❑ You first open the window in the Workspace. ❑ Whenever a Raw file is open. ❑ The Run is open for reanalyzing raw data ❑ At the end of a scan. **Note:** To unlock the editing area, click the Lock Wells button. If appropriate, the Unlock icon will display and the editing icons will be available. **Unlocked** —This mode is available only when you are setting up a live scan. When the icon is unlocked, all editing functions are available (such as Create, Delete, and Edit scan area). **Disabled**—In this mode, the lock button is entirely disabled (grayed out). All scan area functions except Zoom In, Zoom Out, Label, and Grid are disabled. The lock button is disabled when: ❑ You are performing a Test Scan or Scan & Save operation ❑ When you open a Run for rescan
Create Rectangle		Define a rectangular scan area using the mouse. The mouse pointer changes from an arrow to a cross hair. Note that you cannot change direction when creating a rectangle.
Create Ellipse		Define a circular or elliptical scan area using the mouse. The mouse pointer changes from an arrow to a cross hair. Note that you cannot change direction when creating an ellipse.

Table 3-2: Freeform Scan Area Setup Window

Option	Icon	Meaning
Create Polygon		Define a polygonal scan area using the mouse. The mouse pointer changes from an arrow to a cross hair. To finish the polygon, right-click the mouse. A line will be drawn from the last point to the first point in the polygon.
Create Region using Stage Keypad		Allows you to generate a region using the microscope stage keypad.
Import from File		Allows you to import the scan area setup from an event (*.EVT), Raw (*.RAW.XML), or Workspace (*.WS.XML) file. This allows you to use the same scan areas on a set of similar carriers. See “Importing Scan Areas from an Existing File” on page 3-28 for more information.
Import from XY Scattergram		Select regions from an XY scattergram to define a scan area. Before importing the scan area, you can individually define gating regions in an XY scattergram. Import the regions as described in “Importing Scan Areas From an XY Scattergram” on page 3-28.
Select All		If Select Active Well is selected, click Select All to enable all scan areas on the carrier.
Select Scan Areas		Allows you to select a group of scan areas.
Enable Scan Areas		Allows you to pick specific scan areas to scan. Use in conjunction with Select All to designate all scan areas on the slide to be scanned.
Disable Scan Areas		Removes a scan area from those to be scanned. When you disable a scan area, the area is greyed out. When you click Select All to display the scanned data, explicitly disabled scan areas are not selected.
Copy Scan Areas		Creates an exact copy of a selected scan area.
Delete Scan Areas		Deletes any selected scan areas.
Fit		Changes the size of the display to match the actual size of the carrier. Holding the carrier up to the full-size image on the monitor enables you to more accurately select appropriate scan areas.
Zoom In		Zoom in on the image.You can zoom from 25% to 400%.
Zoom Out		Zoom out on the image. You can zoom from 25% to 400%.

Table 3-2: Freeform Scan Area Setup Window

Option	Icon	Meaning
Grid		 *Grid Scale* Fine 1mm Coarse 5mm If you want to lay out scan areas in a precise pattern, Grid displays a grid over the scannable area, as shown: Note that when the carrier window is reduced in size the menu bar adjusts to accommodate the change.
Move Stage to Active Well		Moves the stage to center the objective on the position of subsequent mouse click.
Select Carrier Type		Allows you to specify the carrier type for the scan.
Status Bar		MP—This field displays the X and Y mouse position relative to the top right corner of the carrier. Moving the mouse cursor will change the display. SP—This field displays the X and Y stage position relative to the top right corner of the carrier (black square). %—Displays the carrier display magnification

Right Click on Carrier Window

Fit, Zoom (25% to 400%), Grid and Label can be selected by right-clicking on the Carrier window. Label generates and displays a label for each scan area.

Defining the Scan Area for Freeform Carriers

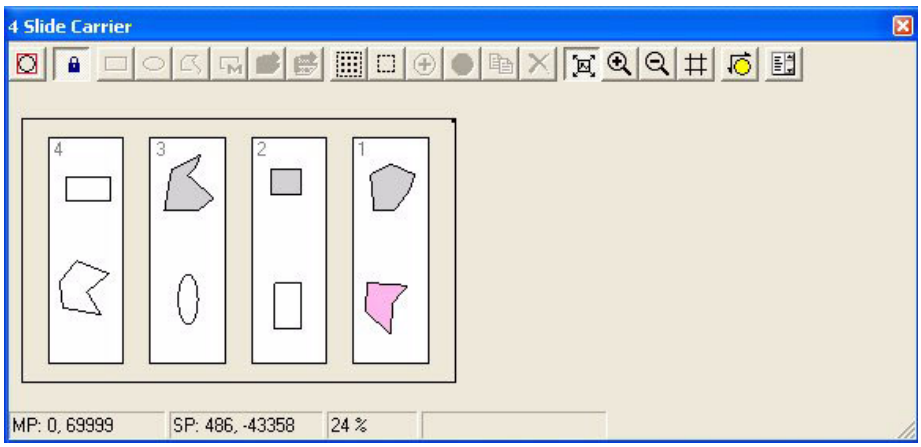
When defining the scan area for freeform carriers, keep in mind the following:

- Scan areas can be selected individually by clicking on them with the mouse.
- When changing to a different specimen carrier all currently defined scan areas are deleted. To save these scan areas, save your Workspace before switching carrier types.

Activating Wells or Scan Areas

The Select Active Well button allows you to choose specific wells or scan areas for data analysis or rescanning. This allows you to rescan a subset of the wells or scan areas on a carrier.

For example, if you have a 4 slide carrier, you may want to rescan some of the scan areas. Following is a sample of what the Scan Area Setup window might look like:



➢ To activate wells (or scan areas):

- ❍ Click Select Active Well
  - ✧ To activate an individual well, click on it with the mouse.
  - ✧ To activate multiple wells, hold the Ctrl key and click on the individual wells you want to select.

❑ If your system does not have the optional AutoFocus feature, **do not** select scan areas in multiple petri dishes. When scanning a petri dish carrier without AutoFocus, you must move the objective turret to the full downward position when moving between petri dishes (or the objective may hit the side of a petri dish).

Select Active Well can be used in two ways, as follows:

- ❍ **Data Analysis**—When you have specific wells activated, the currently open scattergrams and histograms in the Workspace reflect the data only from those wells.
- ❍ **Rescanning**—To rescan activated wells, select Scan Only Active Wells ( ) from the Scan section of the task bar before starting a scan. When you rescan the carrier only those wells you selected are scanned.

Note: When you rescan virtual data, iCys generates a new Raw file of the same name, but with a new Run ID. The new Raw file contains the new data created by the rescan as well as any unchanged data from previous scans. For more information, see Table 3-5, “Field Image Window Icons,” on page 3-68.

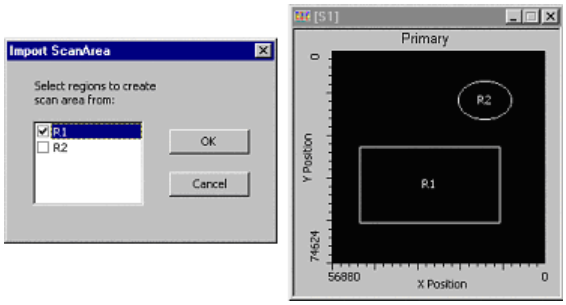
Importing Scan Areas from an Existing File

➢ To import scan areas from a file:

1. Click the Import from File icon ( ).
2. The Open window displays.
3. Browse to and select the Event, Raw, or Workspace file from which you want to import the scan areas. Keep in mind that you probably want the carrier types to be identical.
4. The scan areas from the selected file are imported. Any existing scan areas are deleted.

Importing Scan Areas From an XY Scattergram

1. Click on the Import Regions From XY Scattergram icon ( ).
2. The Import ScanArea dialog box displays. Select the regions you want to import and click OK. Existing scan areas are not affected.



3. If the coordinates of the scattergram are not within the boundaries of the defined slide, the scan area cannot be imported. If this occurs you may need to “normalize” the XY scattergram. To do so, right-click the mouse while the cursor is over the graph. Select Normalize XY from the menu. The graph axes are readjusted to lie within the boundaries of the slide.

Selecting Scan Areas

After you have defined the scan areas in a freeform carrier, you can choose to enable all scan areas in the carrier or to enable specific scan areas.

❍ Enabling a scan area makes it available for scanning

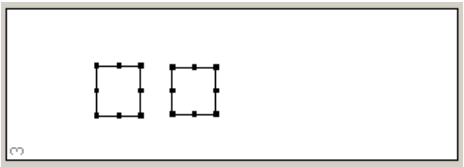
❍ Disabling a scan area makes it unavailable for scanning.

Enabling and disabling scan areas can be a useful technique when you need to rescan a subset of the scan areas in a run.

➢ To enable all scan areas:

1. Click the Enable Scan Areas icon ( )
2. Click the Select All icon ( ). All scan areas are now selected for scanning.

   The selected scan areas will be outlined in black and have handles. For example:



➢ To enable individual scan areas:

1. Click the Enable Scan Areas icon ( ).
2. Click on the individual scan areas that you want to include.
   - ✧ The enabled scan areas will be outlined in black and have handles.
   - ✧ The scan areas that are not selected do not have handles.
3. To select multiple scan areas, hold down the Ctrl key while clicking on the scan areas you want.

➢ To pick a group of scan areas:

1. Click on the Select Scan Areas icon ( ).
2. Then, hold down the left mouse button to draw a box around all the scan areas you want to include in the scan. All scan areas within the box will become enabled.

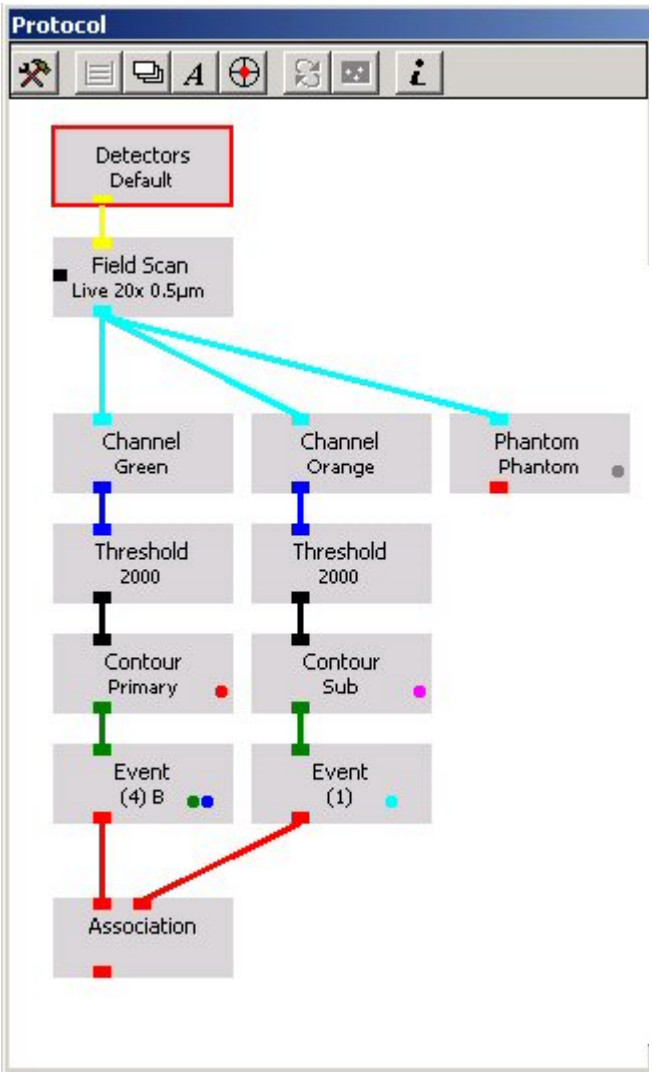
➢ To remove (disable) scan areas from scanning:

1. Click the Disable Scan Areas icon ( ).
2. From here, you can:
   - ✧ Click the Select All icon to disable all scan areas
   - ✧ Click individual scan areas (hold down the Ctrl key to disable multiple scan areas)
   - ✧ Click the Select Scan Areas icon and draw a box around the scan areas you want to disable.

3. Disabled scan areas are greyed out. You can re-select them as described above.

Protocol

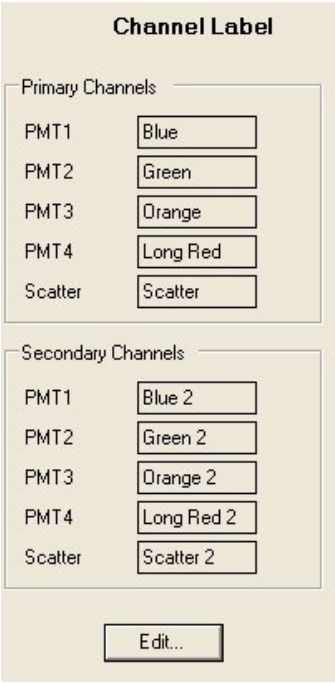
Displays the protocol page, an example of which is seen below.



The protocol page is where you select or create a protocol specifying which data you wish to collect, and how you want to process the data. You may use an existing protocol as is, modify an existing protocol, or create a new protocol.

Protocol Menu Bar

The icons of the protocol menu are summarized in the following table.

Option	Icon	Meaning
Tool Box		The Tool Box provides access to the modules you will use to create a protocol. For additional information see “Creating a New Protocol” on page 3-35.
Repetitive Scan		Optical sectioning scans are only available if your site has the AutoFocus feature. For information on this feature see “Setting Up a Repetitive Scan” on page 3-32.
Channel Label		Displays the channel label menu.  Channel labels can be changed by selecting the Edit button from this menu.
Focus and Performance		Displays the Focus and Performance menu. This allows you to select focusing and scan path parameters. For additional information on this feature, see “Focus and Performance” on page 3-33.
Apply		Applies protocol changes to open image windows on the iCys Workspace. This can be useful when you are adjusting protocol parameters.

Option	Icon	Meaning
View Image		When image modules such as Channel or Threshold are selected, displays the Module Image window. The Module Image window shows the image processed by the selected module and changes when selecting a different module. It is useful for viewing the changes applied by each modules.
Initialize		After all parameters have been defined, loads all values into the protocol.

Setting Up a Repetitive Scan

Use the Repetitive button on the Protocol window to set up repetitive scans.

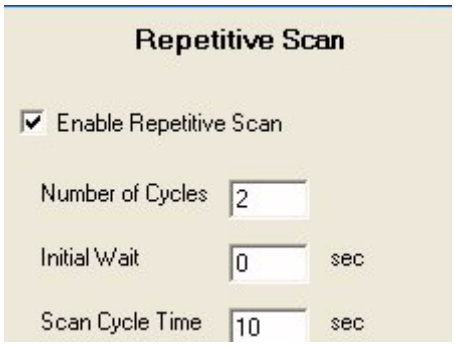
About Repetitive Scans

A **repetitive scan** is the process of scanning the same well or scan area multiple times. This allows you to see the change in a single sample over a period of time (which can be especially useful for live samples).

Generating Repetitive Scans

➢ To generate a repetitive scan:

❍ Click on the repetitive Scan icon (}from the protocol menu. The Repetitive Scan menu appears.



❍ Check the Enable Repetitive Scan box. The other menu options become available.

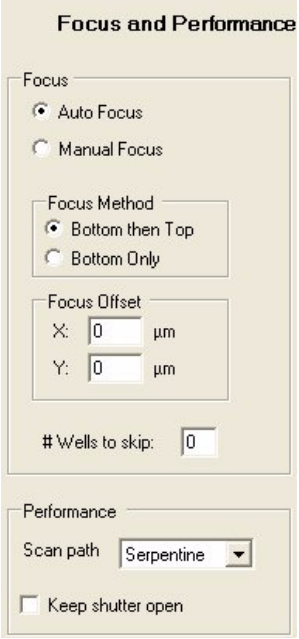
❍ Enter the number of times you want to scan the selected area.

❍ In the Initial Wait field, enter the number of seconds to wait before beginning the scan. This allows you to, for example, specify the time to wait to allow a drug reaction to occur.

- In the Scan Cycle Time field, enter the minimum number of seconds between subsequent scan start times. If the scan finishes before the cycle time has elapsed, iCys will wait until the next cycle to start scanning again. This allows you to, for example, record snapshots at specific intervals of time.

Focus and Performance

Clicking the Focus and Performance icon ( ) opens the following menu.



The Focus and Performance menu functions are described below.

Focus

Allows you to select whether you want to focus the microscope manually or allow iCys to AutoFocus for each scan area:

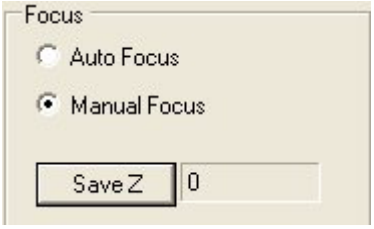
- **AutoFocus**—If your system uses the optional AutoFocus feature, a
  - **Focus Method**—Specify the preferred focus method.
    - **Bottom then Top**—For thicker plastic-bottomed plates (greater than 400µ) especially; iCysiCys will focus first on the plate bottom, then translate up by the bottom thickness to the plate top and refocus.
    - **Bottom Only**—For glass-bottomed or thinner plastic-bottomed (less than 400µ) plates, iCys focuses directly on the bottom then moves up by the bottom thickness plus other offsets to bring the sample into focus.

    If the carrier type supports only one focus method, the supported method will be selected and the unsupported method will be grayed out and unavailable.

- **Focus Offset**—If your system uses AutoFocus and AutoFocus is checked you may shift the sample position where iCys will focus by adjusting the X and Y offsets. The default AutoFocus location depends on the type of carrier, as follow:
  - **For microplates**—The default AutoFocus location is at the center of a scan area (or well).
  - **For other carriers**—The default AutoFocus location is at the start of the first scan field.

  You can use the X and Y offsets to shift the AutoFocus location by a specified number of microns. The X and Y axes should be set independently.
- # **Wells to skip**—Number of wells between refocusing. iCys will automatically refocus after skipping the designated number of wells. This feature is grayed out when a freeform carrier type is selected.

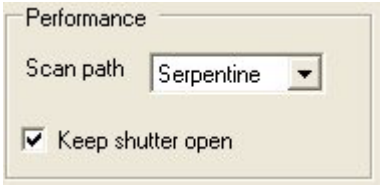
If Manual Focus is checked, the following menu option will appear.



After manually focusing the instrument, click the Save Z button to save the focus setting.

Performance

The performance menu is shown below.



- The pull-down Scan path menu allows you to choose the method of scanning selected scan areas or wells. The options are Left to right, Right to left, and Serpentine (alternating left to right and right to left). Serpentine processing is the fastest of the three options.
- Checking the Keep shutter open box keeps the shutter open between scans.

Note: The “Keep shutter open” option may not be desirable for sensitive dyes and samples, as photo bleaching may occur.

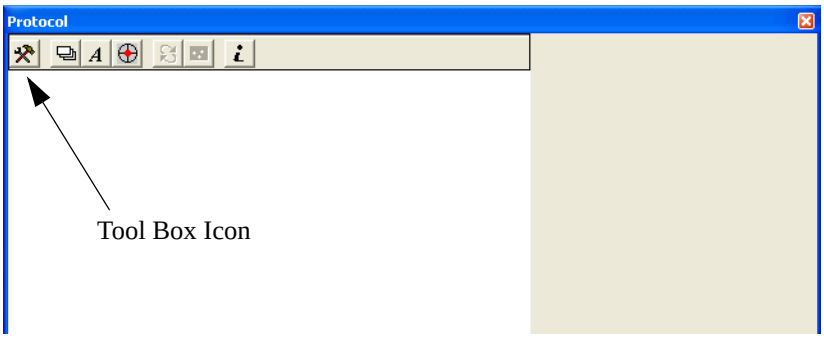
Creating a New Protocol

The following section will walk you through the steps involved in creating a new protocol.

Opening the Protocol window

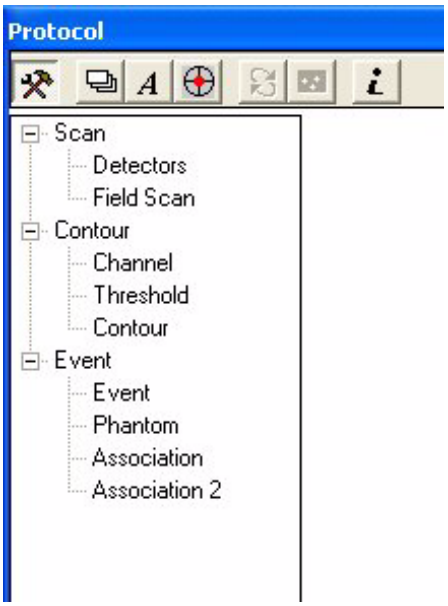
1. Click on New Run in the Task Panel; unless you have made changes to the workspace you wish to save, click No in the Save Workspace dialog box.
2. Select Empty as a Workspace to use. Click OK.

   This will open a blank protocol page.

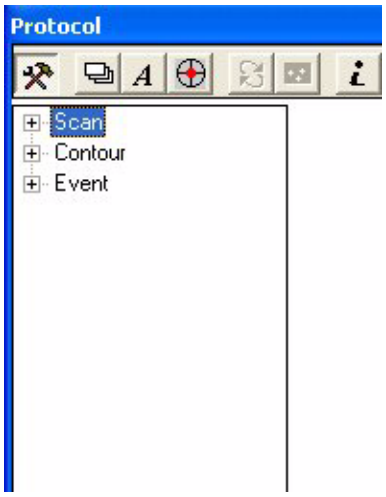


3. Click on the Tool Box icon in the upper left corner of the window. The protocol tools will appear in the left hand portion of the window.

Expanded headings



Contracted headings



4. Click on the "-" symbol next to each of the headings to contract it. Clicking on the "+" symbol next to the headings will expand them and display all the options under that heading.

Tip: Right-clicking on the left hand portion of the page will allow you to expand or contract all headings at once.

Adding Modules

To add a module to the protocol, click on the module in the tool box menu, then click on the center portion of the window. The module you have selected will appear.

A dialog box for that module will appear in the right-hand pane of the window. Each type of module will have a different type of dialog box. Protocol modules are described below.

Deleting Modules

To delete a module, right-click on the module, and select "Delete Module."

Disabling / Enabling Modules

To disable a module right-click on the module, and select "Disable Module."

Note: Selecting "Disable Module" will also disable any modules connected through the output node of the disabled module. To enable a disabled module, right-click on the module and select "Enable Module." This will also enable any modules connected through the output node of that module.

Select All

Selects all modules in the center portion of the Protocol window. Right-clicking again on any of the modules allows one to "Delete Modules" (deletes all modules).

Copy

Not implemented

Print

Not implemented

Protocol Modules

iCys contains several types of modules. These are used as building blocks for protocols. These modules are described below.

Scan Modules

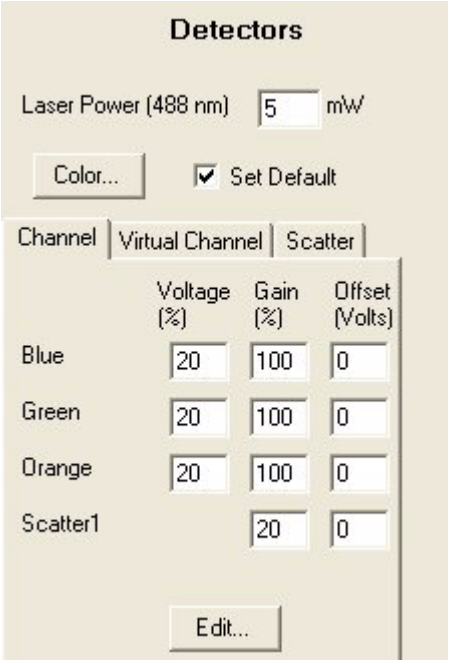
The Scan modules allow you to specify such parameters as lasers, channels, and instrument settings.

Detectors

The Detectors module allows you to select which lasers and detectors you wish to use, establish settings for these, and create virtual channels.

When you add a detector module, or click on an existing one, a dialog box appears on the right portion of the window. There are three tabs on the Detectors window: Channel, Virtual Channel, and Scatter.

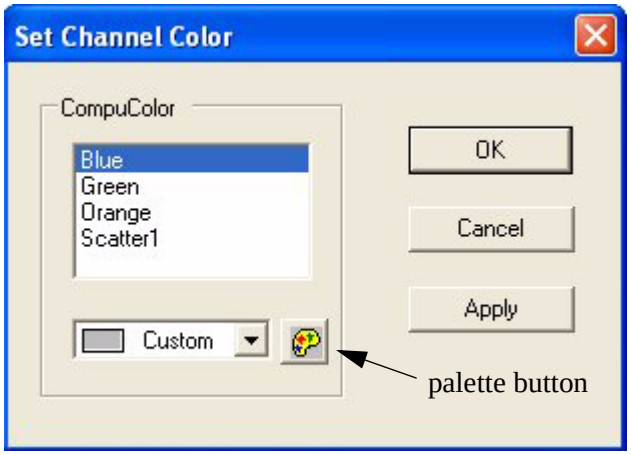
- **Channel Tab**—When the Channel tab is selected, the following menu appears.



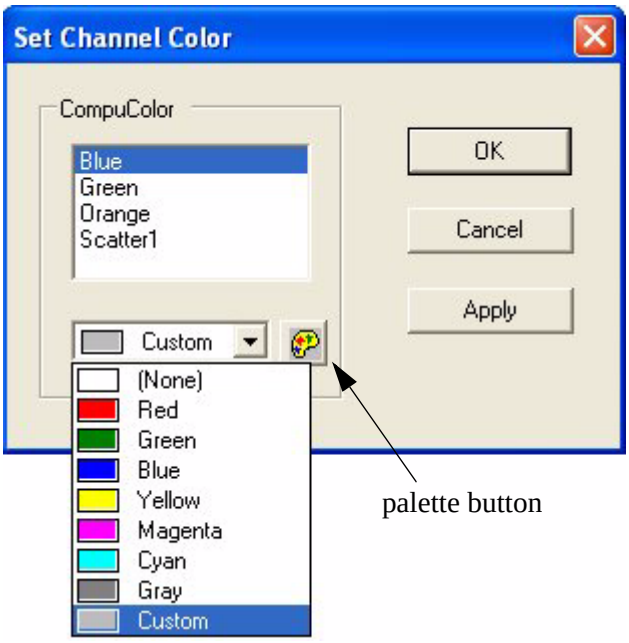
- **Laser Power**—In this field you can enter the (488 nm) laser power you wish to use. For additional information on setting laser power, see “Adjusting Laser Power, PMT Gains, and PMT Offsets” on page 3-82.

Note: When you change a protocol value, the background of the text box changes to red. Press enter or select a different text box to accept the change. If the value entered is invalid, an exclamation point (!) appears next to it. Pointing to the exclamation point will display the error message.

- **Color**—Clicking the Color button opens the following menu.



You can choose a CompuColor for each of the active channels by clicking on the channel name and selecting a color for it from the pulldown menu. Clicking the palette button opens a menu that allows you to create your own colors and apply them to a channel.



❍ **PMT Settings**

The Channel tab menu allows you to adjust the PMT settings. During a Test Scan, you must optimize the PMT voltage, gain, and offset values to scale the fluorescence for the specimen being scanned. Setting these values requires that you open the Field View window in the View menu, and evaluate the pixel values of the events. You can then use the Channel window to set the

PMT voltage and offset values so that the pixel values are consistently within the following ranges:

- **Brightest pixels** (events) – 12,000 to 14,000
- **Dimmest pixel**s (background) – 300 to 500

Optimizing PMT settings

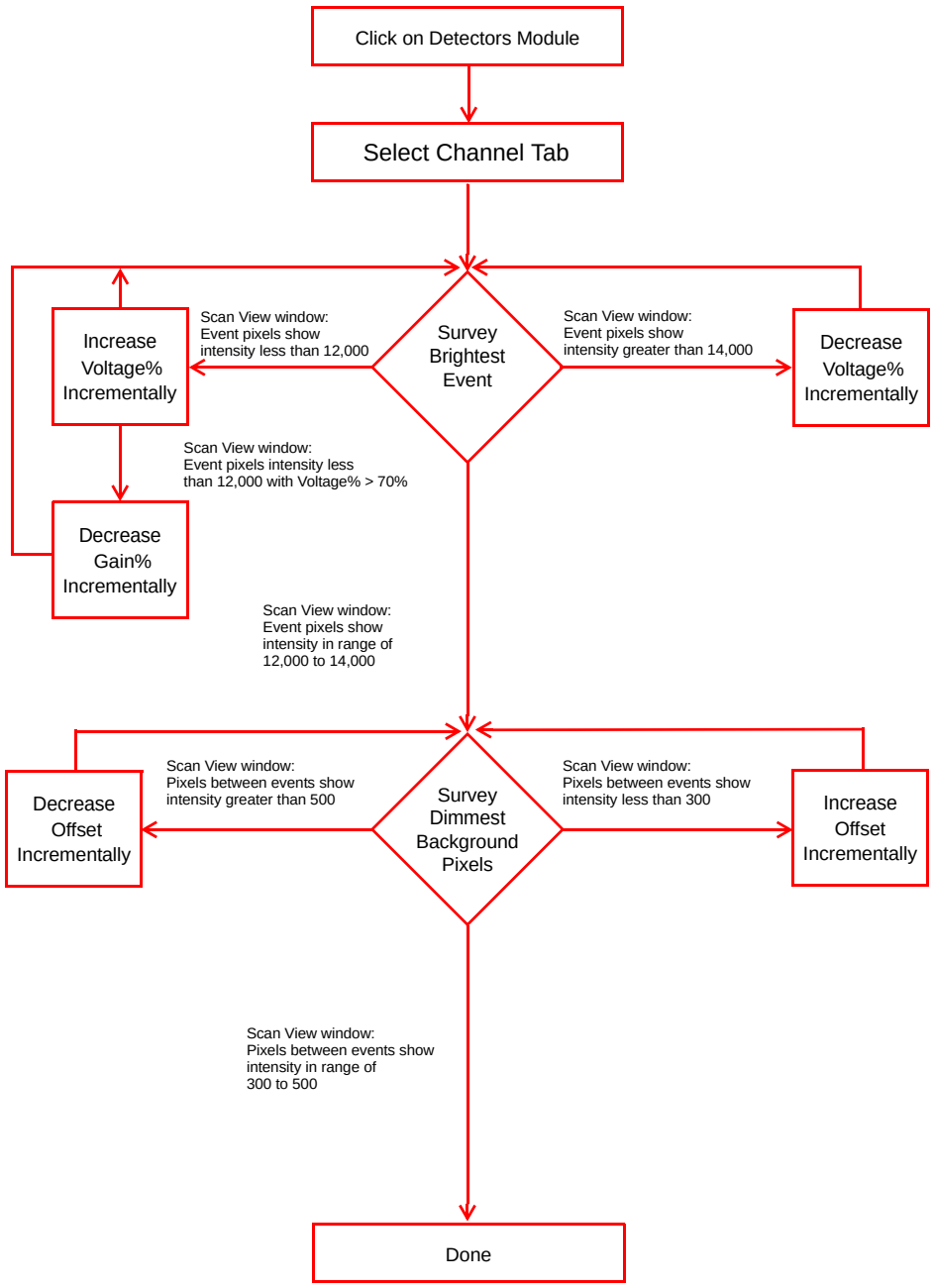
Monitor the pixel values in the scan field while adjusting the PMT settings and re-scanning. Double-click on an event to create an event statistic window that shows values for the event.

Note: Each time you make a PMT adjustment, you need to re-scan the scan field to view the effects of the new settings. Re-scanning the same scan field can cause the fluorescence staining of the sample to fade. To avoid fading, re-scan the same scan field several times and if you are unable to optimize the PMT settings, move on to the next scan field and continue optimizing the settings.

Once you have optimized the PMT settings, check that the PMT settings remain optimized on a new scan field.

The following diagram illustrates the basic tasks involved in optimizing the PMT settings for most samples. Some specimens will require deviations from the guidelines in the flowchart.

Adjusting PMT Channels Flowchart



For detailed instructions on setting PMT parameters, see “Adjusting Laser Power, PMT Gains, and PMT Offsets” on page 3-82.

For further information on performing a Test Scan see “Test Scan” on page 3-80.

Selecting the Lasers and Channels

About the iCys Lasers

iCys comes with equipped with up to three lasers:

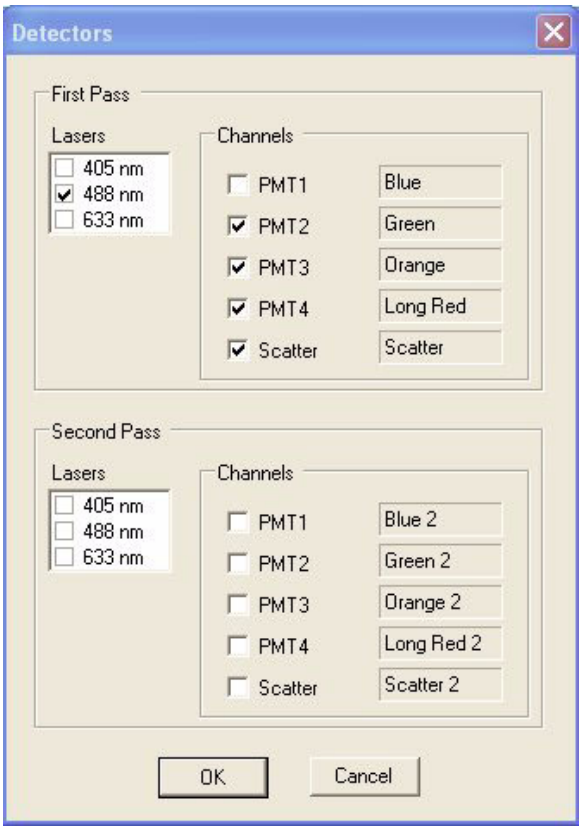
- ✧ Argon Ion, which emits at 488 nanometers.
- ✧ Helium-Neon (HeNe), which emits at 633nm.
- ✧ Violet, which emits at 405nm.

The lasers you select for a given scan will depend on the excitation characteristics of the fluorophores used.

Note: Although you can select any laser or laser combination for either the first or second pass, some laser combinations will not be compatible with the fluorescence emission wavelengths of the dyes you are using. The excitation wavelengths should not overlap the emission wavelengths in which you are interested for any one pass.

Selecting Data Acquisition Channels

- ❍ **Edit**—When the Edit button on the Channel tab is selected, you can specify the lasers and channels to be used in both the first and second passes of the scan by clicking the Edit button. The following menu will appear.



Use the Channel checkboxes to select which data channels you want to use. iCys can acquire real data from up to five detectors, called PMT1 to PMT4, plus the scatter channel.

Site-specific names of each PMT are shown in the Channel dialog box. These names are site-dependent, but can, for example, include Blue, Green, Orange, or Long Red. If your site uses fewer than 4 PMTs, the unused PMTs are greyed out and unavailable.

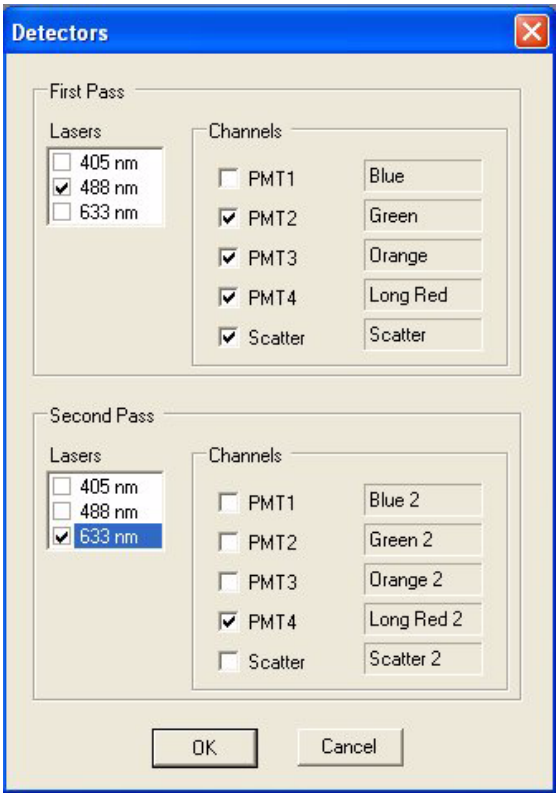
Scatter does not use a filter cube. The scatter image is generated from transmitted light passing through the sample and striking the scatter detector.

The wavelengths detected by each PMT depend on the filter cubes installed. The standard configuration for filter cubes is:

- ✧ PMT1 = Blue
- ✧ PMT2 = Green
- ✧ PMT3 = Orange
- ✧ PMT4 = Long Red

The filter cube arrangement is site-dependent. Your site may use different filter cubes.

If you are using multiple lasers in two passes, each selected detector will constitute a separate data channel. For example, PMT4, with a Long Red cube installed, will detect propidium iodide during an Argon laser pass, and allophycocyanin (APC) during a HeNe laser pass, thus generating two channels of data from the same PMT. In this case, you will see both a Long Red channel and a Long Red 2 channel.



The choice of data acquisition channels will be determined by the emission characteristics of the fluorophores, which laser is used to excite them and the filter cubes installed.

Check the boxes next to the lasers and channels you wish to use for each pass of the scan. Clicking OK enters your selections into the protocol.

Tip: This menu can also be opened by double clicking on the detector module box.

For details on setting voltage, gain, and offset levels, see “Adjusting Laser Power, PMT Gains, and PMT Offsets” on page 3-82.

- **Set Default**—Checking this box sets the oscilloscope settings to their default values. For information on using the oscilloscope, see “Using the Oscilloscope Feature” on page 3-90.

Creating Virtual Channels

iCys has up to five detectors, each of which can be used to acquire data from any of the three lasers. The output of these detectors comprise the “real” data channels. Virtual channels can be created by combining these actual data channels using various operators. Thus, virtual channels can be created that, for example, sum the Green and Orange channels, subtract the Orange channel from the Long

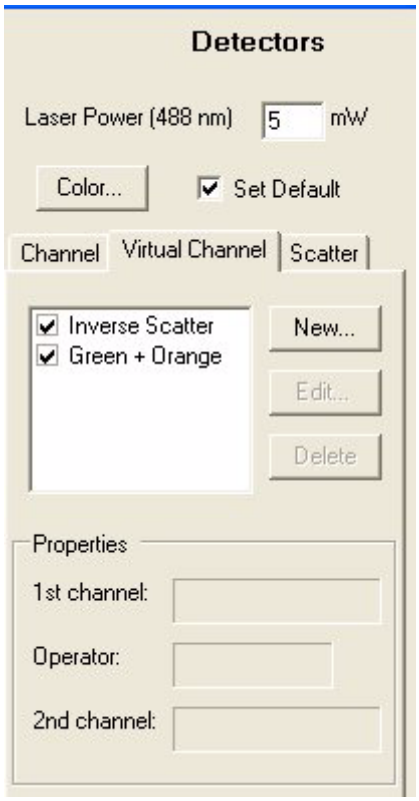
Red channel, or double the output of the Blue channel (by either adding Blue to Blue or multiplying Blue times 2).

Within the Virtual Channel tab, if the box to the left of the virtual channel name is checked, that virtual channel is selected and will be calculated during the scan.

Highlighting a virtual channel name displays the definition for that channel in the properties dialog box.

If one or both of the constituent detector channels is not selected in the Instrument Channels tab, the virtual channel will not remain selected when the menu is closed.

- **Defining a Virtual Channel**
  - **Virtual Channel Tab**—This tab allows you to create virtual channels by manipulating or combining existing channels. When this tab is chosen, the following menu appears.



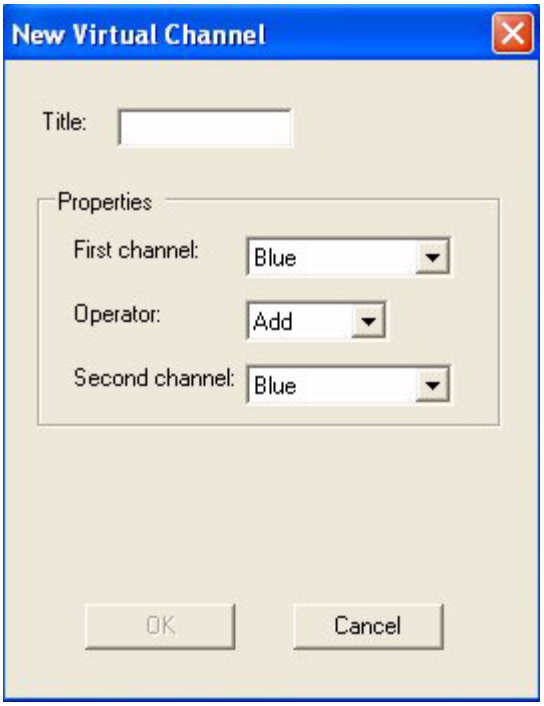
The window in this menu displays previously defined virtual channels. Checking the box next to a virtual channel activates it for the experiment. If you do not wish to use a particular virtual channel in the current experiment, uncheck this box.

If you highlight a virtual channel on the list, its properties will be displayed in the lower part of the menu.

✧ Creating a Virtual Channel

To create a virtual channel

1. Click the New button. The following menu will appear.



2. Enter the name for the virtual channel in the Title field.
3. Select the first channel to be used from the pulldown menu. You can choose from any defined channel, including previously defined virtual channels.

4. Next, select an operator from the pulldown menu. The following operators are available:

   - **Add**
     Creates a virtual channel by adding the first and second channel.
   - **Subtract**
     Creates a virtual channel by subtracting the second channel from the first.
   - **Multiply**
     Creates a virtual channel by multiplying the first channel by the factor entered in the operand field.
   - **Max**
     Creates a virtual channel of the maximum of channel one or two.
   - **Min**
     Creates a virtual channel of the minimum of channel one or two.
   - **Invert**
     Creates a virtual channel by inverting the value of the first channel. No second channel is used.
   - **ShiftPeak**
     Shifts the pixel values to change the brightness of a image. ShiftPeak works by computing the histogram of the intensity of an image. The peak value of the histogram is usually the average value of the background. To shift the peak value, enter the new peak value into the Reference field (note that, for ShiftPeak, the Second Channel field changes to Reference). If the new peak value is higher than the original value, the entire image will be

brighter.

ShiftPeak is particularly valuable for use with Scatter to correct for background intensity changes between scan fields.

✧

5. Select the second channel (or reference or operand) for this virtual channel.
6. Click OK. The New Virtual Channel dialog closes and the virtual channel is saved.

✧ **Edit**—Allows you to edit the selected virtual channel.

✧ **Delete**—Deletes the selected virtual channel.

❍ **Working With Virtual Channels**

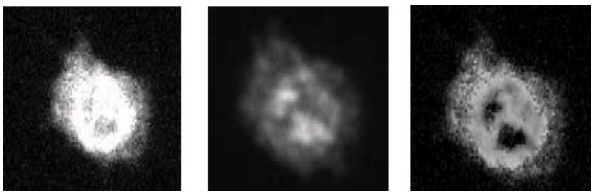
✧ **Nesting Virtual Channels**

Note that virtual channels can be nested by choosing a previously defined Virtual Channel as one channel in the definition of a new virtual channel. For example, you can create a Virtual Channel consisting of (Green+Orange) - LongRed, where Green+Orange is an existing Virtual Channel.

✧ **Using Virtual Channel Scan Images**

Virtual channel scan images may be selected for display and storage in any of the Image or Gallery windows, just as actual data channels are.

Below are scan field images of the same cell visualized in the Green and Long Red real channels, and the Green minus Long Red virtual channel.

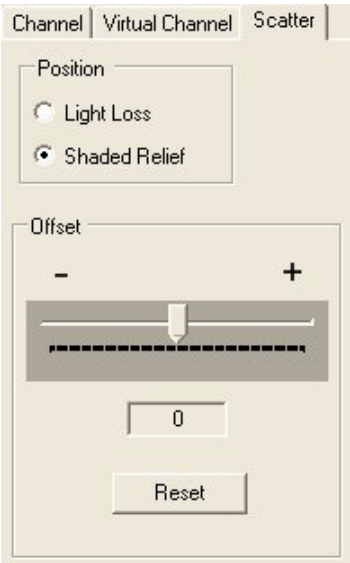


Green Long Red Green minus Long Red

Scatter Tab

The Scatter tab is available only if your instrument is equipped with the motorized scatter assembly.

Clicking the Scatter tab opens the following menu.



❍ **Selecting the Forward Scatter Method**

Forward Scatter allows iCys to generate images that are much different from fluorescence images. When Scatter is selected as a channel, you can select the Forward Scatter method you want to use:

✧ **Light Loss**—iCys centers the detector in the forward scattered beam. This position emphasizes the effects of sample absorption, refraction, and other light not picked up by the scatter detector.

✧ **Shaded Relief**—iCys positions the detector so the light hits the edge of the detector. In this position, with the offset at 0, half of the forward scattered light falls on the detector, while the other half misses the detector. When performing a Test Scan, you can adjust the Shaded Relief Scatter offset, described on “Setting the Shaded Relief Scatter Offset” below.

✧ **Setting the Shaded Relief Scatter Offset**

If you select Shaded Relief Scatter, the Shaded Relief Scatter offset section displays the Offset slider. Changing the Scatter offset changes the sensor angle so that the shading is more or less prominent. Moving the slider to the right catches more scatter signal, making the shading less prominent; moving the beam to the left makes the shading more prominent.

✧ **To set the Shaded Relief offset:**

During a Test Scan, display scatter images.

Use your mouse to move the slider left or right, then rescan and check the image. As you move the slider, the numerical offset is shown in the display window.

Continue making changes and rescanning until you are satisfied with the quality of the scatter images.

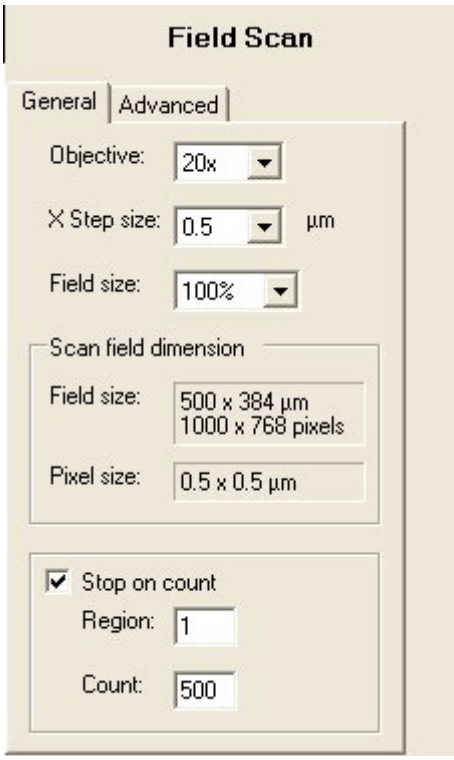
When you save or exit the Workspace, the offset is saved with other iCys settings.

Tip: The slider can be reset to 0 by clicking the Reset button.

Field Scan

The FieldScan module allows you to set up a scan that scans, segments events, develops event features, and displays data on a scan field by scan field basis.

Creating or clicking on a Field Scan module opens the following menu.

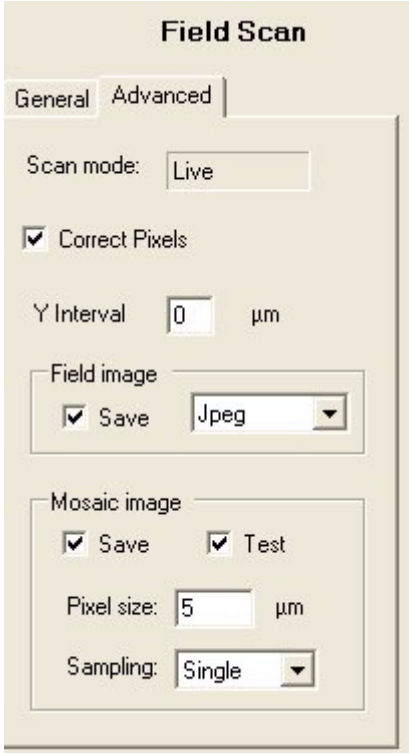


- **General Tab**—Under the General tab you can specify the objective you are using, the X-step size in microns for the scan, and the field size. These selections will be reflected in the Field size and Pixel size fields.
- **Objective**—Allows you to select which microscope objective lens you will use for this run. When not actively scanning, the objective is moved out of the way of the motorized stage to prevent a carrier from being bumped by the objective.
- **X Step size**—Determines the size of a single stage step in the X direction. The default value for X Step is 0.5µm for 20X and 40X objectives, and 1.0µm for the 10X objective. Select the size of the X step increment. From the X Step pull-down, select any value from .5µm to 20µm (in .5µm steps.)
- **Field Size**—Determines the number of X steps that iCys will take when scanning. You can choose a percentage (100%, 50%, or 25%) or to adjust the

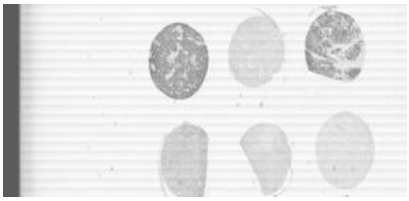
number of X Steps to generate a square scan field. The scan field and pixel size are determined by the dimensions you select and are displayed in the Scan field dimension window below.

- **Scan field dimension**—Displays the pixel size and size of the scan field based on the parameters you have selected.
- **Stop on count**—Checking this box will stop the scan when the number of events in the designated Region reaches the designated count.

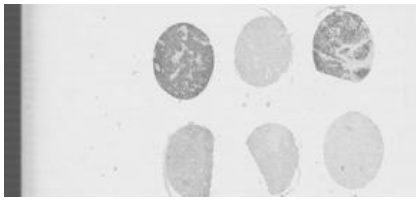
  Note that when the count has been reached, the system might scan one extra field since events are analyzed while scanning the next field. Events from the extra field will be ignored when stop on count has been reached.
- **Advanced Tab**—Clicking the Advanced tab opens a menu in which you can specify whether or not to apply pixel correction, the Y interval in microns, whether or not to save field images, and, if so, in what format. You can also choose to save mosaic images for a Test Scan, a scan and save, or both.



- **Scan Mode**—Describes current scan (Virtual, Live, Bypass)
- **Correct Pixels**—Checking this option enables scanline correction to account for variations in collection efficiency along the scanline. This is performed on each of the images as they are acquired, and is shown in the figure below.



Uncorrected laser scan image exhibiting roll-off



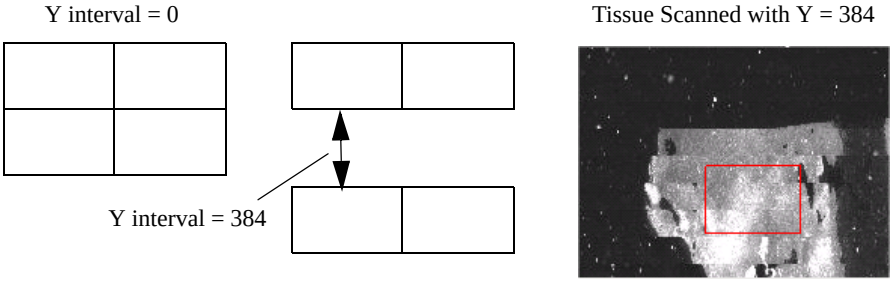
Corrected laser scan image

- **Y Interval**—Allows you to specify a gap, in microns, to add between scan field rows.

  If the distance = 0, the scans are contiguous.

  Scan strips (that is, scan field rows) are 384µ wide. Therefore, if you set the Y interval to 384, you can perform a fast scan by scanning every other row.

  For example:



  In the example above, you can clearly see the scan strips in the tissue.

- **Field image**—Choose whether or not to save a field image, and in what format.
  - **JPEG**—If Raw files are saved in Jpeg format (.jpg extension), some minor differences may be seen between these files and the live data, however, the size of the files is greatly reduced with Jpeg files.
  - **Flat**—ImagePro flat files (.flf extension) store the identical pixel values as are used in the live scan, however, flat files are very storage intensive.
- **Mosaic image**—Choose whether or not to save a mosaic image, or to view a mosaic image during a Test Scan.
- **Pixel size**—Select the pixel size in microns for the mosaic image. A smaller pixel size gives better image resolution. A larger pixel size decreases storage space needed for the image.
- **Sampling**—Determines the property used to create the mosaic image.
  - Single creates the image based on the first pixel in the sampling bin.
  - Average creates the image based on the average value of the pixels in the sampling bin.
  - MaxPixel creates the image based on the pixel in the sampling bin with the maximum value.

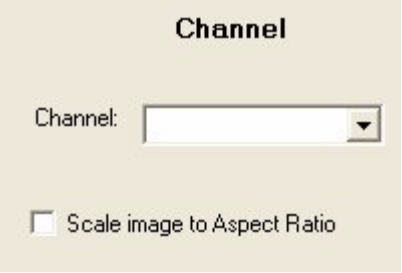
Contour Modules

The Contour section of the Menu Bar allows you to define Contour parameters, including channel, threshold, color, and name. Additional information on defining Contours can be found in “Types of Contours” on page 4-3.

Channel

Creating or clicking on a Channel module opens the following menu

.



- **Channel**—The pulldown Channel menu allows you to choose a contour channel from a list of active channels, including virtual channels.
- **Scale image**—Checking this box will scale the image to the aspect ratio of the scan.

If selected, the new image is created so that the aspect ratio is always 1 to 1.

If cleared, the new images aspect ratio is based on the X-Step and objective.

The aspect ratio must be selected when the subsequent modules include shape-sensitive operations such as Watershed and morphological filters.

Threshold

Threshold represents the minimum amount of pixel fluorescence required to generate a contour for an event.

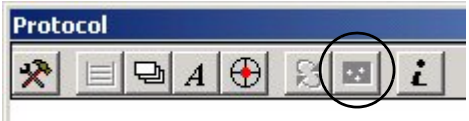
When setting the threshold you can choose Auto, which will automatically set the threshold, or you can set it manually using a slider.

The threshold module will display either the word Auto or the numerical setting you have entered.

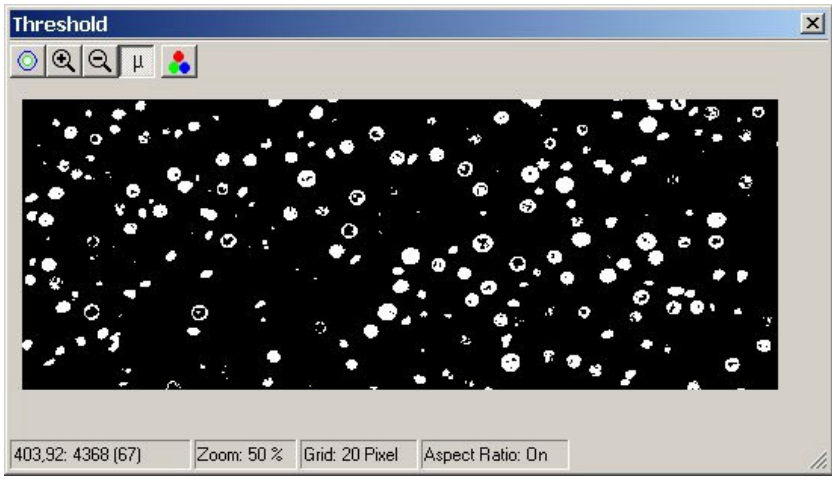
There are two methods you may use to manually adjust the threshold.

Pixel survey method—From the Field image (or if you are using tools from the iNovator Toolkit, the Field or Well image windows) survey the pixel values at the edge of a number of representative events about which you want the system to draw contours. Set the threshold value to a rough average of these values.

Image window method—The image window allows you to interactively view the effects of changing the threshold setting. With the threshold module selected, bring up, the image window by Clicking on the View Image icon ( ) in the Protocol Tool Box window.

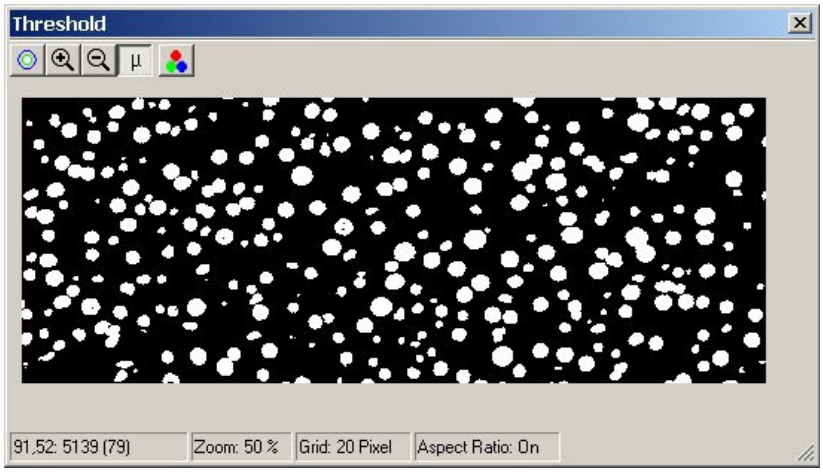


This will open up the Interactive Threshold window as shown below.



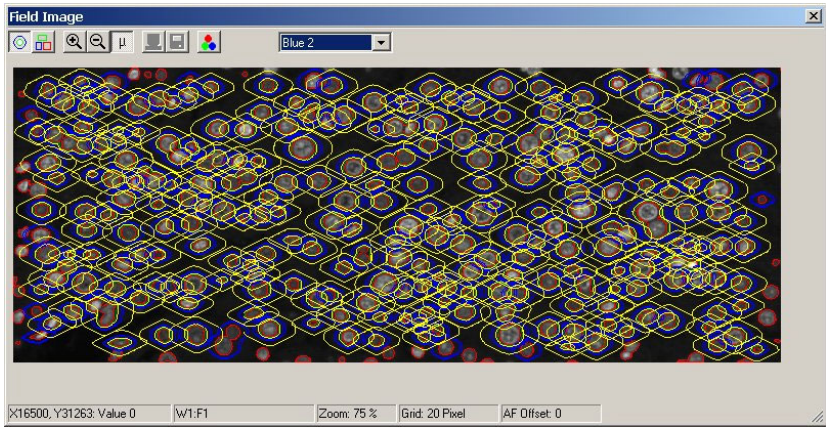
The Interactive threshold window will display a binary image of the scan field where pixel values above the threshold are bright and pixel values below the threshold are dark. In the above example, the threshold value is 5000.

Use the slide bar in Threshold module to decrease the threshold value until dimmer nuclei can be seen in the Interactive Threshold window. The example below is at a threshold value of 2500.



Refresh the contours to view the cellular segmentation in the Field View window.

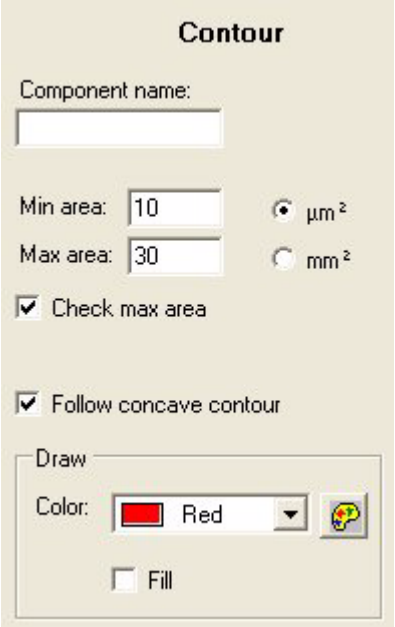
Continue adjusting the threshold value to achieve optimal segmentation.



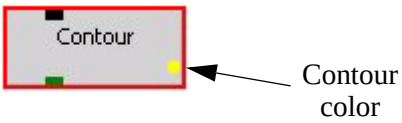
Contour

Allows you to generate the primary (threshold) contour for events by contouring the input image.

Creating or clicking on a Contour module opens the following menu.



- **Component name**—Enter the name for the contour component here. This component name will carry through to other analysis windows such as scattergrams, histograms, and run statistics.
- **Min area**—Specifies the minimum area an event must have to be considered for contouring in either μm^2 or mm^2.
- **Check max area**—When this is checked, the Max area box appears. Here you can specify the maximum area an event may have and still be contoured.
- **Follow concave contour**—Checking the Follow concave contour box instructs the software to continue tracing a contour through a concave shape. Use this option only when necessary, as it is computationally intensive and can increase your scan and analysis time.
- **Color**—Use this to select the color displayed in the image windows for contoured events. When you have selected a color for the contour this will be reflected in the small box on the lower right of the contour module.



- **Fill**—Check this box to fill the contoured events with the selected color in the scan image window.

Event Modules

Event

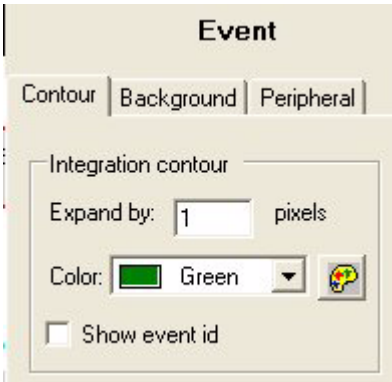
Options under the Event menu allow you to specify events, create Phantom events, and create Associations.

The Event module creates events that are defined by a threshold contour. You can set up the integration peripheral and background contours.

Contour Tab

Selecting the Contour tab from the Event menu opens the following menu.

From the Contour tab, you can set up the integration peripheral and background contours.



Integration contour—Defines the boundary within which the total fluorescence of the event will be calculated (integrated).

Expand by—Specifies the number of pixels to generate the integration contour outside the threshold contour. That is, if you specify Expand by 3, the integration contour is drawn 3 pixels outside the threshold contour.

Color—Select the color of the peripheral contour line. If color is set to None, no visible contour will be drawn.

Show event id—Displays the event ID for each event in the scan image.

Background Tab

Selecting the Background tab opens the following menu.

Use Dynamic Background—If checked, the system uses two boundary lines to define an average background value that is subtracted from the fluorescence based features for each contoured event.

Distance—Specifies the distance between the integration contour and the first background contour. Set the first background contour away from the integration contour such that it is away from the event fluorescence.

Width—The distance between the first and second background contour. Set the width such that the outer contour line includes the dimmest pixels adjacent to an event.

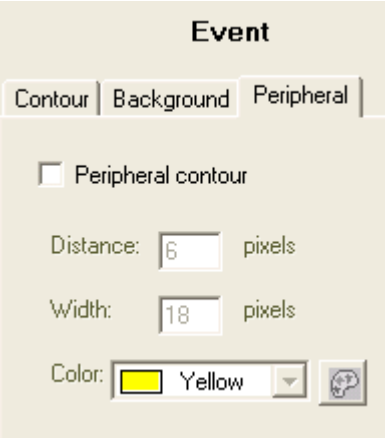
Use pixels between—When determining the background, you usually want to use those pixels that represent the mean pixel value, such as 30-70%. If, however, the background is very bright, you might want use a lower threshold, such as 20-50%.

Color—Select the color of the background contour lines. If color is set to None, no visible contours will be drawn.

Corrected Channels—Allows you to select the individual channels that will use the background correction.

Peripheral Tab

Selecting the Peripheral tab opens the following menu.



Peripheral contour—Defines two boundary lines that are used to determine the periphery of an event.

Distance—Specifies the distance between the integration contour and the first peripheral contour. Set the first peripheral contour such that it picks up the brighter peripheral pixels.

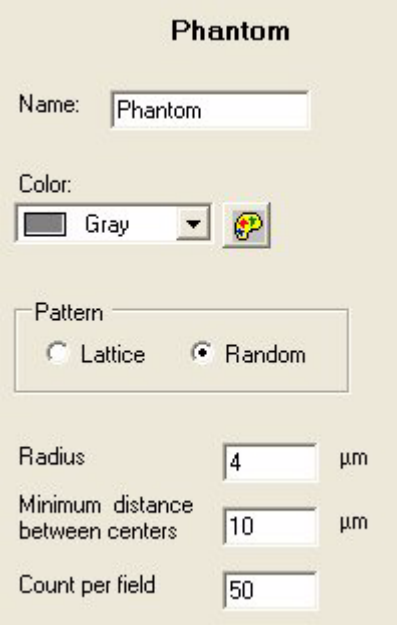
Width—Specifies the distance between the first and second peripheral contour. Set the width such that the outer contour line includes the dimmer pixels that are still within the periphery of an event.

Details on the Contour, Background, and Peripheral tabs are found in “Integration Contours” on page 4-7, “Background Contours” on page 4-8, and “Peripheral Contours” on page 4-9.

Phantom

Phantom contours are events generated by iCys from circular contours applied over the laser-scanned image.

Creating or clicking on a Phantom module opens the following menu.



Name—Allows you to change the name of the Phantom contours. This can be especially useful if you want to generate multiple phantoms.

Contour color—Select the color of the phantom contour line. If color is set to None, no visible contour will be drawn.

Pattern—Specify the type of phantom contours you want to generate:

> **Lattice**—Creates an evenly spaced field of phantom contours. Specify the Radius (in microns) and Minimum distance between centers (in microns).
>
> **Random**—Generates a random pattern of contours. Specify the Radius, Minimum distance between centers, and the Count per field parameters.

Radius—The size of each contour. Phantom contours are always circular.

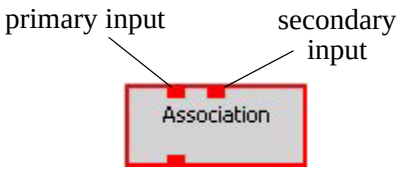
Minimum distance between centers—If you space the phantom contours very closely, you will generate more data. The phantoms will overlap one another if the minimum distance is smaller than the phantom diameter.

Count per field—The maximum number of phantoms iCys will generate per scan field.

Phantom contours are discussed in detail in “Generating Phantom Contours” on page 4-12.

Association

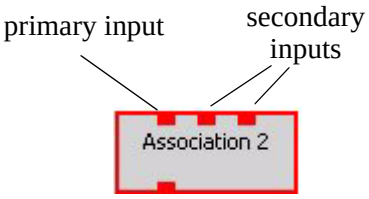
An Association module allows you to associate two event components generated with either an Event Module or a Phantom Module. The Association module has two inputs. The input on the left is the primary input. The input on the right is the secondary input.



The secondary input is used for an event component that will become “sub-events” within the primary event. If the primary and sub-events overlap each other, iCys associates the sub-events with the primary event.

Association 2

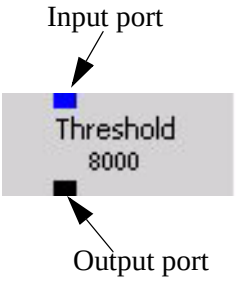
The Association 2 module allows you to associate two subevent components with a primary event component. The Association 2 module has three inputs. The input on the left is the primary, the other two are secondary.



For more information about sub events, see “Scattergram and Histogram Axis Tab” on page 4-23, “Scattergram and Histogram Event Type Tab” on page 4-26, and “Event Window Details” on page 4-36.

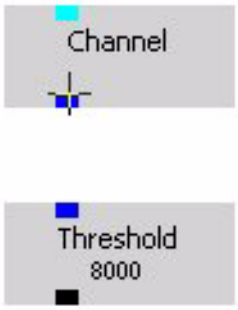
Connecting Modules

As you create modules, you will connect them to graphically create a macro that establishes the flow of data for your experiment. Modules have color-coded input and output ports. Input ports are on the top of the module, output ports on the bottom.

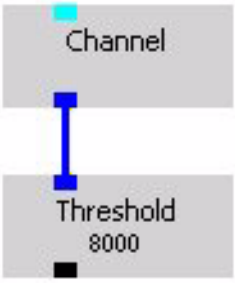


To connect two modules:

1. With your mouse, point to the output port you want to connect. The cursor will change to a cross hair.

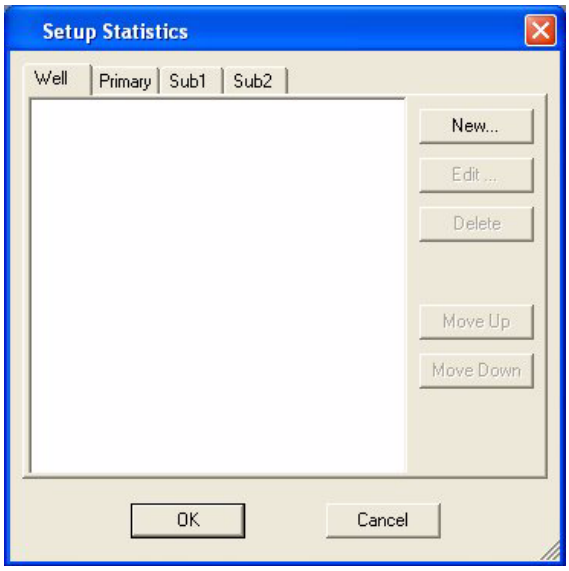


2. Click and drag to the desired input port. Remember, it must be of the same color as the output port.
3. When you release the mouse, a colored line will connect the two ports.



Statistics

Clicking the Statistics button opens the following window.

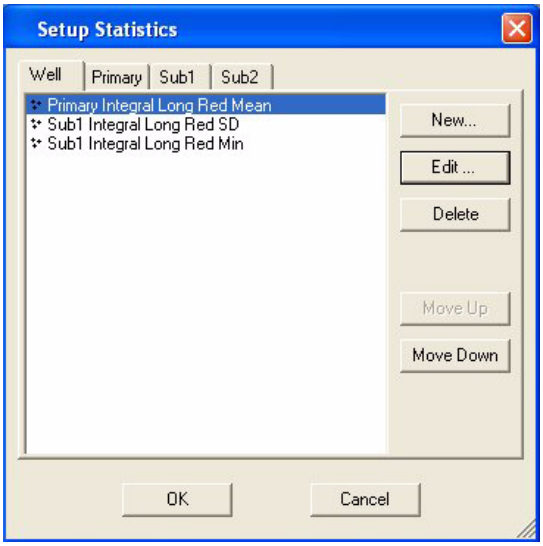


This window allows you to specify the statistics reported for a run. You can elect to create statistics based on an entire well or any event components you have defined in your protocol. The tabs available will depend on what event components you have defined in the protocol, and the labels on the tabs will reflect the labels you entered for those components.

Note: After making changes to the protocol, you must initialize the protocol again for the changes to be reflected in the Statistics tabs.

Well Tab

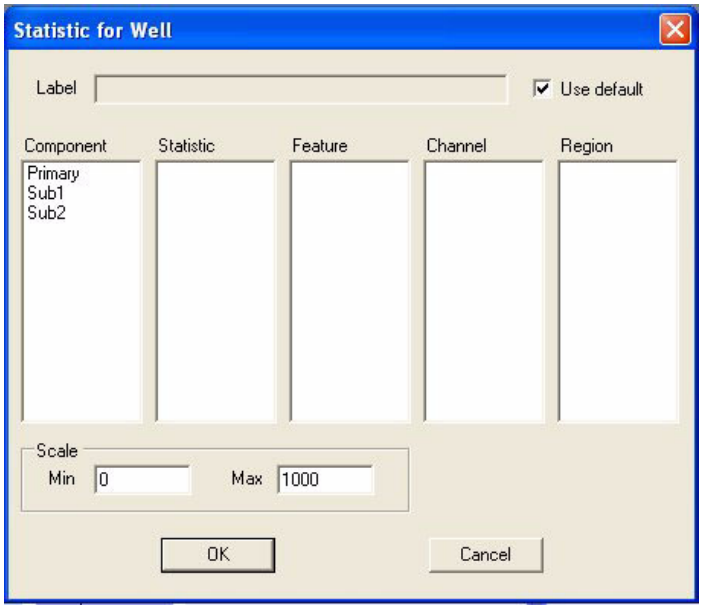
The Well tab is always available on the Setup Statistics menu. When it is selected, the menu displays any Well Statistics previously defined for this protocol. An example is shown below.



Creating a Well Statistic

To create a new Well Statistic, click the New button.

The following menu appears:



- **Component**—Any contour specified in the current protocol can be used as a component. Select the component for which you want statistics calculated.
- **Statistic**—After a component is selected the Statistic field will be populated.

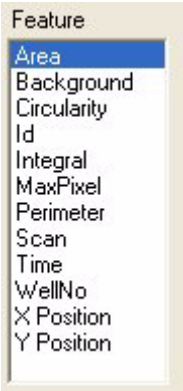
The following table summarizes the Statistic options:

Table 3-3: Statistics Options

Parameter	Meaning
Count	Number of events associated with component
Mean	Arithmetic mean of the component for the well or scan area.
SD	Standard deviation of the component for the well or scan area.
CV	Coefficient of variation of the feature for the well or scan area.
Min	Minimum value of the feature for the well or scan area.
Max	Maximum value of the feature for the well or scan area.
Sum	Sum of the feature values for the well or scan area.
FWHM	The measurement of the full width at half maximum for the well or scan area.

Note: You can select more than one statistical parameter by holding down the Ctrl key while clicking on the parameters. This also allows you to deselect unwanted parameters. If you click on a parameter, holding down the Shift key and clicking on a second parameter selects those two parameters and all parameters between them.

- **Feature**—If you select any Statistic other than Count, the Feature field will be populated, as seen below.



The following table summarizes the options in the feature field:

Table 3-4: Feature Options

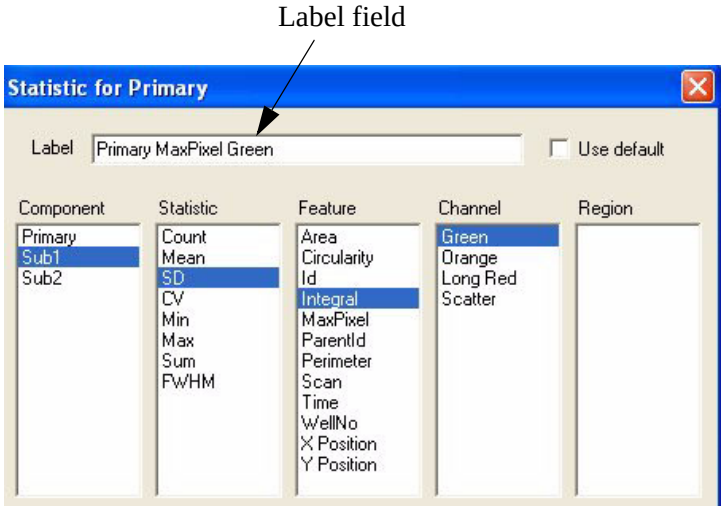
Parameters	Meaning
Area	Area in square microns of each event.
Background	Background fluorescence or signal nearby each event.
Circularity	How close the event is to a perfect circle. The circularity is defined by: perimeter^2/area Therefore, the circularity of a perfect circle is 4π (12.566). The larger the circularity number, the less circular it is. Note that, due to normalization, the circularity of an event may be less than 12.566.
Id	The Id number of the event. Each event is individually tagged with an Id number.
Integral	Integrated fluorescence per event for the selected channel.
MaxPixel	Brightest pixel value per event for the selected channel.
Perimeter	The perimeter of the event in microns.
Scan	Pixel number, from 0 to 768, indicating the y-position of the scanning mirror where the event was detected.
Time	Time, in seconds, at which the event was acquired. When rescanning a Raw file, the Time parameter contains the Well Number of this scan area.
WellNo	Well number (or scan area number) of event.
X position	X position in the carrier for each event.
Y position	Y position in the carrier for each event.

- **Channel**—If you have chosen Background, Integral, or MaxPixel, the Channel field will be populated with the channels in this protocol. You must select which channel to use for analysis.

- **Region**—If you have created any regions on a scattergram or histogram display, you can choose to create statistics based on any of these regions. If no regions have been created, this field will remain blank.

  Note that regions are specific to event components. That is, if a Region 2 is defined in a scattergram for Primary events it will be available for statistics for Primary events but not for other components.

- **Label**—If the Use Default box is checked in the Label field, the Statistic will be automatically labeled according to the parameters selected. Unchecking the box allows you to enter your own label.



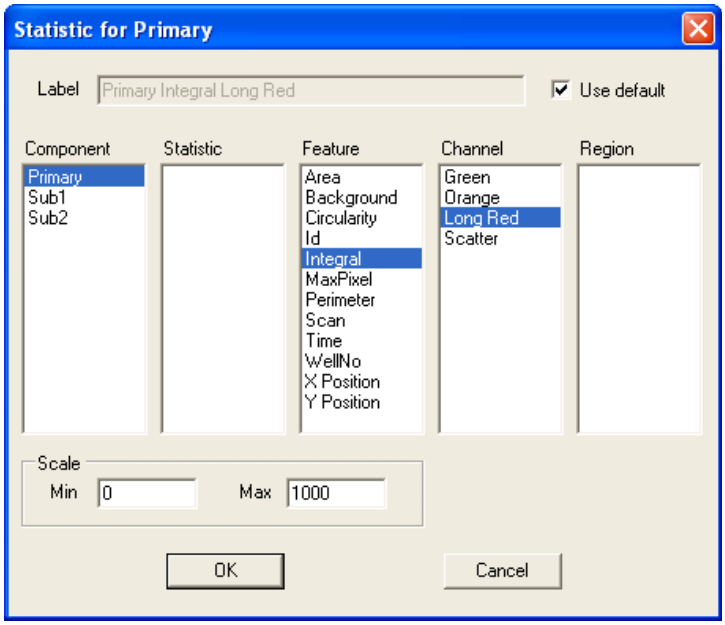
- **Scale Min Max**—These values determine the scale used when showing expression levels in the Microplate window (see “Showing Expression Levels” on page 3-20).

Note that the statistics are calculated only for components that are lower in the association hierarchy than the component (or well) tab. For example in the example below the component hierarchy (established using the Association2 module) is:

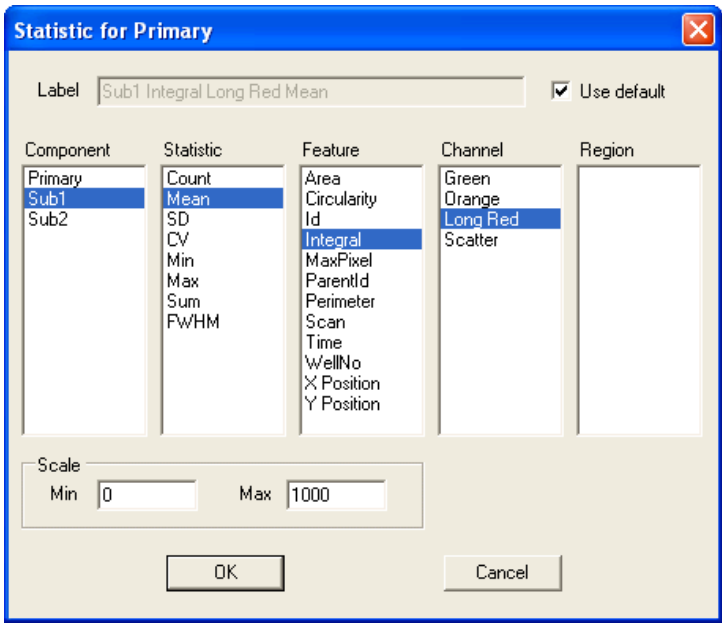
- Well
  - Primary
    - Sub1
    - Sub2

Run statistics for a Well can be calculated on Primary component events or either of the two Sub component events. For the selected component, Well statistics are calculated using all of the events of that component within each well (and within the specified gating region.)

Statistics for a component event has no meaning because there is only one component event. Thus for the example below, the Long Red Integral value will be reported for each primary event, but there is no aggregate of events on which to calculate statistics.



Because the Sub1 component is lower in the association hierarchy than the primary component (sub-event are within primary events) statistics are valid for sub-events per primary events. In the example below, a mean Long Red Integral will be calculated for the sub1 events within each primary event.

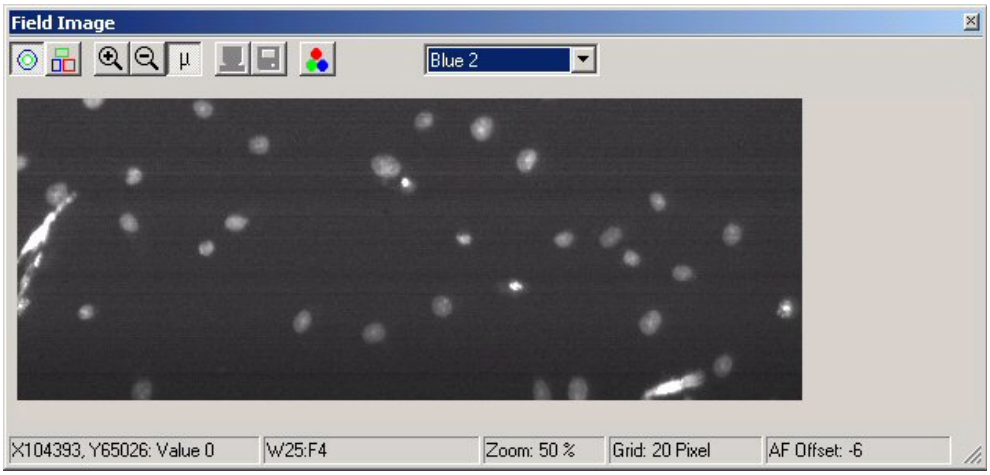


View Section

Field Image

Opens the Field Image window, which allows you to control and monitor the scanning of your specimen.

Click **Field Img** from the task bar to display the **Field Image** window, pictured below. This window displays an image of the current scan for the selected channel.



The Field Image window icons are described in Table 3-5, “Field Image Window Icons,” on page 3-68.

The Field Image Window Icons

Table 3-5 describes the functions of the Field Image window.

Table 3-5: Field Image Window Icons

Option	Icon	Meaning
Draw contours		Displays any segmentation contours you defined including phantom contours
Highlight		Highlights events that are within colored regions. Use the scattergram and histogram functions to set colors for regions.
Zoom in		Zoom in on the image. Magnification range is from 25% to 1600%. To zoom in on a specific area, hold down the shift key (the cursor will turn to magnifier) then click on the desired area.
Zoom out		Zoom out on the image. Magnification range is from 25% to 1600%.
Display in microns	µ	Selects whether the X and Y cursor positions are displayed in microns or in pixels. Cursor positions are reported at the bottom left of the window.
Adjust Focus Offset		Allows the user to adjust the sample focus. For information on setting the focus offset, see “Setting the User Focus Offset” on page 3-69.
Set Focus Offset		Sets the adjusted offset for use in the current Workspace. Note that this does not save the new offset to disk. For more information, see “Setting the User Focus Offset” on page 3-69.
Show CompuColor		Displays an iCys-generated color for each channel. The colors are overlaid so you can see all the detectors together to get color coding. This is useful for scanning with multiple detectors.
Channel	Green	Drop down menu allows you to select which channel to view.

Setting the User Focus Offset

Although the iCys AutoFocus is designed to correctly focus on the specimen to produce sharp, focused laser scanned images, some specimens may require an offset from the system setting. In this case, if your system uses the optional AutoFocus feature, you can manually adjust the focus as described below.

Note: If your system does not have AutoFocus, then the User Focus Offset is not applicable. For more information, see “Using the Microscope without AutoFocus” on page 2-8.

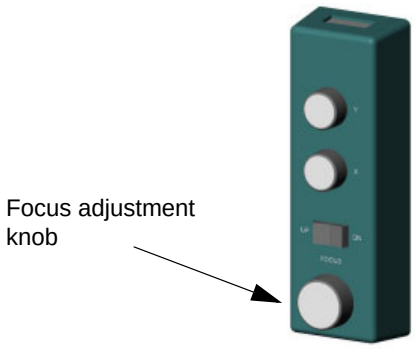
Adjusting and Setting a User Focus Offset

➢ To adjust the User Offset:

1. Click on Field Img to open the Field Image window. There are two icon buttons that control the User Offset:

   ✧ **Adjust Focus Offset**—Enables the Focus Adjustment Knob on the Remote Control unit (as shown in Figure 3-1).

Figure 3-1: Remote Control Unit — Focus Adjustment Knob



   ✧ **Set User Offset**—When your scan is properly focused, click Set User Offset to use the new setting for the remainder of the scan.

   Prior to scanning, the Set User Offset button is unavailable and is grayed out.

   The current value of the User Offset is shown at the bottom of the Field Image window.

2. Click Single Field Scan( ) to begin a scan.

3. Using the Channel Selection in the Field Image window, select the scatter detector, or a fluorescence detector with fairly bright and distinct staining.

4. Click Adjust Focus Offset ( ). The Set Focus Offset button will become active.

5. While the scan is paused, you can set the focus offset by rotating the focus adjustment knob on the Remote Control unit.Then click Refresh Current Scan

( ) to check the focus. Keep changing the focus and refreshing the scan until you are happy with the results.

The displayed value on the bottom of the Field Image window (AF Offset:) changes as you adjust the focus knob.

6. When the scan image appears as desired, click the Set Focus Offset button to set the current value of the User Offset.

   The new User Offset is used for all scans in the current Workspace. However, the new value is not saved to the Workspace file until the Workspace is saved.

Note: If the scan completes before you click the Set Focus Offset button, the offset returns to zero, the Set Focus Offset is deactivated, and the Adjust Focus Offset button is cleared. Additionally, the focus knob is disabled while scanning.

Saving a User Focus Offset

After the User Offset has been set, click on Save Workspace to preserve the new value in the Workspace File. If you exit iCys without saving the Workspace file, the new offset will be lost, and the next time you load that Workspace file the previous value will be loaded.

The Scan View Status Bar

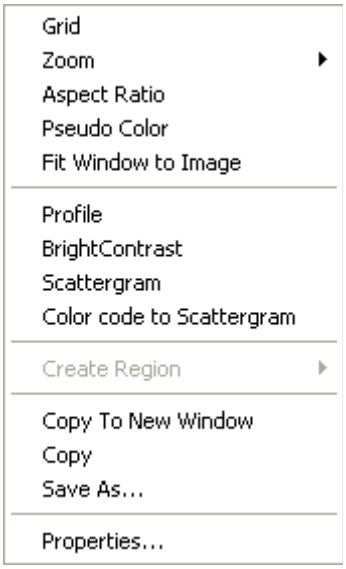
The Status bar is at the bottom of the Scan View window. It provides the following information:

Table 3-6: Scan View Status Bar

Status Detail	Meaning
Cursor position	The XY coordinates of the cursor position either in microns or pixels.
Pixel value	The intensity of the pixel over which the cursor is hovering. Values can range from 0 to 16383.
Scan number	The number of the current scan.
Zoom value	The magnification factor as set by the Zoom in and Zoom out buttons.
Grid Size	Displays the Grid size in pixels
AutoFocus offset	If applicable, the offset established using the Adjust focus offset and Set focus offset buttons.

The Field Image Properties Menu

If you right-click on the Field Image window, the following menu displays:



The options available on this menu are:

- **Grid**—When Zoom is set to greater than 400%, overlays the scan area with a pixel by pixel grid:

  If you Zoom out to less than 400%, the grid disappears.

Note: In the grid example above, the aspect ratio is on. Therefore, the pixels appear to be rectangular rather than square.

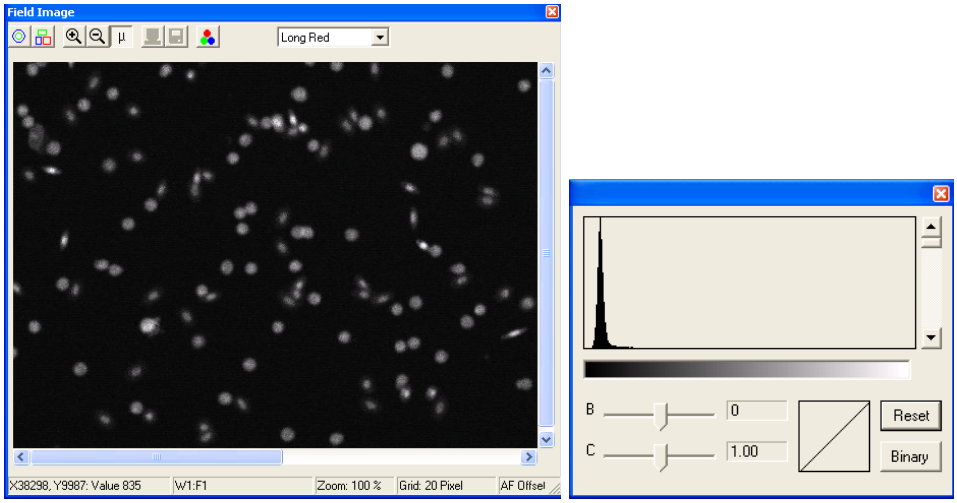
- **Zoom**—Allows you to zoom directly to the magnification of your choice between 25% and 1600%. Like the Zoom In and Zoom Out icons.
- **Aspect Ratio**—The same as Display in Microns. Selects whether the X and Y cursor positions are displayed in microns or in pixels. If Aspect Ratio is selected, the view is in Micron mode.
- **Pseudo Color**—Displays the Field image in color based on pixel intensity. The scale used to determine the color also displays:

  You cannot have both the Pseudo Color and CompuColor color schemes selected for the same window. If one is selected and you select the other, the first color scheme is deselected.

Note: The pseudo color scale is set within iCys and cannot be changed.

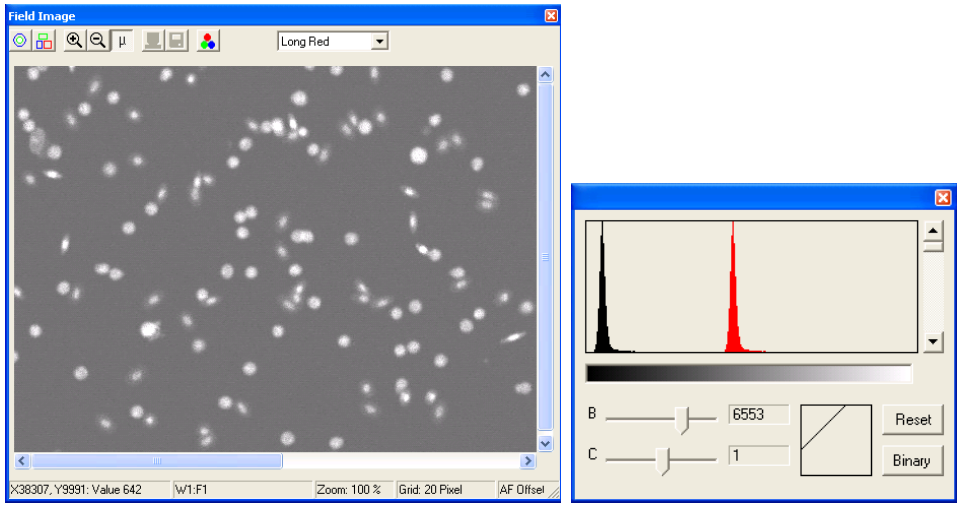
- **Fit Window to Image**—When you generate a sub-window from the Scan View window (by using the Copy command), use Fit Window to Image to make the image fit to the size of the sub-window.

- **Profile**—Displays the Profile View window which allows you to display the pixel intensity across a line on the image. For information on creating a profile view, see “Creating a Profile View” on page 3-76.
- **BrightContrast**—The BrightContrast function opens up the following window that allows you to manipulate the Brightness and Contrast of the Field Image. Brightness may be manipulated to bring out dim objects from a dark background. Contrast may be manipulated to better resolve objects that are of close brightness levels.



In the histogram in this dialogue box plots the distribution of pixels in the image. The horizontal axis represents expression level or pixel value and the vertical axis plots count of pixels at a given expression level.

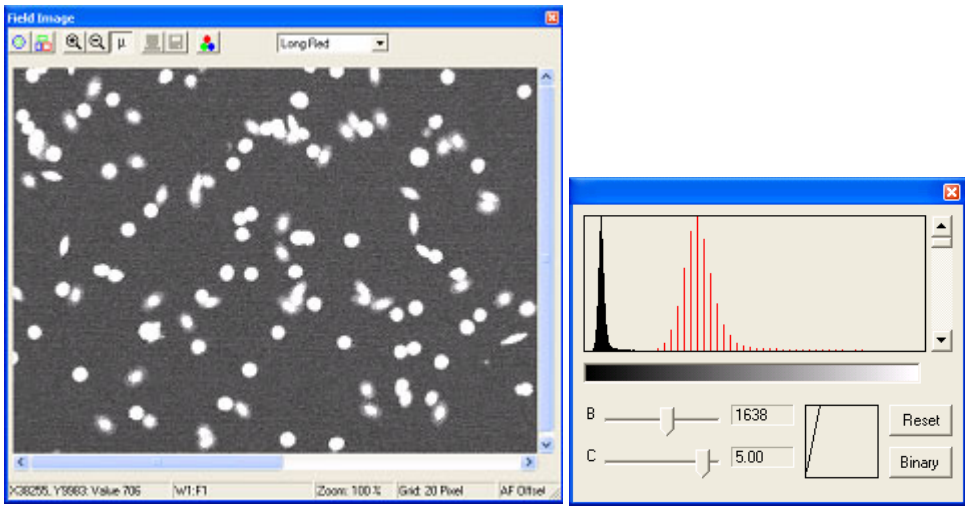
Adjusting the brightness slider shifts the population of pixels along the horizontal axis. Thus, in the example below the red population represents the shifted pixels. The brightness value, 6553, represents the amount of the shift in expression counts (-16,383 is completely dark, 16,383 is completely white.) All pixels in the field image have become brighter.



The graph in the lower right corner represents the transfer function for the pixel population transformation. The horizontal axis plots the input (or starting) pixel values and the vertical axis plots the output (or finished) pixel values. Comparing the graph immediately above to the original shows a vertical transformation of the transfer function line corresponding to the 6553 pixel value shift.

Adjusting the contrast slider broadens or narrows the pixel, population as shown below

.

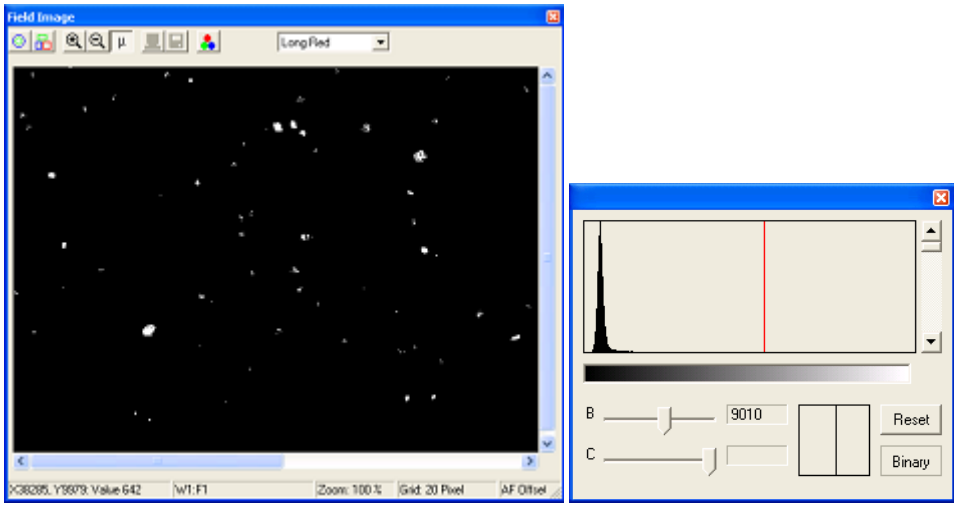


Here the red population can be seen to be broader that the original (black) population. This serves to enhance the resolution of objects with similar but distinctly different brightness values. The effect on the transfer function is to increase it's slope.

The reset button restores the image to its original state.

The binary button makes all the pixels above a certain level completely white and the entire pixel range below that level complete dark. The level of this

demarcation is the threshold value. With the binary button selected, the contrast slider sets the threshold level. Note that the transfer function shows infinite slope.

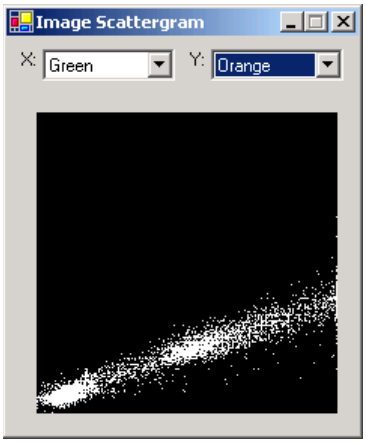


These adjustments only affect the field image that is displayed. Saved image data and calculated event features are not affected by these adjustments.

❑ **Scattergram**

Image Scattergrams

An image scattergram is an XY graph of all the image pixels. For example:



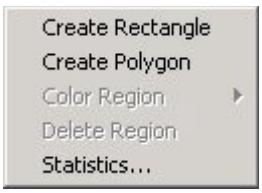
You can use the image scattergram to:

❍ Select the channels you want to display. All valid channels, real or virtual, are available from the X and Y pulldown lists.

❍ Select regions and add color to show the actual channel expression.

➢ To add a region:

1. Right-click on the Image Scattergram window. The Image Scattergram menu displays:



2. Select Create Rectangle.
3. Create a rectangle as described in “Defining the Scan Area for Freeform Carriers” on page 3-26.
4. Right-click on the Image Scattergram again and select Color Region (which will now be available).
5. Select a color for the region. Within the Well Image window, the color shows the portion of the image that has expression levels within the region on the Image Scattergram.

- **ColorCode to Scattergram**—Colors the image to match the pixels that are within the colored regions on the image scattergram.
- **Copy to New Window**—Displays the current image in a new, unnamed window in the iCys Workspace. You can add a name to the window from the Properties dialog, described below. However, keep in mind that this represents a snapshot. If you are performing a scan, the window does not change.
- **Copy—**Copies the current image to the clipboard, which allows you to paste the image to the application of your choice.
- **Save As**—Allows you to save a snapshot of the Scan View window as a graphics file in one of the following formats:
  - JPEG (*.jpg)
  - Bitmap (*.bmp)
  - TIFF (*.tif)
  - Image Pro Flat File (*.flf)
- **Properties**—Displays the properties window, which display information about the image and allows you to manipulate certain miscellaneous parameters.

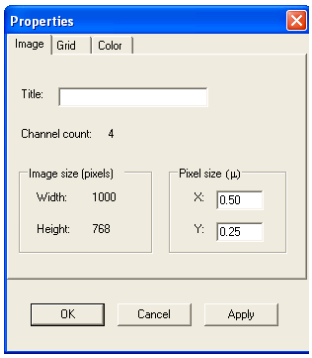
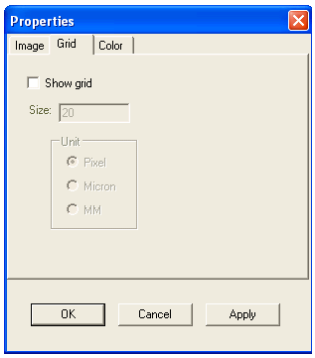


Image tab—Allows you to change the pixel size in the display. Shown below the X pixels represent .5 µm and the X pixels represent .25 µm, the default setting for the 40x objective lens.



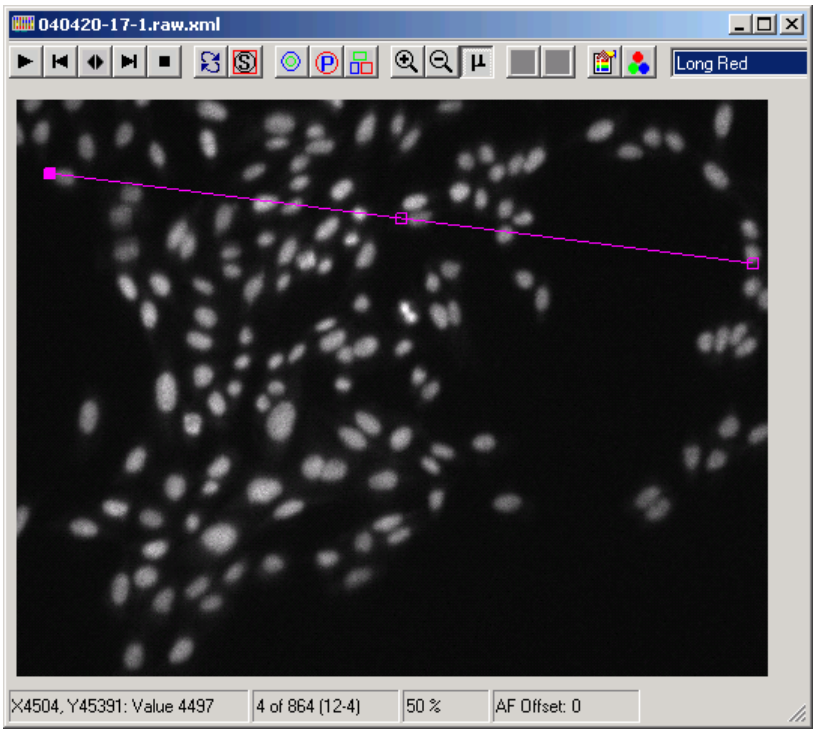
Grid tab—Allows you to change the default spacing and units for the grid.

Creating a Profile View

The Profile View tool allows you to see the pixel intensity across a single line, or profile, in any image window.

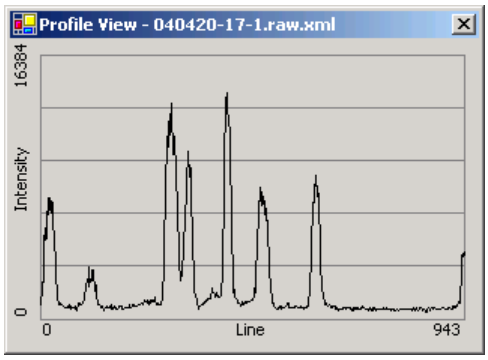
➢ To use the Profile View:

1. Select Profile. A blank grey window displays.
2. Holding the left button on your mouse down, draw a line across the scan area in any window in which the scan displays (the Scan View, Well Image, or Scout Image windows). A magenta line appears:



Tip: Note that as you draw the profile line, it begins with a solid box and ends with an open box. The solid box always represents the left-most data in the Profile View window, even if the line is drawn from left to right.

3. When you lift up the mouse button, the Profile View window displays the pixel intensity across the line in the scan area:



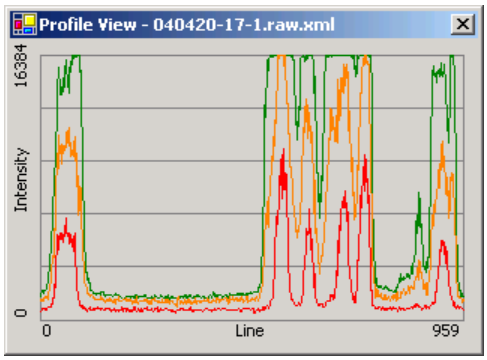
4. As a new scan area is generated by iCys, the Profile View will change to reflect the new image. The Profile View window displays the intensity and the

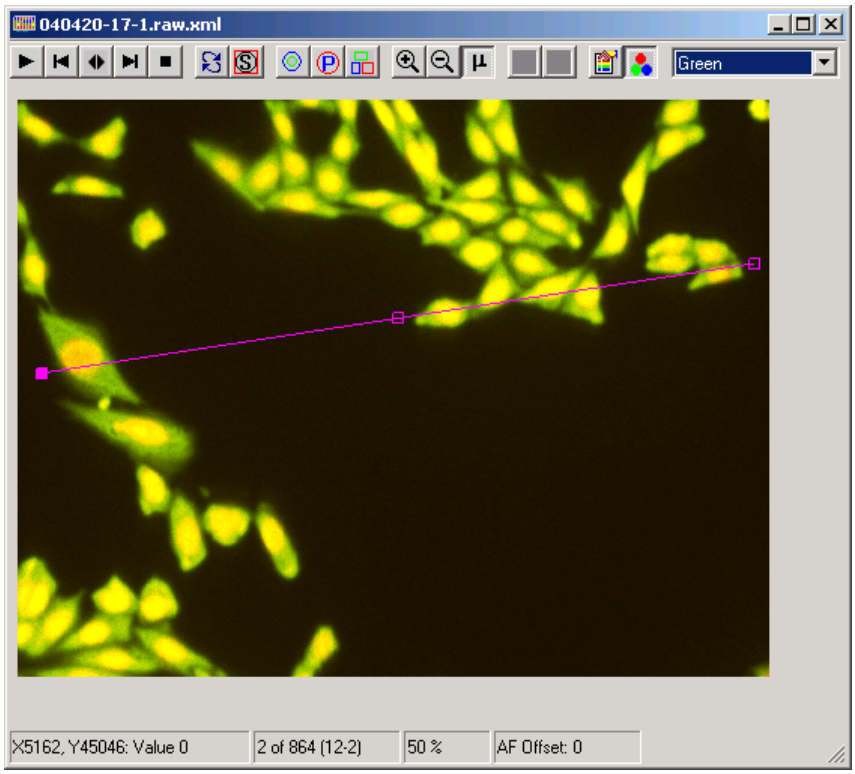
length of the profile line, in pixels. You can change the profile line by redrawing it at any time. However, iCys only allows you to have a single profile line in each window.

While only one Profile window can be open, you can have profile lines in multiple image windows. When you select an active window, the Profile View will reflect that window. If you have a profile line in, for example, both the Scan View and the Well Image window, the Profile View will change to reflect the selected window.

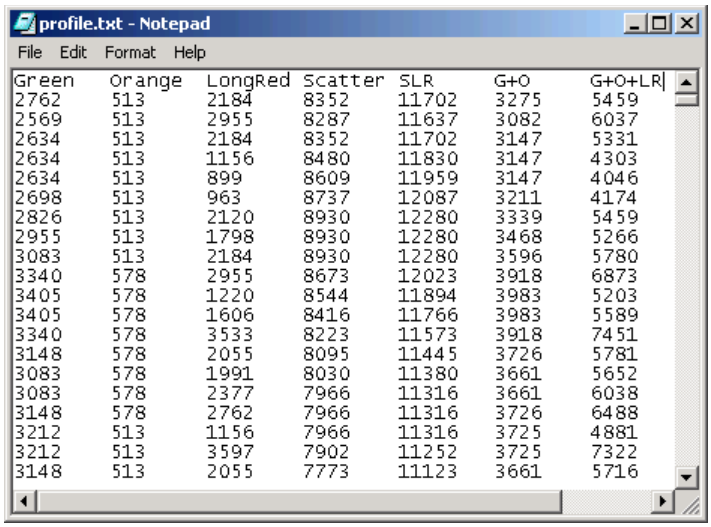
When you select a new channel, the Profile View will change to reflect the intensity for that channel.

5. To view the profile values for all selected channels (both real and virtual), select CompuColor from the scan window. A profile line is displayed for each channel. Keep in mind that you may need to select colors for the channels as described in “Color” in the “Scan Modules—Detectors” section on page 3-37. For example:





6. You can copy or save the data in the Profile View window by right-clicking on the Profile View window. The Copy and Save As options display:
   - **Copy—**Copies the current image to the clipboard, which allows you to paste the image to the application of your choice.
   - **Save As**—Allows you to save the data in the Profile View window in one of the following formats:
     - JPEG (*.jpg)—Saves the image as a graphic in .JPG format.
     - Bitmap (*.bmp)—Saves the image as a graphic in .BMP format.
     - Text (*.txt)—Saves the data used to generate the image as tab-delimited text. There will be one column per color and one row for each pixel where the value is the pixel intensity. For example:


profile.txt - Notepad

File Edit Format Help

Green	Orange	LongRed	Scatter	SLR	G+O	G+O+LR
2762	513	2184	8352	11702	3275	5459
2569	513	2955	8287	11637	3082	6037
2634	513	2184	8352	11702	3147	5331
2634	513	1156	8480	11830	3147	4303
2634	513	899	8609	11959	3147	4046
2698	513	963	8737	12087	3211	4174
2826	513	2120	8930	12280	3339	5459
2955	513	1798	8930	12280	3468	5266
3083	513	2184	8930	12280	3596	5780
3340	578	2955	8673	12023	3918	6873
3405	578	1220	8544	11894	3983	5203
3405	578	1606	8416	11766	3983	5589
3340	578	3533	8223	11573	3918	7451
3148	578	2055	8095	11445	3726	5781
3083	578	1991	8030	11380	3661	5652
3083	578	2377	7966	11316	3661	6038
3148	578	2762	7966	11316	3726	6488
3212	513	1156	7966	11316	3725	4881
3212	513	3597	7902	11252	3725	7322
3148	513	2055	7773	11123	3661	5716

7. To turn off Profile View, right-click again in a scan window, and click on Profile. When Profile has been cleared in all scan windows, the Profile View window will close.

Well Image

Opens the Well Image window. A Well image is a mosaic image of all scan fields for a given scan area.

The Well Image window has the same features as the Field Image window (see Table 3-5, “Field Image Window Icons,” on page 3-68) with the following additions.

Table 3-7: Window Icons Specific to the Well Image

Option	Icon	Meaning
Show Label		Selecting Show Label overlays a grid that represents the boundaries of the constituent field images that have been assembled to form the well image.
Create Region		This feature is available only with the iNovator Toolkit.

Region Image

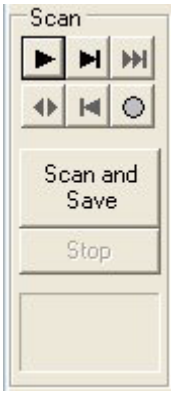
Displays an image of the region being scanned. This feature is available only with iNovator Toolkit.

Refresh

Updates and redraws all images.

Scan Section

Use the Scan section of the task bar to run either a Test Scan or a full Scan & Save operation.



This section discusses the following topics:

- ❑ Test Scan, page 3-80
- ❑ Scan and Save, page 3-84

Test Scan

Performing a Test Scan enables you to adjust all of your iCys settings before executing a Scan & Save operation. Test Scan performs a scan, which allows you to see and adjust your PMT sensors (and other settings) but does not save any data from the scan.

Additionally the Test Scan operation allows you to analyze the data and define regions in scattergrams and histograms before you perform a Scan & Save.

The six buttons at the top of the Scan section of the task bar control the Test Scan function. These are described in the table below.

Table 3-8:

Start/Pause/Resume	,	Use this button to: ❑ Start a Test Scan ❑ Pause a scan that has been started ❑ Resume a scan that has been paused.
Single Field or Next Scan		Acquires first field and pauses, or advances to and scans the next scan field.
Next well		Advances to and scans the next well
Rescan		Rescans current field
Previous field		Returns to and scans previous field
Scan Only Active Wells		If selected, iCys scans only those scan areas selected in the Carrier window. For more information about the Carrier window, see “Setting up a Microplate Scan” on page 3-17 and “Setting up a Freeform Carrier Scan” on page 3-22.
Stop scan	Stop	Stops the current scan

During a Test Scan the Scan Area Setup window displays the scan status of the wells or scan areas. However, you cannot select any of the icons from the toolbar. Additionally, the following options are disabled from the task bar:

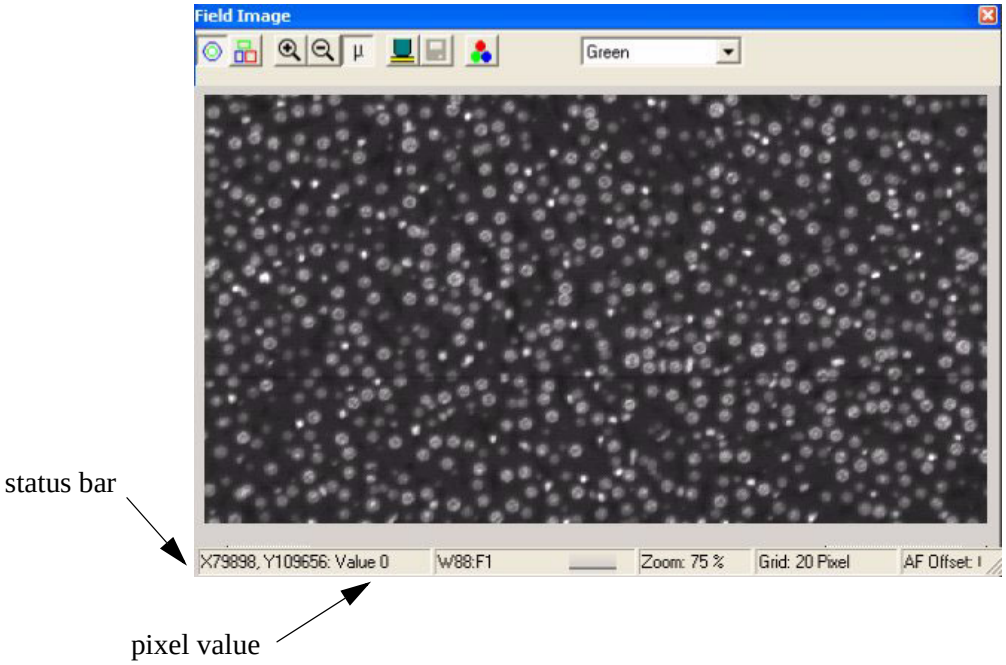
- ❑ New Run
- ❑ Open Run
- ❑ Load
- ❑ Statistics
- ❑ Refresh

Note: To access any of the disabled functions, stop the Test Scan by selecting the Stop button on the iCys task bar.
Note that it will take a few moments for the scan to stop. iCys completes the current scan field before stopping.

Adjusting Laser Power, PMT Gains, and PMT Offsets

➢ To adjust the PMT voltage and offset values:

1. Click the Start button on the task bar to begin a Test Scan.
2. Pause the scan by clicking the Pause icon on the Task Bar to make adjustments on the active scan field.
3. From the Channel tab on the Detectors menu, you can change the following settings:
   - Laser Power—Sets the amount of power (in milliwatts) used by the 488 nm laser. The laser is adjustable between 5 and 20 mW. In general, CompuCyte suggests that you do not change the laser power. There may be instances where it is necessary to do so, but first try to adjust the PMT settings.
   - Voltage%—Sets the percentage of PMT sensitivity. For a good quality sample, the Voltage should be somewhere in the range of 30-70%.
   - Gain%— Sets the amount of signal amplification. The gain should be left at 100%.
   - Offset (Volts)—Sets the background sensitivity.
4. Set the laser power to a level appropriate for your experiment.
   Fluorescent dyes absorb a certain amount of energy, so increasing the laser intensity (power) will cause the dye to fluoresce more strongly, but only up to a point. Once the dye is saturated, there will not be any increase in fluorescent brightness. The saturation point is dye specific, but generally, 5mW of Argon is a good intensity (this corresponds to approximately 1mW at the objective).
5. Set the Voltage%:
   In the field image window, move the cursor over the brightest pixels and read the pixel values located in the status bar.



If the brightest event pixels have pixel values greater than 14,000, incrementally decrease the PMT voltage value and re-scan the scan field by selecting rescan () icon. If the brightest event pixels have pixel values less than 12,000, incrementally increase the PMT voltage value and re-scan the scan field again (by first pausing the scan and then rescanning).
Make sure that none of the brightest pixels are saturated (reading 16383) and the brightest pixel values are predominantly in the range of 12,000 to 14,000.

6. To help determine if you have sufficient pixel intensity, you may want to use the bright contrast dialog in the field image view.

7. Set the Gain%:
For very bright samples requiring very low voltage settings (below 10%), you can decrease the Gain. This will allow you to get a better signal at a lower Voltage. For changes in Gain to be effective, you need to make fairly large changes in the parameter; try starting with a 20% change.

8. Set the Offset:
In the field image window, move the cursor over the dimmest pixels and read the pixel values located in the status bar at the bottom of the field image window, as shown in the previous figure.
If the background pixels have values less than 300, incrementally increase the PMT offset value and re-scan the scan field. If the background pixels have values greater than 500, incrementally decrease the PMT offset value and re-scan the scan field by selecting the rescan icon. The offset is very sensitive, you may only need to change it by 0.1 or 0.2 percent. Offset values are usually negative. Decrease the offset to decrease the background values.

Make sure that none of the dimmest pixels are reading 0 and the background pixel values are consistently in the range of 300 to 500.

Note: Offset range is between -5 and 5 volts. Changing offset to correctly adjust background pixels will also affect bright pixels. Check bright pixels after offset adjustments, and adjust voltage if necessary.

Note: Staining often varies across the specimen; review multiple scan fields to ensure that settings are appropriate.

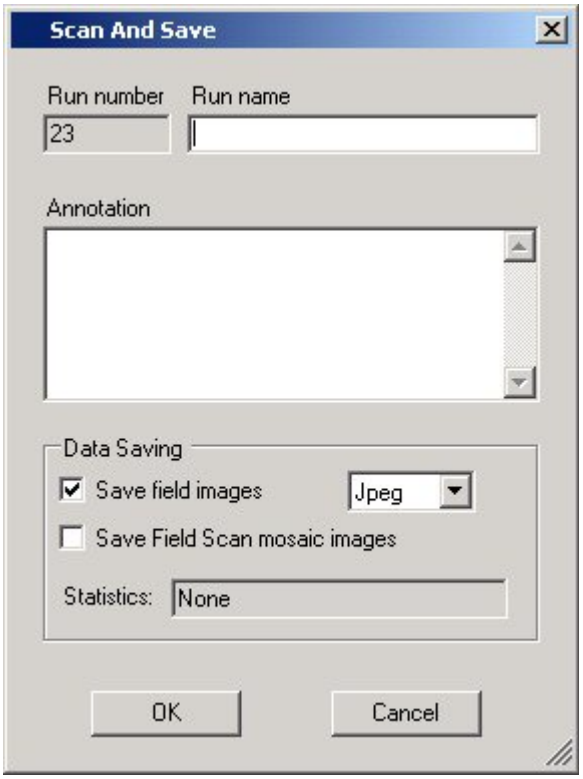
9. When you are satisfied with the settings, you can confirm that they are optimized by using the bright contrast tool in the field image window.

Scan and Save

Once you are satisfied with the settings for your scan, you can click the Scan and Save button. Scan and Save begins the formal scan process.

➢ To Start a Scan and Save:

1. Click Scan and Save from the Task Bar. The Scan and Save dialog displays:



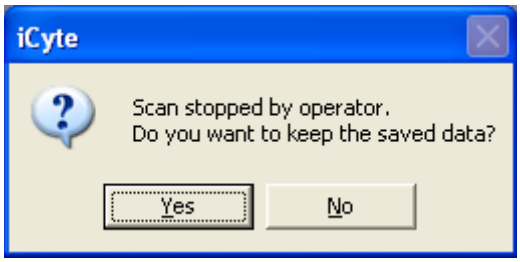
2. Enter a Run name.

3. Enter a Run Annotation if desired

4. Check appropriate Data Saving entries. Save images as either Jpeg or Flat.
5. Click OK. The window closes and the iCys scan begins.

Keep in mind that during Scan and Save, most options are disabled.

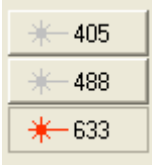
When you stop the scan before all scan areas have been scanned, you will be given the chance to keep the data that has been already saved:



If you select No, the all run data already saved will be deleted. The run number will not be reused, so the number for the next saved run will be incremented.

Instrument Section

Laser Shutter buttons



The laser shutter buttons allow you to manually open laser shutters. When the laser shutter is open, the button appears pressed and the laser graphic is colored. During scan the buttons update to reflect the current shutter status.

Objective pulldown

Use the Objective pulldown to let the software know which objective is currently selected in the turret.

Data Structure

Workspaces and Workspace Files

Configuring for an experiment involves setting up the instrument for data acquisition, organizing the data displays for subsequent analysis, and storing selected information about each well on a plate or slide. All of these instrument and display settings are saved in a Workspace file, which always has a.WS.XML extension.

The Workspace contains three main sections, as described below.

Workspace Protocol Settings

The Workspace protocol settings include information about the scan settings and analysis settings, including:

- ❑ Carrier and scan areas
- ❑ Protocol settings from the protocol window
- ❑ Run annotations
- ❑ Data saving options

Note: The protocol settings are also saved in the Raw and Event files.

Workspace Display Protocol Settings

The display protocol settings, including gating and graph settings such as:

- ❑ The features for each graph as well as its position and status
- ❑ The relationships among gating regions of the scattergrams, histograms, 2-parameter histograms, and cell analysis windows
- ❑ Run statistics, galleries, scan field images, and histogram files that you wish to have automatically saved during the scanning process

Workspace Desktop Settings

Workspace Desktop settings include the size and window positions of the standard windows. The information includes:

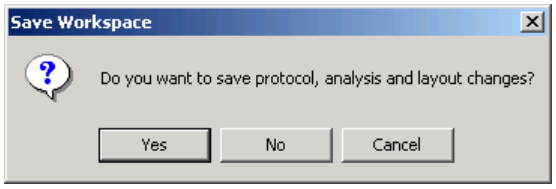
- ❑ Tabs
- ❑ Window positions and sizes

Using Workspaces

You can change, delete, or add new analysis windows to your desktop. Any changes can be saved either by overwriting the current Workspace file or saving the Workspace under a new name.

Saving Workspaces

When you make changes to the Workspace and then perform any action that closes the current Workspace, prompts you to save your changes as follows:



The Save Workspace message indicates that Workspace elements were changed since the last time the Workspace was saved. If no changes were made, the Workspace closes without displaying the Save Workspace message.

- If you click **No**, iCys discards your changes.
- If you click **Cancel**, the action is canceled.
- If you click **Yes**, iCys saves the Workspace. Depending on the action that causes the Workspace to close, iCys takes one of the following actions:
  - **Open Run**—Displays the Open Run window to allow you to open a new Run (which includes the Workspace associated with that Run).
  - **Open Workspace**—Displays the Open Workspace window to allow you to open an existing Workspace.
  - **Save Workspace**—Saves the current Workspace, which remains open.
  - **Save Workspace As**—Displays the Save Workspace As window to allow you to save the Workspace under a new name or into a new directory.
  - **Close Workspace**—Opens the default.WS.XML Workspace.
  - **Exit**—Closes iCys.

Note: When you save a read-only Workspace (such as a template or a Workspace associated with a Run file), the Save Workspace As window displays and the new Workspace will be saved in the WorkFiles directory.

Any open Raw or Event files remain open.

The Save Workspace and Save Workspace As messages inform you of what changes have been made to the Workspace, as follows:

- **Protocol** —Changes to the scan protocol, such as channels, PMTs, and other items, as described in “Workspace Protocol Settings” on page 3-86 and “Workspace Display Protocol Settings” on page 3-86.
- **Analysis**—Changes to scattergrams, histograms, galleries (including window positions), and run statistics.
- **Layout**—Changes to positions for windows such as the Scan View window, Carrier, iNovator, and Well Statistics windows.

If your system is using the iNovator Toolkit, described in Appendix B, Using the iNovator Toolkit," then the macro associated with the Workspace is saved when you save the Workspace.

➢ To save a Workspace:

Select **File** ⇒ **Save Workspace**. The Workspace is saved with the current name. If the Workspace already existed, any changes you made to it are saved.

➢ To save a Workspace file under a different name:

1. Select **File** ⇒ **Save Workspace As**. The standard Microsoft **Save As** window displays. If the Workspace is read-only, the WorkFiles directory automatically opens.
2. Save the Workspace under a different name in the WorkFiles directory.

When you open the Workspace file later, the entire experiment is loaded, including instrument settings and the configuration of data displays on the screen.

Creating A New Workspace

To create a new workspace:

1. Open an existing Workspace, such as default.WS.XML.
2. Make the changes you want for the new Workspace.
3. Select **File** ⇒ **Save Workspace As** and save the Workspace with a new name into the WorkFiles directory. The Workspace is saved.

Opening an Existing Workspace

If you are doing a series of similar experiments, you may want to set up a single Workspace and reuse many or all of the same parameters. Using an existing Workspace allows you to do so.

➢ To open an existing Workspace:

1. Select **File** ⇒ **Open Workspace**.
2. If desired, save the current Workspace and then browse to the Workspace you want to open, and click **Open**. The new Workspace opens. It contains the histograms, scattergrams, PMT settings, and other settings that were saved with that Workspace.

   If your site is using the iNovator Toolkit, described in Appendix B, "Using the iNovator Toolkit," then the macro associated with the Workspace becomes available when you open the Workspace and select **iNovator** ⇒ **Initialize**.

Other File Types

iCys creates and uses several file types, and stores them in specific locations. These are summarized below.

Run files

- Run.xml files—Contains run metadata and indexes to all other files. Stored in the Run folder.
- Workspace files (*.ws.xml)—The workspace used to generate the run. Stored in the run folder.

Field images

Generated by Field Scans, stored per scale in two folders:

- Acquired field images (*.jpg or *.flf) in the carrierX\scanX\FLD subfolder
- Computed field images (*.jpg or *.flf) (for virtual channels and compucolor) in the carrierX\scanX\FLDC subfolder

Mosaic images

Generated by Mosaic Scans or Field Scans if “Save Mosaic” checked. Stored per scale in two folders

- Acquired mosaic images (*.jpg) in the carrierX\scanX\MOS subfolder
- Computed mosaic images (*.jpg) (for virtual channels and compucolor) in the carrierX\scanX\FMOSC subfolder

Event files (*.evt)

Stored per carrier in the carrierX\EVT subfolder

Run statistics files (*.stat.txt)

One file per component in the run folder, generated according to the Statistic Setup. Stored in the run folder.

Saved Graphs

All defined graphs are stored per data unit (well or scan area event) for each carrier.

- Scattergrams (*.bmp) in the carrierX\SCA subfolder
- Histograms (*.his) in the carrierX\HIS subfolder
- 2-parameter Histograms (*.h2s) in the carrierX\H2S subfolder

Galleries (*.jpg)

All defined galleries are stored per data unit (well or scan area event) for each carrier in the carrierX\GAL subfolder

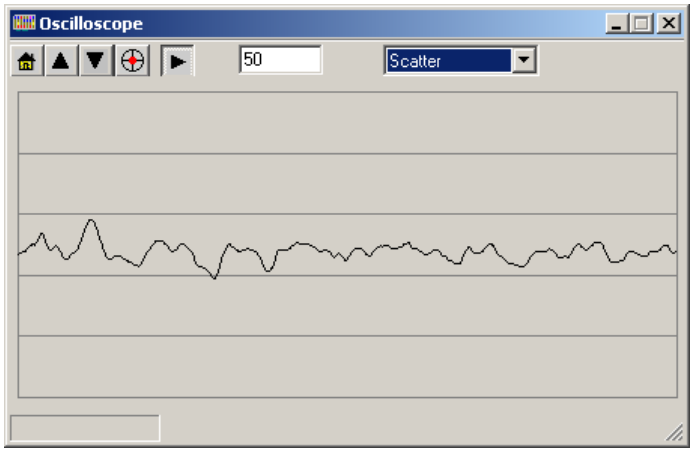
Specialized Features

Oscilloscope

Using the Oscilloscope Feature

The oscilloscope menu is accessible from the Instrument menu on the menu bar. It is shown below.

The iCys oscilloscope feature displays a waveform of the collected signal for one or more PMTs across a scan line. The oscilloscope will use the detector settings marked as default in the protocol window.



Note: Home, Objective Up, Objective Down, AutoFocus, and Objective Step will be available only if your system has the optional AutoFocus feature. If your system does not have AutoFocus, these buttons are greyed out and unavailable.

The Oscilloscope window has the following icons:

Table 3-9: Oscilloscope Icons

Option	Icon	Meaning
Home		Moves the objective to Home.
Objective Up		Moves the objective up the number of microns specified in the objective step box.
Objective Down		Moves the objective down the number of microns specified in the objective step box.
AutoFocus		AutoFocuses the objective. Only available if AutoFocus is selected, as described in “Focus and Performance” on page 3-33.
Play or Stop		Opens or closes the selected shutter, and activates the display. Select appropriate laser(s) on task bar.

Table 3-9: Oscilloscope Icons (Continued)

Option	Icon	Meaning
Objective Step	50	Specifies the number of microns to move the objective up or down when you click the Objective Up or Objective Down icon.
Channel	Green	Allows you to select the channel with which to use the oscilloscope. If you select All Channels, the oscilloscope displays overlapping lines; one for each selected channel, as described in “Selecting the Lasers and Channels” on page 3-41.

Merging Runs

The Merge Runs feature allows you to merge field image data from two similar Runs into a third virtual Run that contains the data that you specify. Merging runs can be useful to study the change in a cell over time or to virtually create more channels. You can, for example, modify a previously scanned sample by staining it with a different dye or treating it with a different drug, and then rescanning so that the changed cells can be compared directly to their originally scanned counterparts.

To merge two Runs, both Runs must have been generated using similar parameters, as follows:

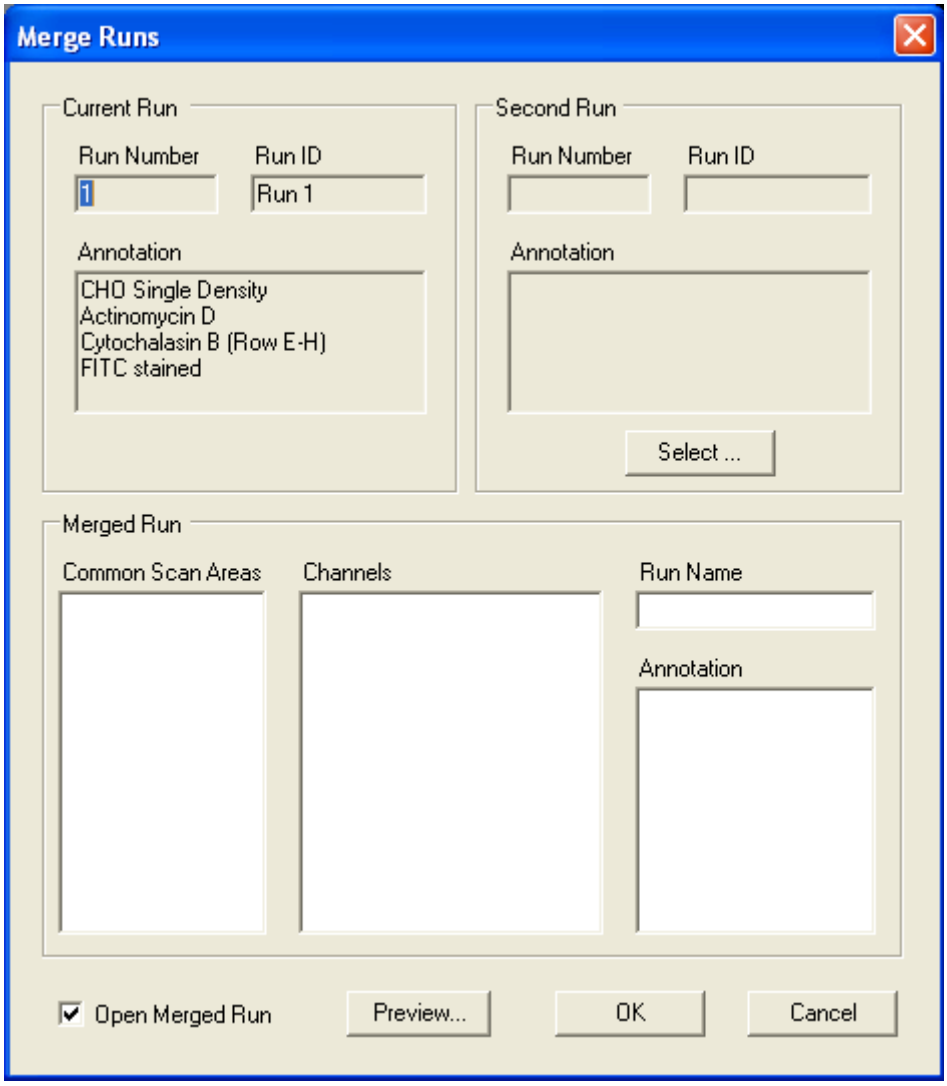
- ❑ Both Runs must have been created using one Field Scan with saved field images that use the same format (either Jpeg or Flat).
- ❑ The Runs must have the same carrier type and contain only one carrier.
- ❑ The Runs must have been generated using the same objective and resolution.

Additionally:

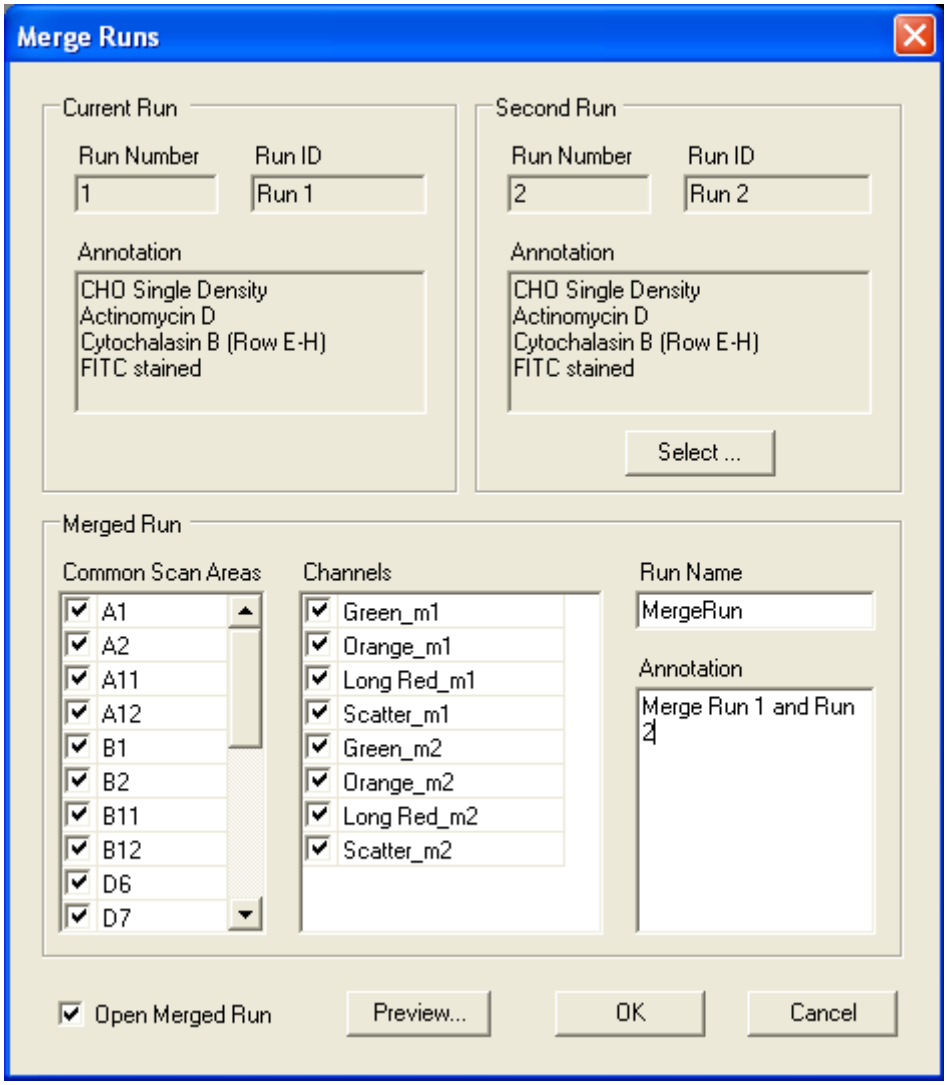
- ❑ Only scan areas with the same ID and size will be included in the merged Run.
- ❑ For partially scanned scan areas, the common scan fields will be included in the merged Run

To merge two Runs:

1. Open the first Run you wish to merge.
2. Select **File** ⇒ **Merge Runs**. The Merge Runs window displays with information about the current run in the Current Run section:



3. Click the Select button. The Open Run window displays. Select the Run you want to open as the Second Run. The information from the second run is entered into the Merge Runs window:



When the second run is opened, the following information is automatically entered:

- In the **Second Run** section: Run information for the second run.
- In the **Merged Run** section:
- **Common scan areas**—Scan areas for which there is similar data in both carriers. Only the common scan areas can be merged.
- **Channels**—All real channels from each run. Virtual channels are not included. For duplicate channels (that is, where the channel exists in both runs), the letter m (for merged) is appended to the end; that is, for the first Run, the channels are listed as Orange-m1, Blue-m1, Blue 2-m1, and so on; for the second Run, the channels are Orange-m2, Blue 2-m2, and so on. If you then merge a previously merged run, the merge number is incremented; for example Orange-m3, Long Red 2-m3, and so on. If desired, you can change the name of the channel from the

Channels list. Select the name of the channel you want to change and type in a new name.

4. In the **Common Scan Areas** section, all common scan areas are automatically selected for merging. If desired, clear the checkbox for any scan areas you do not want merged.

5. In the **Channels** section, all channels are automatically selected. Clear the checkbox for any channels you do not want included in the merged run. If desired, you can change the name of any channel.

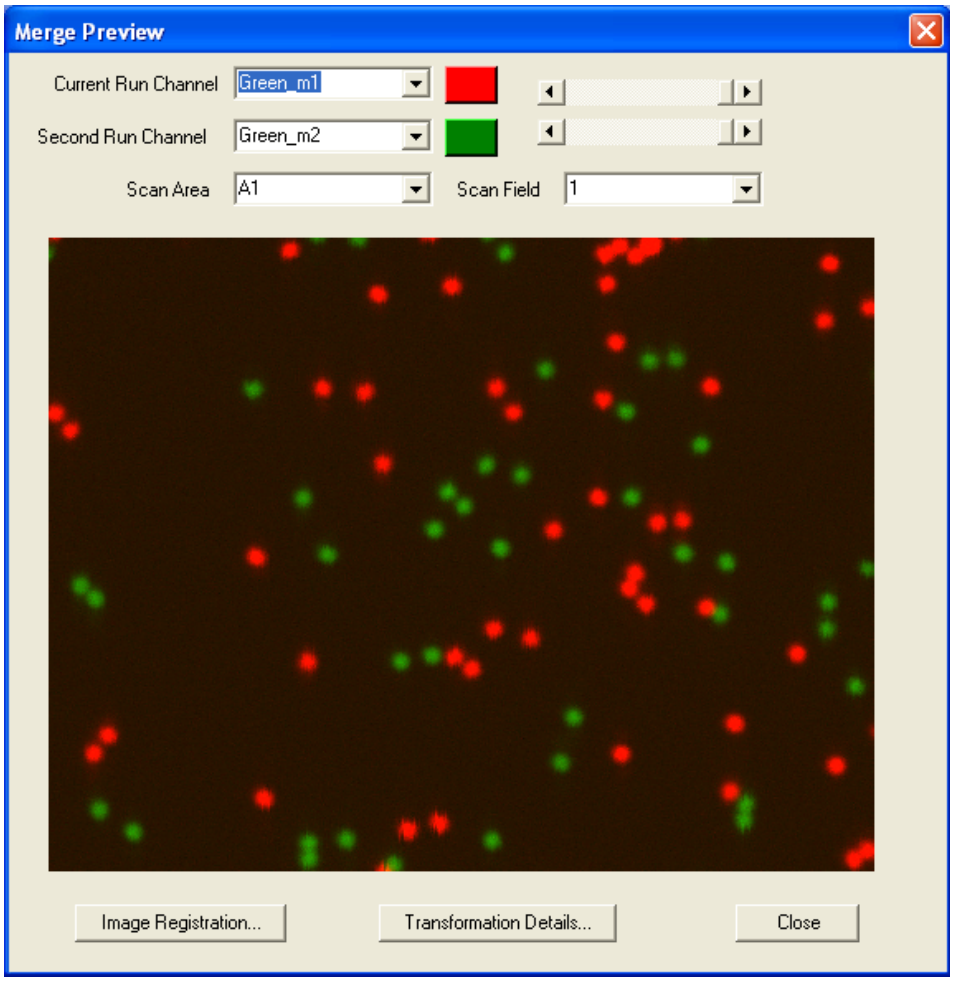
6. Enter a new **Run Name** and, optionally, an **Annotation**.

7. If you want the new, merged run to open automatically, select **Open Merged Run**.

8. Optional Image Registration:

   When you generate runs for merging, you may find that the events for each run do not line up exactly due to slight positioning discrepancies. You can apply “Image Registration” to corresponding images so that they are properly aligned.

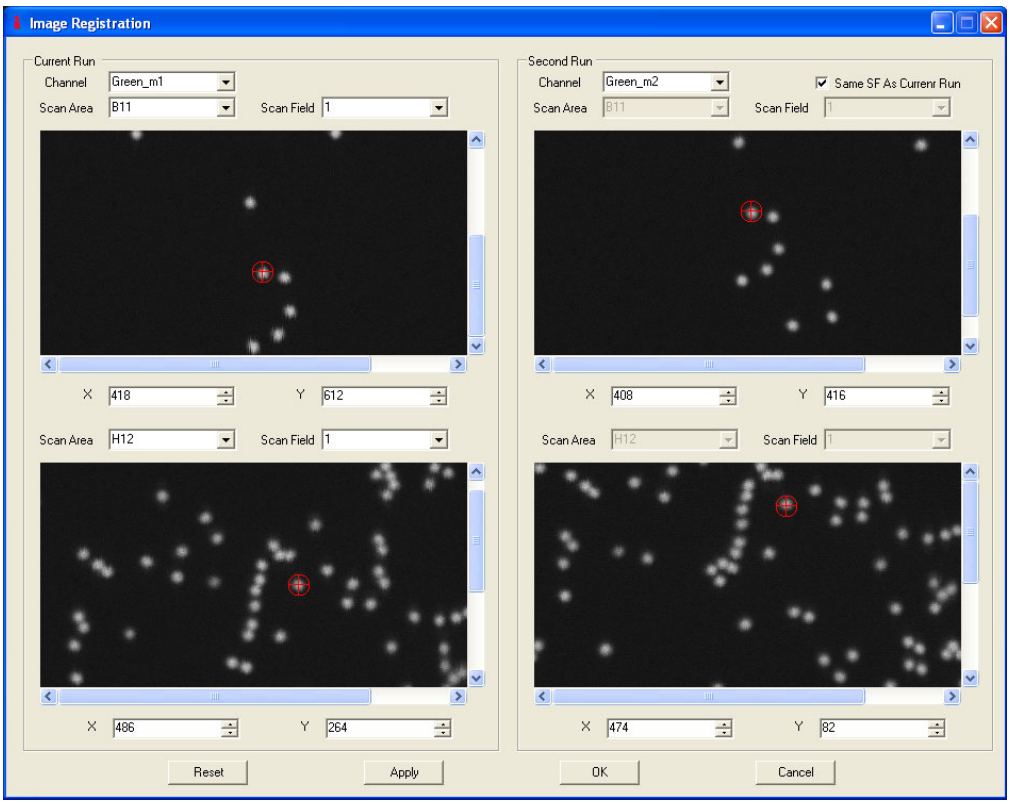
   To preview how these two runs' images match, click the Preview button. The Merge Preview window opens:



A combined field image from two runs is shown in the window. You can adjust the color and intensity for each run's image to best identify the cells of each run. You can also choose to view different channel or scan field.

If images of the two runs match well close the window.

If there is a discrepancy between the runs you can apply image registration by clicking on Image Registration button. The Image Registration window displays:

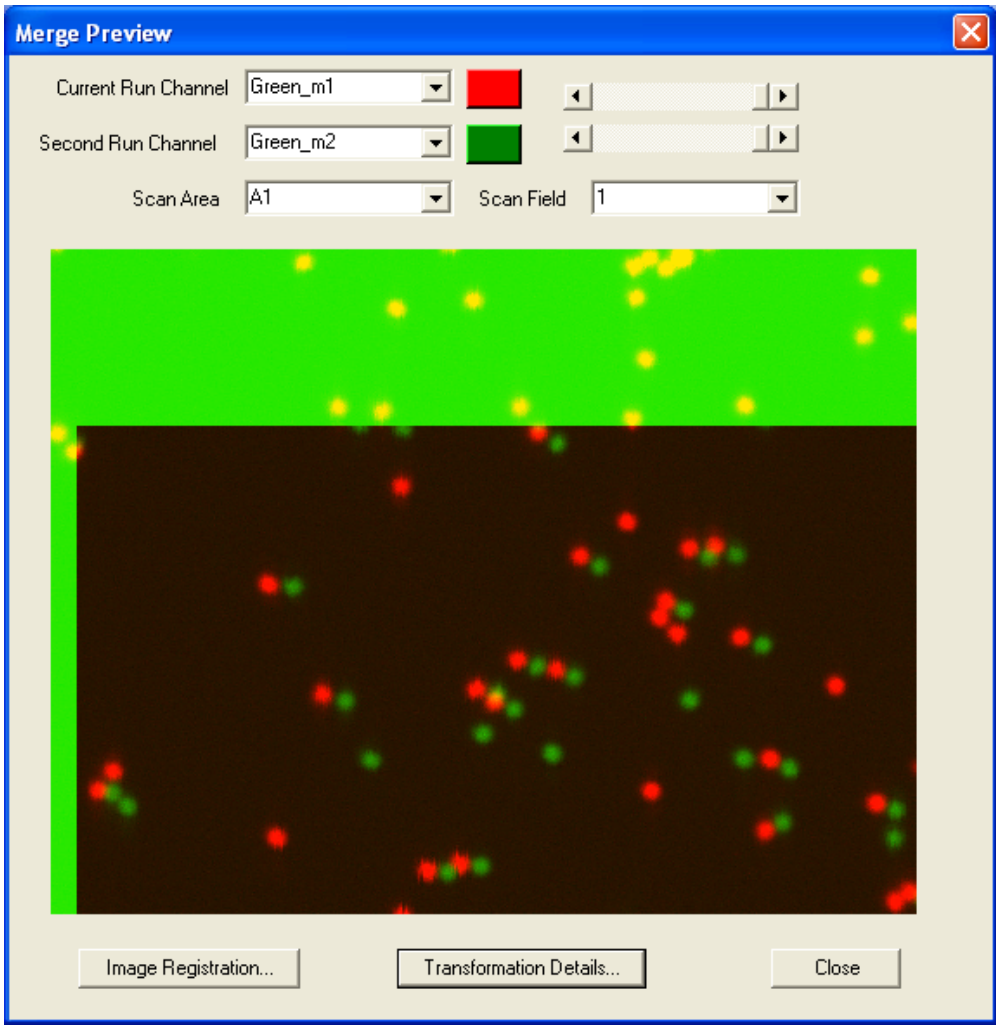


In this window you select two points in the first carrier and two points in the second carrier that you want to align. To select a point, choose a channel, scan area and scan field that shows an easy identifiable location and then move the marker to that location.

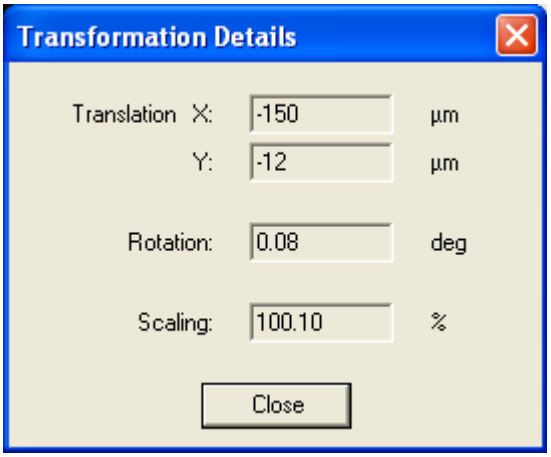
Most likely the corresponding location appears in the same scan field of the second run. If not, clear the checkbox “Same as the first run” and choose the correct scan field.

Note: When selecting the second pair of points, choose a scan area that is physically far away from the first one. A long distance between the points gives the most accurate result.

Once the four points have been selected, click Apply to see the results in the Merge Preview window.



To view the transformation data for the image registration, click the Transformation Details button



If you are not satisfied with the result, open the Image Registration window and click the Reset button to clear the transformation data. You can then reselect points.

If you are satisfied with result, close the Merge Preview window.

9. Click OK on the Merge Run window to start the merge, a message, *"Merging files, please wait"* displays.

When the merge is complete:

- ❑ If you selected **Open Merged Run** and there were changes to the current Run Workspace:
- ❑ The Save Workspace window displays. Click the appropriate button, as described in "Saving Workspaces" on page 3-87.
- ❑ When the merge is finished, a new Run opens that contains only the merged data.
- ❑ If you did not select Open Merged Run, the new Run is saved, but the original Workspace and Run remains open.

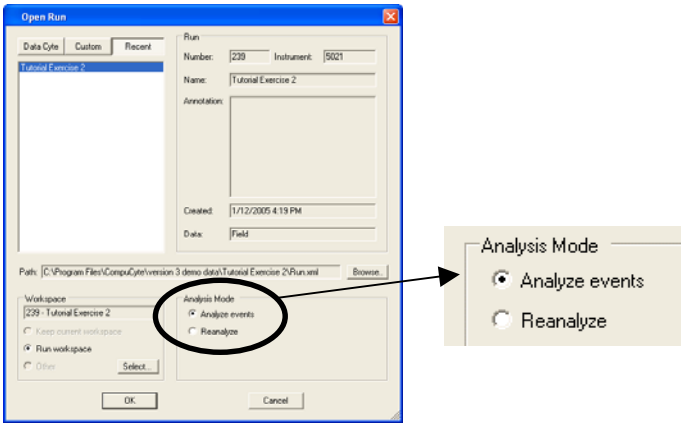
10. In the new Run, only the field image data and the Workspace are saved. After the Runs have been merged, you will need to perform a Scan & Save using the new merged data to generate new analytical data for the merged Run.

Working With a Merged Run

- ❑ After you generate new data, the merged channels are very similar to any other virtual channels (for more information about virtual channels, see "Working With Virtual Channels" on page 3-47).
- ❑ Merged channels are available in the merged run for analysis and for creating new virtual channels.
- ❑ Any virtual channels from the first run are brought forward (along with the other Workspace settings of the first run) into the merged run's Workspace.
- ❑ Virtual channels, and other Workspace information, from the second Run are not brought forward into the new merged Run.
- ❑ When using Dynamic Background, channels merged in from the second run will be background-corrected by default.

Reanalyzing Runs

There are two modes available for reanalyzing saved Runs: Analyze Events and Reanalyze. These modes are selectable in the Open Run dialogue window from the Task bar.



Analyze Events

In the analyze events mode the events for the saved Run are may be displayed in existing or newly defined scattergrams histograms and expression maps. Gating regions may be created, deleted or manipulated. Run Statistics may be altered. Events may be relocated in their Field or Well images.

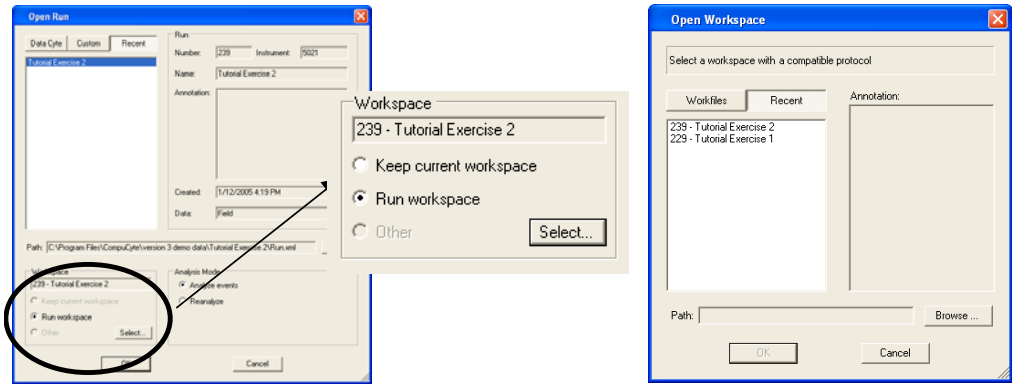
Reanalyze

In the Reanalyze mode the saved field scan images may be re-processed using the protocol. Parameters in the protocol (other than hardware parameters such as PMT settings, focus settings, objective selection, scan areas, etc.) may be modified. These include contour channels, threshold values, virtual channel definitions, associations, etc. In addition to these protocol changes, all of the event analysis options available in the analyze events mode are available in the Reanalyze mode as well. Runs may test-scanned or scan-and-saved. Scan and saved runs are saved as a separate runs which itself can be reanalyzed.

Reanalysis and Workspaces

When selecting a run for Analyze Events or Reanalysis the default is to open the run with the Workspace that was saved with that run. You also have the option to open the run with a different Workspace. This allows you, for example, to modify a given workspace and then use that modified Workspace to analyze multiple Runs. The Workspace and the Runs need to be compatible.

To select a Workspace, click on the Select button to display the Open Workspace Dialogue. From here navigate to and select the desired Workspace.


Open Run
Workspace
239 - Tutorial Exercise 2
Keep current workspace
Run workspace
Other
Select...
Open Workspace
Select a workspace with a compatible protocol
Workfiles
Recent
Annotation:
239 - Tutorial Exercise 2
229 - Tutorial Exercise 1
Path:
Browse ...
OK
Cancel

Chapter 4

Generating Analytical Data

This chapter discusses how to set up analysis parameters in the iCys™ Cytometric Analysis Software.The iCys software is designed to allow you to create and analyze the data you want. Using iCys, you can define contours, scattergrams, histograms, and other analytical tools.

This chapter describes the analysis parameters and how they are used in iCys.

Included in this chapter:

- Contours, page 4-2
- Displaying Run Statistics, page 4-16
- Displaying Region Statistics, page 4-18
- Defining and Creating Galleries, page 4-20
- Creating and Changing Scattergrams, page 4-22
- Creating and Changing Histograms, page 4-38
- Locating and Viewing Events in the Field Image Window, page 4-45

iCys™ RESEARCH IMAGING CYTOMETER

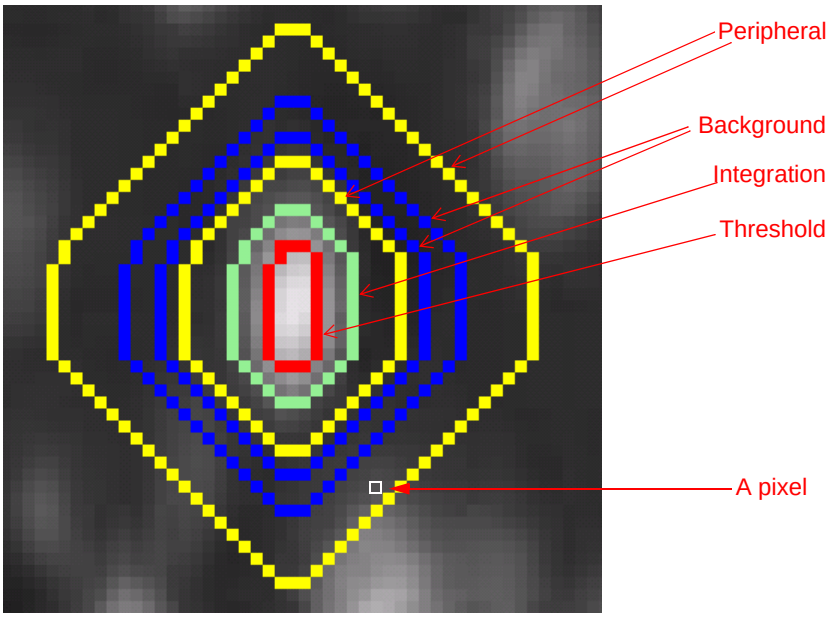
Contours

Contouring is the automated process of setting boundaries to segment and define individual events. The Channel, Threshold, and Contour sections of the Protocol ⇒ Contour menu and the Protocol ⇒ Event menu allow you to define contours. (see “Protocol” on page 3-30.) The features of these menus are summarized below.

- **Channel**—The first step in defining a Contour is choosing the Channel on which to base the definition. Select a Channel from the Protocol ⇒Contour ⇒ Channel menu. For more information on selecting a channel see “Channel” on page 3-52.
- **Threshold**—The Threshold and Channel determine what qualifies as an event. The Threshold defines the contiguous pixels whose values are equal to the calculated background for the event plus the threshold value for that channel. This contour defines the presence of the event at that location. Threshold settings are available in the Protocol ⇒ Threshold menu. Primary (Threshold) contour settings are found in the Protocol ⇒ Contour menu. For more information on setting the Threshold see “Threshold” on page 3-52.
- **Integration**—Defines the boundary within which the total fluorescence of the event will be calculated (integrated). Settings for the Integration contour are found in the Contour tab of the Protocol ⇒ Event menu. For additional information on setting the Integration contour, see “Defining the Integration Contour” on page 4-7
- **Background**—Defines two boundary lines that are used to calculate the background fluorescence for an event. Setting for the Background contour are found in the Background tab or the Protocol ⇒ Event menu. For information on defining the Background contour see “Generating Background Contours” on page 4-8
- **Peripheral**—Defines two boundary lines that are used to determine the periphery of an event. For additional information on Peripheral contours, see “Peripheral Contouring and Background Correction” on page 4-13
- **Phantom**—Defines contour lines based on non-event criteria. Phantom contours are described in “Phantom Contours” on page 4-11.

The figure below shows a magnified view of an event with the various contours displayed. Note the gradations of grayscale colors in the individual squares. Each of these squares is a pixel. By moving the cursor around in the Field Image window, you can read the individual values of each pixel in the status bar.

Figure 4-1: Contour Lines



Types of Contours

iCys allows you to generate a number of different types of contours, which were briefly described above. This section describes contours in greater detail.

Defining Contours

Threshold Contours

As described above, iCys considers the Threshold contour the primary contour. In iCys, the Integration, Background and Peripheral contours are all generated a specified number of pixels out from the Threshold contour.

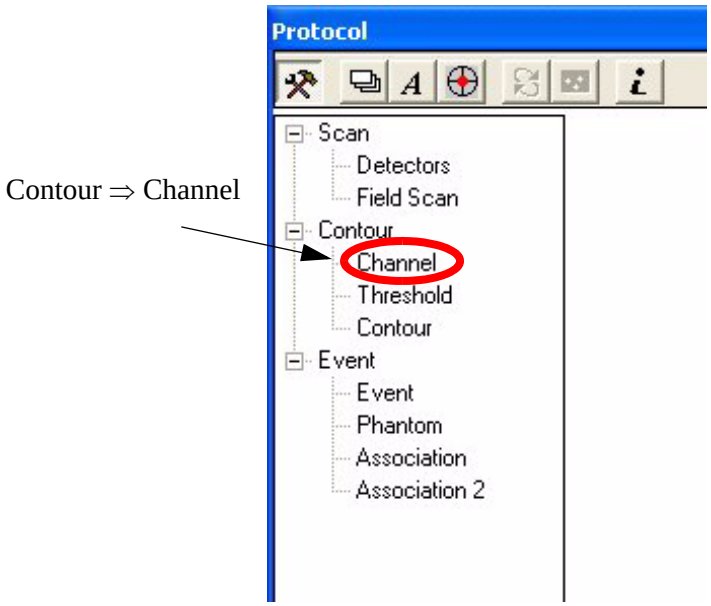
Generating the Threshold Contour

To set a primary contour, you need to assign a channel and then set the parameters for the contour.

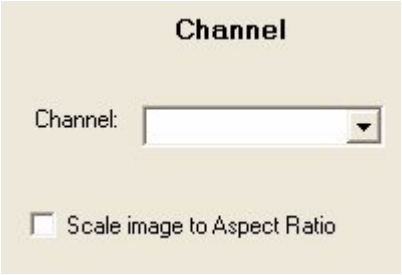
Assigning Contouring Channels

➢ To assign contour channels:

1. From the Protocol window, choose Contour ⇒ Channel and click in the field to the right of the Protocol menu to create a new Channel module, or click on an existing Channel module.



The following module settings will be displayed.



2. .Select the channel, real or virtual, that you want as the contour channel from the Channel pull-down menu.

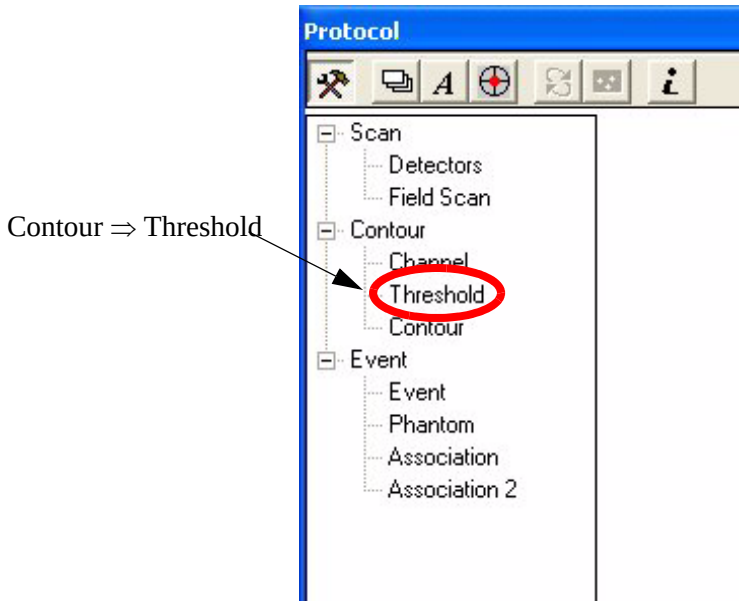
Setting the Threshold

The Threshold represents the minimum amount of pixel fluorescence required to generate a contour for an event.

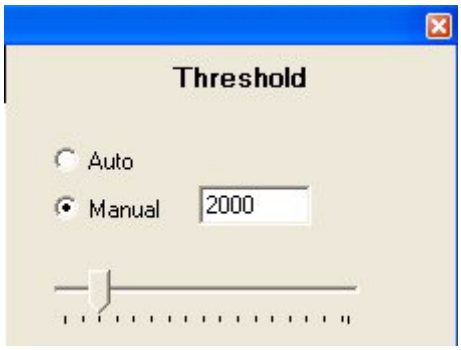
Settings for the Threshold contour are accessible from the Protocol ⇒ Threshold menu.

➢ To set the Threshold:

1. From the Protocol window, choose Contour ⇒ Threshold and click in the field to the right of the Protocol menu to create a new Threshold module, or click on an existing Threshold module.



The following module settings will appear.



When setting the threshold you can choose Auto, which will automatically set the threshold, or you can set it manually using a slider.

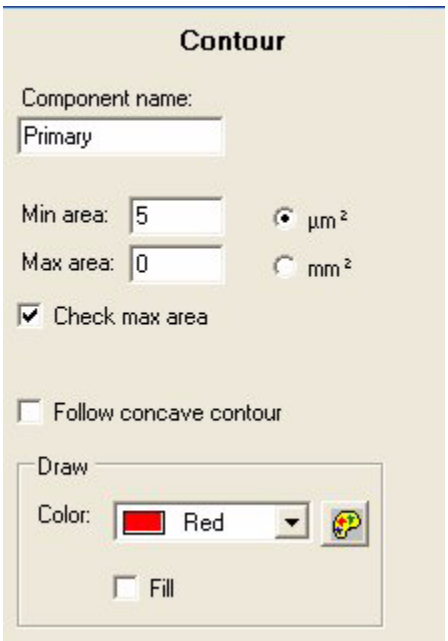
The threshold module will display either the word Auto or the numerical setting you have entered, as seen below.

To select an appropriate threshold value, open the Well image window, and move the cursor over the pixels at the edges of events. Read the pixel values located in the status bar, and use those numbers to estimate a threshold value.

Note: Any event that touches the border of the scan field cannot be contoured.

Additional Contour Parameters

Creating a new Contour module from the Protocol ⇒ Contour ⇒ Contour module, or clicking on an existing Contour module brings up the following module settings.



- **Component name**—Enter the name for the contour in this field.
- **Minimum Area**—The minimum area that an event must have to be considered for contouring. This can be specified either as μm^2 or mm^2.

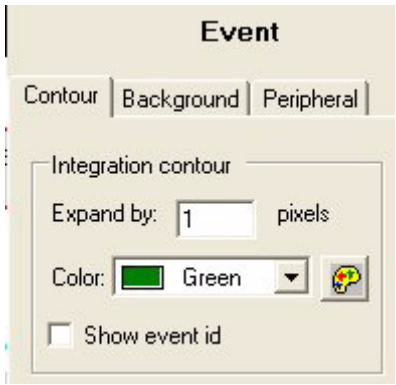
Note: To be contoured, all the pixels within the area must be brighter than the threshold level.

- **Check Maximum Area**—If this box is checked, the Max area field appears. Events with a greater area than that specified in this field will not be contoured. This feature is useful for microarrays where each core has a known size.
- **Follow concave contour**—Checking this box instructs the software to continue tracking along concave segments of a contour.
- **Color**—Select the color for the contour using either the pulldown menu or the color palette ( ).
- **Fill**—Check this box if you want the elements in the interior of the contour to be colored.

Integration Contours

An Integration contour defines the area within which fluorescence will be integrated for a given event. The integration contour is intended to capture more of the fluorescence associated with an event by adding adjacent fluorescence just below the threshold value.

An integration contour is specified in the Event module (Protocol ⇒ Event ⇒ Contour tab):

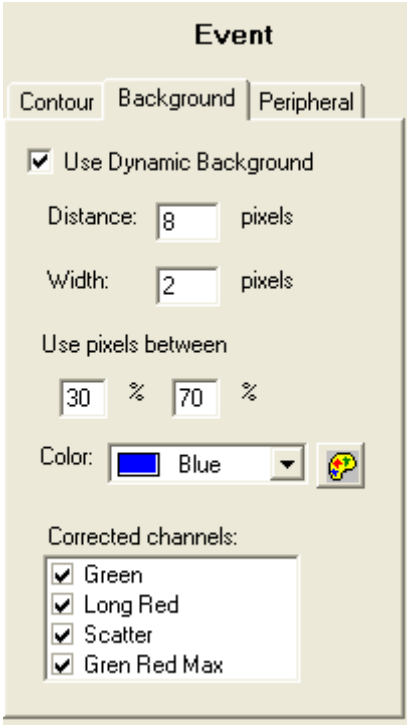


Defining the Integration Contour

- **Expand by**—Adds a specified number of pixels between the threshold contour and the integration contour. Keep in mind the following:
  - If Added Pixels is set to 0, the Integration contour overwrites the Threshold contour (that is, you only see the Integration contour line).
  - If Added Pixels is set to 1, the integration contour is adjacent to the threshold contour.

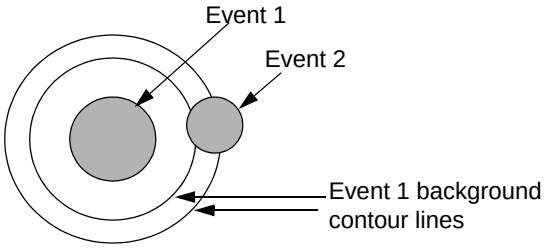
Background Contours

Background contours are available from the Protocol ⇒ Event ⇒ Background menu, shown below.



Background contour lines are used to capture an approximate value that represents the background fluorescence surrounding an event. The value of the background is calculated by taking the pixels between the **Use Pixels Between** boundaries, and determining a mean background value. This background value is then subtracted from each event pixel.

In the following example, the background annulus of Event1 overlaps Event2, which will skew the Event1 background higher. By setting **Use Pixels Between**, you can limit the effect of these kinds of non-background anomalies.



Generating Background Contours

Determine whether you want background contours.

➢ To add background contours:

1. Click the Background tab of the Protocol ⇒ Event window.
2. Check the Use Dynamic background box. Background contouring will be inactive unless this box is checked.
3. Set the following parameters:
   - **Distance**—The distance between the integration contour and the first background contour. Set the first background contour away from the integration contour such that it is away from the event fluorescence.
   - **Width**—The distance between the first and second background contour. Set the width such that the outer contour line includes the dimmest pixels adjacent to an event.
   - **Use Pixels Between**—When determining the background, you usually want to use those pixels that represent the mean pixel value, such as 30-70%. If, however, the background is very bright, you might want use a lower threshold, such as 20-50%.
   - **Color**—Select the color for the Background contour using the pulldown menu or the color palette.
4. Once the background value has been determined, you can use **Corrected channels** to subtract that value from each event pixel, thereby compensating the selected channels for the background noise.

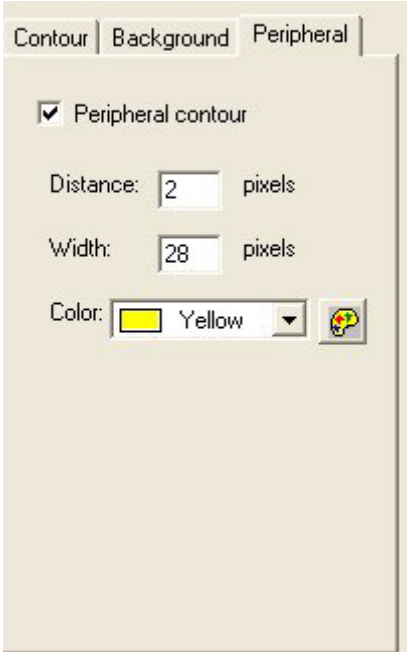
   When using dynamic background, subsequently added channels (such as virtual or merged channels) will be background corrected by default.

   For a discussion of when you might want to do this, see “Peripheral Contouring and Background Correction” on page 4-13.

Peripheral Contours

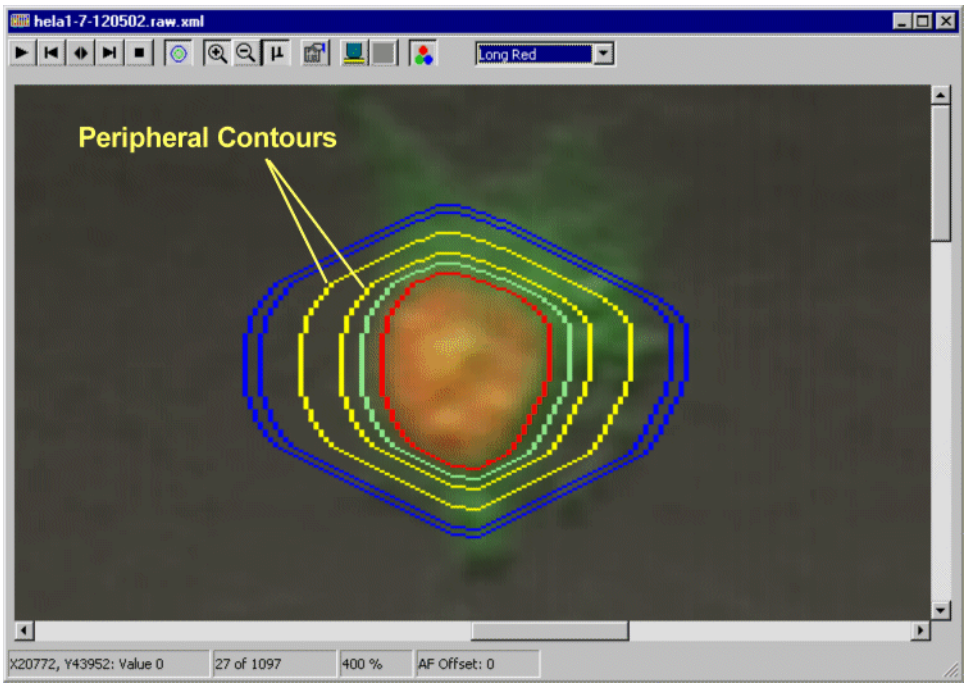
You can use additional contour functions to add more information to your data file. For example, Peripheral contours give you a measure of the fluorescence in a fixed annulus outside of the threshold contour, which is useful for sampling the cytoplasm when contouring nuclei with nucleic acid dyes.

Peripheral contour settings are available from the Peripheral tab of the Protocol ⇒ Event ⇒ Event menu, shown below.



➢ To add peripheral contours:

1. Check the Peripheral contour box. Peripheral contouring will be inactive unless this box is checked.
2. In the **Distance** field, enter the number of pixels between the threshold contour and the inner annulus of the Peripheral contour.
3. Enter the width in pixels of the Peripheral contour in the **Width** field.
4. Select a color for the Peripheral contour.



When you use Peripheral contouring (yellow in the above example), the integral value generated is expressed as the Peripheral integral feature. It is independent of the Integral contour, expressed as the Integral feature. MaxPixel data can also be measured in the Peripheral contour, and is expressed as the Peripheral Max feature.

Phantom Contours

Phantom contours are artificial events that are generated by the iCys software from circular contours that are applied over the laser-scanned image. These phantom contour events have the following features:

- Integral
- MaxPixel
- X Position
- Y Position
- Area
- Time
- Scan

Phantom contours may be laid down within a Field Image in a random fashion or in a lattice (two-dimensional array.) They are typically used in applications where you want to assess and quantify the fluorescence expression of the image, independent of the actual events.

Generating Phantom Contours

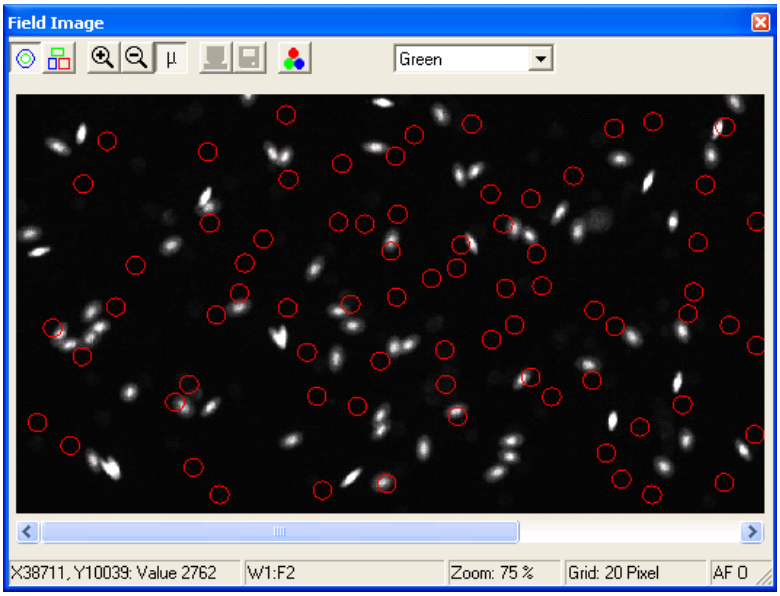
Creating a new Phantom module from the Protocol ⇒ Event ⇒ Phantom menu, or clicking on an existing Phantom module displays the following menu.

➢ To generate Phantoms:

1. Enter a name for the Phantom Component.
2. Select a color for the Phantom.
3. Select Random or Lattice to define the desired pattern of placement:
   - ❍ **Lattice**—Creates an evenly spaced field of Phantom contours
   - ❍ **Random**—Generates a random pattern of contours.
4. Enter the Radius in microns for the Phantom contours.
5. Enter the minimum distance between centers for the Phantom contours.
6. Enter the count per field. The Count per field parameter specifies the maximum number of phantoms per scan field.

Displaying Phantom Contours in Field or Well Images

To view phantoms in any of the Field or Well Image select ( ) The phantoms in the figure below are randomly placed.



Using Contouring

Following are some examples of how contouring can be used.

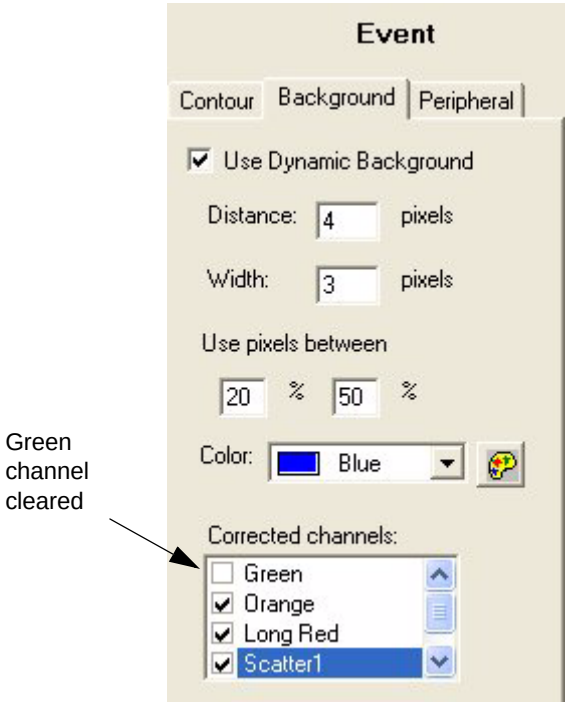
Comparing the Size of the Nucleus to the Amount of Cytoplasm

You can set up contours to compare the size of the nucleus to the amount of cytoplasm. The key here is that by generating the contours, you can determine the integral areas for both the nucleus and the cytoplasm, as follows:

- Threshold contour isolates individual cells around the nuclear stained area.
- Integral contour begins a specified distance from the threshold and integrates fluorescence in the nucleus.
- Peripheral contours begin farther out from the threshold contour, and need not extend to the end of the cytoplasm. Pixel values within the peripheral contours are integrated, providing a measure of cytoplasmic fluorescence.
- Background contours begin just outside the peripheral contours for background sampling.

Peripheral Contouring and Background Correction

When using peripheral contours, you usually do not want to subtract background from the detector that measures the cytoplasmic stain of interest. For example, consider a specimen where the primary contour is set to segment events based on the Blue detector and the cytoplasmic staining is measured in the Green detector. To prevent the background value, which is determined for each detector by the pixels in the event's background contours, from subtracting too much from your peripheral integral, deselect the Green detector in the Corrected Channels list box on the Background tab of the Event module.



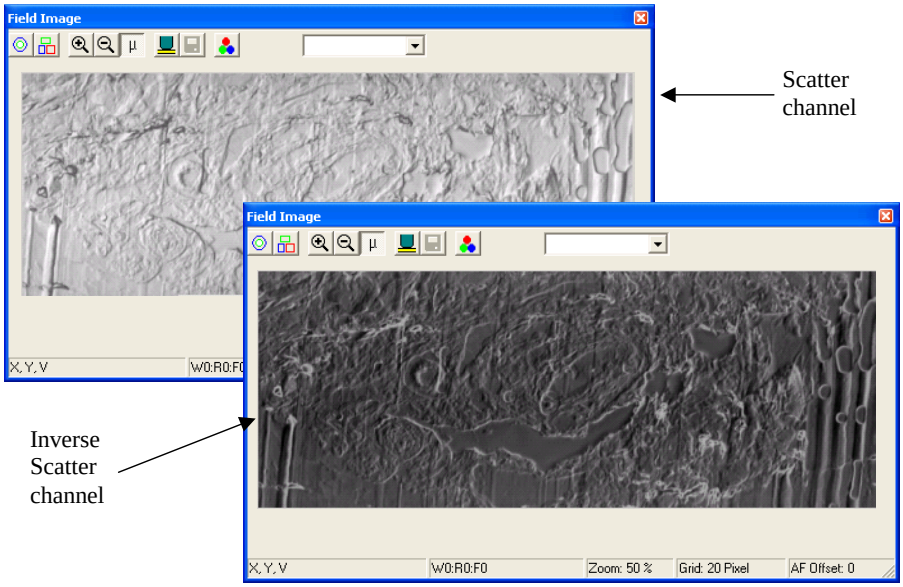
Using Inverted Scatter for Contouring

Using the Inverted Scatter channel for contouring allows you to generate precise contours on events with little or no fluorescence. When you use the Light Loss scatter position, events appear dark on a bright background (light loss due to absorbance and refraction is measured). Therefore, using Inverted Scatter generates bright events with a dark background. With appropriate settings, the bright events can be contoured.

➢ To use Scatter for contouring:

1. In the Protocol ⇒ Detectors window choose the Virtual Channel tab.
2. Create a virtual channel using Inverse Scatter. Give the channel a meaningful name (such as ScatterInvert). For details on creating a virtual channel, see “Creating Virtual Channels” on page 3-43.
3. In the Scatter tab of the Detectors window, select Light Loss as the Scatter position.
4. In the Protocol ⇒ Detectors ⇒ Virtual Channel tab, check the name, e.g. ScatterInvert, of the new virtual channel.
5. Create a new channel module using the inverse scatter virtual channel. For additional information on channel modules see “Channel” on page 3-52.

When you run iCys, the contoured events will appear bright on a dark background, as shown:


Field Image
Scatter channel
Field Image
Inverse Scatter channel
X, Y, V
W0:R0:F0
Zoom: 50 %
Grid: 20 Pixel
AF Offset: 0

Displaying Run Statistics

➢ To open the Run Statistics Window, click View ⇒ Run Statistics

The Run Statistics Window displays the statistics that are defined for each component:

Run Statistics

Well | Primary

#	Well	Primary Count	Primary Count R1	Primary Count R2	Primary Count R3	Primary Integral Long Red Mean R1
1	A1	257	12	0	0	5,904,857
2	A2	361	14	1	0	5,985,822
3	A3	327	7	0	0	5,564,663
4	A4	293	8	0	0	6,129,214
5	A5	546	57	0	0	5,906,051
6	A6	912	213	0	0	5,727,108
7	A7	725	168	0	0	5,812,676
8	A8	1,159	317	1	0	5,716,103
9	A9	748	207	0	0	5,864,259
10	A10	1,015	238	0	0	5,845,347
11	A11	887	237	0	0	5,712,835
12	A12	858	210	0	0	5,823,786
13	B1	288	12	0	0	6,239,678
14	B2	382	10	0	0	6,015,150
15	B3	397	10	0	0	6,187,858
16	B4	363	13	0	0	6,007,000

Defining Run Statistics

For information how to define the statistics see “Statistics” on page 3-60. You can also open the Setup Statistics window by double-clicking on the window. Changes to the Statistics Setup become effective immediately and you can view the updated values for all available events.

Tabs

A tab will be available for each component for which statistics have been set up. In this example, the Well tab shows a row for each well, and the Primary tab shows a row for each Primary event.

Columns

There are typically three sets of columns:

- ❑ **Row number (#)**—A sequential number for the row. Clicking on the # returns the rows to the original sort order.
- ❑ **Row identification**—(Carrier, Room, Well, <Parent Component Name>, <Component Name>) At the minimum there will be a <Component Name> column (such as Well or Primary), to identify the item on the row. Additional columns such as Carrier, Room, and/or <Parent Component Name> will be displayed if needed.
- ❑ **Statistics**—one column for each defined run statistic.

Sorting the Data

The data in the Statistics window can be listed in either ascending or descending order by any column. To change the order of the data, click on the column name on which you want to sort. Click once to change the order of the data to descending; click a second time to reverse the order. To return to the original sort order, click on the # column.

Exporting the Statistics

Right-clicking on the statistics window brings up a context-menu from which you can export the tabular data to third party applications such as Excel for further analysis:

- **Copy**—copies all rows of the current tab to the clipboard from where they can be pasted into Excel for example.
- **Save As…**—allows saving the values tab-delimited to a text file.

Displaying Region Statistics

➢ To open the Region Statistics window, click **View** ⇒ **Region Statistics**.

The **Region Statistics** window displays detailed statistics about each scattergram, histogram and region in your Workspace, including statistics for the events in each region:

Region Statistics

Region	Label	Param	Mean	CV %	STD	FWHM %
S1 (4892:100%)		X Position	38958.97	1.48	576.64	2.16
		Y Position	10981.05	5.40	592.93	7.89
S2 (4807:98%)		Green Integral	1742627.22	121.08	2109970.76	16.08
		Long Red Integral	1960439.44	62.14	1218280.41	12.38
R1 (2261:46%)		Green Integral	111474.77	49.64	55335.90	16.48
		Long Red Integral	1517324.30	10.54	159898.79	10.50
R2 (1065:21%)		Green Integral	2975854.66	8.92	265538.45	8.00
		Long Red Integral	1071677.73	13.96	149609.48	5.56
R3 (162:3%)		Green Integral	5926816.40	6.72	398383.92	4.02
		Long Red Integral	2154944.81	9.26	199571.97	6.76
R4 (264:5%)		Green Integral	220894.16	42.17	93156.13	16.80
		Long Red Integral	3040285.59	10.13	307862.26	7.06
R5 (347:7%)		Green Integral	3063551.22	7.48	229037.11	7.45
		Long Red Integral	2560149.58	7.09	181422.27	5.18
R6 (87:1%)		Green Integral	5993238.86	5.93	355341.52	3.94
		Long Red Integral	3572569.43	5.50	196329.30	3.83
S3 (4825:98%)		Green Integral (Sub1)	1679765.26	131.18	2203483.26	8.71
		Long Red Integral (Sub1)	795279.91	132.99	1057624.21	8.83

Tip: If no regions have been set, you can still invoke region statistics to see statistics on total events in each graph.

The Region Statistics window displays the following information:

Table 4-1: Region Statistics Window Statistics

Statistic	Description
Region	The id of this scattergram (S1, S2, for example), histogram (H3, H4), or region (R1, R2) along with: ❑ The number of data points in the defined area. ❑ The percentage of data points currently displayed in the graph as compared to the entire population of events. For a scattergram or histogram, the percentage should be 100% (assuming that both axes are normalized and the graph is not gated from another graph).
Label	If you labeled this region, as described in “Labeling Regions” on page 4-34, the name appears here.

Table 4-1: Region Statistics Window Statistics

Statistic	Description
Param	The parameter(s) used in this region, as described in "Scattergram and Histogram Feature Parameters" on page 4-24. ❑ For scattergrams, and regions within scattergrams, there are two parameters, one for each axis. ❑ For histograms, and regions within histograms, there is one parameter, reflecting only the X axis.
Mean	The mean of the events in this region.
CV%	The coefficient of variation for events in this region. The CV% is Standard Deviation/Mean.
STD	The standard deviation for events in this region.
FWHM%	The measurement of the full width at half maximum for each parameter.

For more information about scattergrams, see "Creating and Changing Scattergrams" on page 4-22.

For more information about histograms, see "Creating and Changing Histograms" on page 4-38.

Defining and Creating Galleries

Galleries are a series of images centered on events in a specified region. Galleries may be generated in one of two ways:

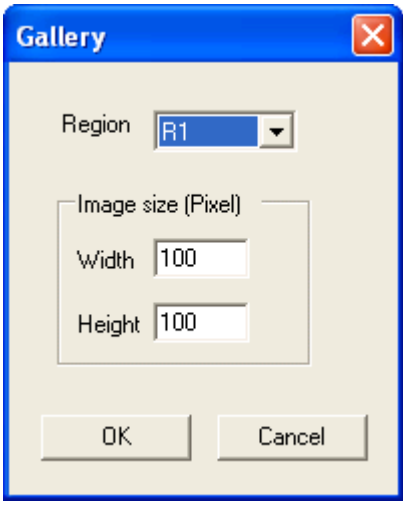
- ❑ Select **View** ⇒ **New Gallery** from the iCys menu bar to create gallery images that are automatically saved. This method is described in “Creating a Gallery from the View Menu” on page 4-20.
- ❑ By clicking on **Create Gallery** from the View Cells window as described in “Viewing Cell Details” on page 4-35. This method is described in “Creating A Gallery From the View Cells Window” on page 4-20.

Creating a Gallery from the View Menu

Galleries created from the View menu can be viewed during a scan. As each scan area is scanned, the gallery updates to show the first 25 events in the region.

➢ To create a new gallery from the View menu:

1. Select **View** ⇒ **New Gallery** from the iCys menu bar.The Gallery dialog displays:



2. Select the region for which you want to define the events to be included in the gallery. When the gallery appears, you can choose which channel you wish to see by selecting the appropriate tab at the top of the gallery.

Note: To display a gallery you must specify a region that defines the events to be included. If you have not created any regions in your Workspace the **New Gallery** selection is grayed out.

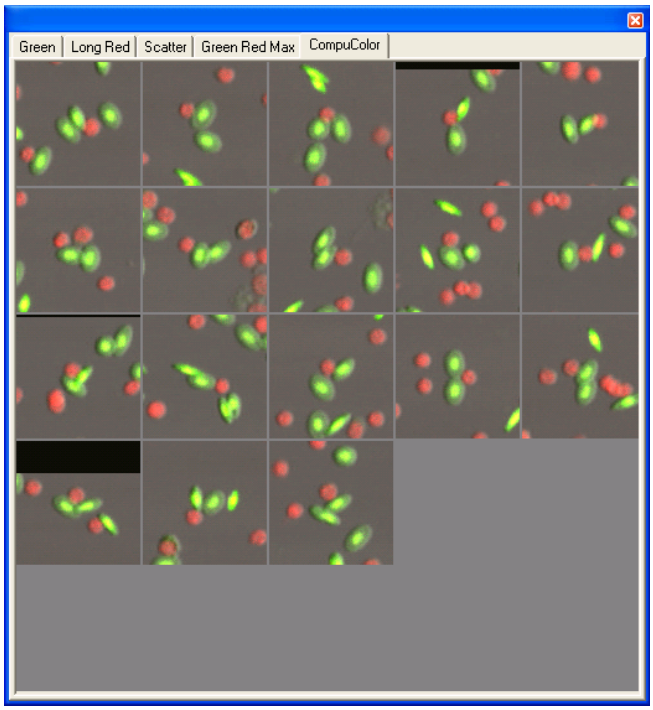
Creating A Gallery From the View Cells Window

Galleries created from the View Cells window have the following properties:

- These gallery images are not automatically saved. To save a gallery image, right-click on the image. When you click on Save Gallery Image, the Save As window displays. Gallery images can only be saved in JPG format.
- By clicking on the tabs at the top of the gallery, you can display the events for all active channels.

➢ To create a gallery from the View Cells window, click the Create Gallery button.

The images displayed in the gallery begin with the selected event (or the first event, if no events are selected). Each window in the gallery displays the next event in the list until all the windows are filled or iCys reaches the end of the events list. For example:



Copying a Gallery

You can copy a picture of a gallery to any application that accepts data from the Windows clipboard.

➢ To copy a gallery:

1. Right-click on the gallery.
2. Select Copy from the pop-up menu.
3. Open any application that accepts data from the clipboard (such as WordPad, Word, or Excel), and use the Paste function from that application to paste the graphic into the application.

Creating and Changing Scattergrams

This section contains the following topics:

- About Scattergrams, page 4-22
- Using the Graph Property Window, page 4-23
- Setting Scattergram Regions, page 4-30
- Setting Region Attributes, page 4-31
- Viewing Cell Details, page 4-35
- Other Scattergram Information, page 4-37

About Scattergrams

Scattergrams are two parameter graphs that allow you to view and save a wide variety of data during an iCys scan. Each spot on a scattergram represents an event that corresponds to the attributes you have selected.

Within an iCys scattergram, you can create one or more regions. A region is a subset, or area of interest, within the scattergram from which you can:

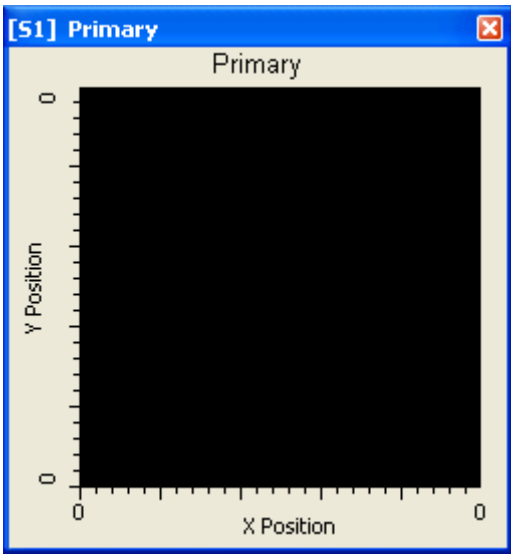
- Create relationships between scattergrams and histograms so that the data in one graph is derived from events that are within regions in other graphs. This is called "gating," and is described in greater detail in "Scattergram and Histogram Event Type Tab" on page 4-26.
- Generate statistical analyses of the events in regions. For more information, see "Viewing Cell Details" on page 4-35 and "Displaying Region Statistics" on page 4-18.
- Add color to regions to allow you to easily identify or compare subpopulations of events that appear in different analysis graphs. For more information, see "Changing Region Color" on page 4-32.

You can make changes to the existing scattergrams or add more scattergrams.

➢ To create a new scattergram, select **View** ⇒ **New Scattergram** from the menu bar or right-click on the Workspace and select **New Scattergram**.

Note: Scattergram and Histogram windows are numbered consecutively. Scattergrams have an S prefix, histograms have an H prefix.

A new scattergram is added to your Workspace.



Using the Graph Property Window

➢ To adjust the scattergram, right-click on the scattergram to open the **Graph Property** window. The **Graph Property** window contains three tabs:

- ❑ Axis Tab
- ❑ Event Type Tab
- ❑ General Tab

This section discusses the tabs in the **Graph Property** window.

Scattergram and Histogram Axis Tab

The Axis tab for both the scattergram and histogram windows lets you choose what data to display and how to display it. The only difference between the Axis tabs for these graphs is that the Y-axis for histograms is always set to Count and cannot be changed.

For more information about histograms, see “Creating and Changing Histograms” on page 4-38.

You can set the following parameters on the **Axis** tab:

- ❑ **Axis for Scattergram**—Select the axis (X or Y) to which you want to make changes.
- ❑ **Ticks—**Specify a number that determines the number of tick-marks displayed on this axis.

Note: When the parameter is X or Y position, iCys flips the axes (so the 0,0 is in the upper right-hand corner. This causes an event’s position on the scattergram to parallel its position on the carrier.

- **Grid—**If selected, overlays a grid on the display for the axis you have selected. The number of grid lines is based on the number of tickmarks selected in the **Ticks** parameter.
- **Label**—Specify a label for this axis. The name is displayed along the axis you selected. If **Use default** is checked, the axis will be labeled according to its parameter.
- **Feature—**Select the measured parameter for each axis. The features that are available depend on the type of event. If the feature is not appropriate for the event (for example, Parent ID for a Primary Event), it will not appear in the Feature pulldown.

Tip: For features designated with a (Sub *), a selection will display in the Features pulldown for each subcontoured channel. For example, if you have both Green and Orange subcontours, you will see the following:

Area (Sub Green)
Area (Sub Orange)

Depending on the event type, the available features are:

Table 4-2: Scattergram and Histogram Feature Parameters

Feature	Meaning
Area	Area in square microns of each event.
Area (Sub *)	For primary events if there are subcontours—The total area, in square microns, of all sub events associated with the primary event.
Array XPos	For tissue arrays, the x position in the array
Array YPos	For tissue arrays, the y position in the array
Background	When using Dynamic Background, the background fluorescence or signal nearby each event.
Circularity	How close the event is to a perfect circle. The circularity is determined by: $\text{perimeter}^2/\text{area}$ Therefore, the circularity of a perfect circle is 4π (12.566). The larger the circularity number, the less circular it is. Note that, due to normalization, the circularity of an event may be less than 12.566.
Count (Sub *)	For primary events if there are subcontours—A count of the number of sub events associated with the primary event.

Table 4-2: Scattergram and Histogram Feature Parameters (Continued)

Feature	Meaning
Cycle	For repetitive scans, the cycle number within the series of scans.
Integral	Integrated fluorescence per event for each saved channel.
Integral (Sub *)	For primary events if there are subcontours—The Integrated fluorescence per sub event for each saved channel.
MaxPixel	Brightest pixel value per event for each saved channel.
Parent ID	Subcontours only—The WellID of the primary event to which this subcontour is related.
Perimeter	The number of microns that comprise the perimeter of the event.
Peripheral Integral	Peripheral contours only —The integral of the fluorescence in the area defined by the peripheral contour.
Peripheral Max	Peripheral contours only —The MaxPixel in the area defined by the peripheral contour.
Scan	Pixel number, from 0 to 768, indicating the y-position of the scanning mirror where the event was detected.
Time	Time, in seconds, at which event was acquired. When rescanning a Raw file, the Time parameter contains the Well Number of this scan area.
Well #	Well number (or scan area number) of event.
X position	X position in the carrier for each event.
Y position	Y position in the carrier for each event.
Z position	Z position in the carrier for each event.

Tip: You can combine parameters by choosing a numerator as well as a denominator parameter. The resulting parameter will reflect the value of the numerator divided by that of the denominator.

- **Channel** —Assign the channel to display for features that are channel-specific.
- **Log scale**—If checked, displays this parameter in a base 10 logarithmic scale (that is 0= 10^0, 1= 10^1, and so on). This can be useful if the data for this parameter has a very broad range. For example, integral data may display better in log scale.

- **Normalize when applied**—If checked, automatically scales the axis for this parameter so that all datapoints are included. The axis will be normalized only when OK or Apply is pressed, not continuously as the data changes.
- **Range**—Allows you to specify the range of the value displayed in the graph by entering specific values.The Range fields default to settings appropriate for the parameter chosen for the axis.

Scattergram and Histogram Event Type Tab

This tab allows you to specify the events that will trigger a spot on a scattergram or histogram.You can set the following parameters on the **Event Type** tab:

- **Component**—Select a component defined in the protocol. Events of this component will be displayed.
- **Gating**—Specify whether you want to include all events in this graph or only those that conform to a specific region, or "gate" on another graph. You can combine two regions into a gate by selecting the first region, and combining it with a second region using the Boolean operators AND, OR, or NOT.

The **Gate type** field indicates the type of gate specified in the first (left-hand) field:

- **Normal**—The graph from which you are gating (that is, the current graph, which can be either Primary or Sub) is of the same event type as the graph in which the region(s) lie.

  For normal gating, make sure that you only gate between events contoured using the same component. If you create gates between different components, the results are undefined.
- **ParentToChild**—The graph on which you are gating (that is, the current graph) is a Sub event graph and the region lies within a Primary event graph.
- **ChildToParent**—The graph on which you are gating (that is, the current graph) is a Primary event graph and the region lies within a Sub event graph.

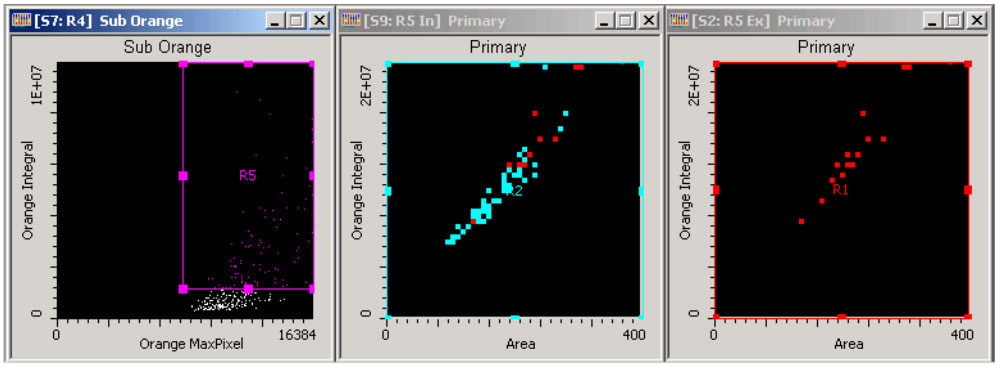
- **Child to Parent Mode**—When the Gate type is ChildToParent, as described above, you may have a situation as follows;

  You can select one of the following options:

  - **Inclusive**—Include those events that have any sub events in the region.
  - **Exclusive**—Include those events that contain only the selected sub events in the region.

  In the following example:

- The first scattergram to the far left is gated to another scattergram (not shown) such that it only displays events that contain two orange subevents.
- The second scattergram is inclusive and shows all events that within the region that contain two subevents. Note that the exclusive events are also shown in this scattergram (in red).
- The third scattergram is exclusive and shows only those events for which the two subevents are relatively large (that is, for which the integral is high).



Child to Parent Gate | Inclusive Gating | Exclusive Gating

Tip: The Inclusive and Exclusive scattergrams are shown in low resolution, as described in “Scattergram General Tab” on page 4-27.

For more information about creating regions for scattergrams, see “Setting Scattergram Regions” on page 4-30.

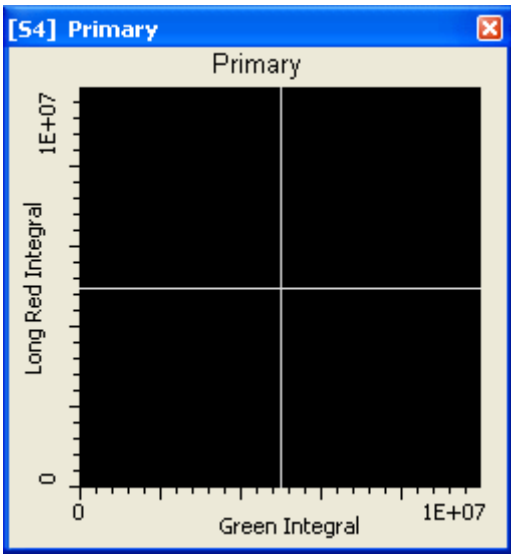
For more information about creating regions for histograms, see “Setting Histogram Regions” on page 4-41.

Scattergram General Tab

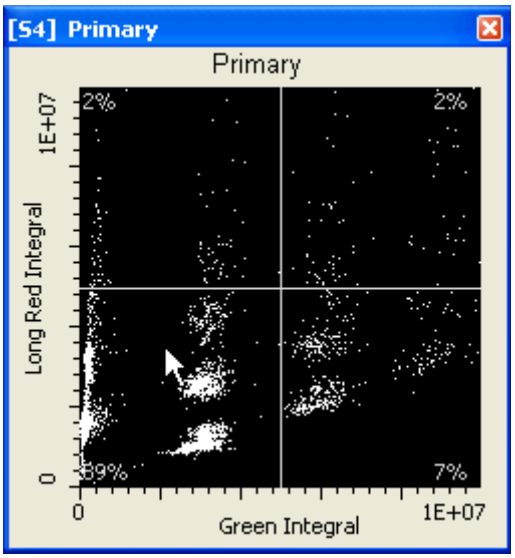
The General tab allows you to set a number of miscellaneous parameters. The general parameters for scattergrams and histograms are different; for more information about the histogram parameters, see “Histogram General Tab” on page 4-39.

The parameters available from this tab are:

- **Title**—Enter a title for this graph. The title appears at the top of the graph.
- **Display (Global)**—Specify whether you want to display scattergram and histogram data on a white or a black background. Note that this setting affects all scattergrams and histograms in this Workspace, not just the current graph.
- **Resolution**—Specify the display resolution. You can select either high or low resolution.
- **Quadrant statistics**—Displays a quadrant on the graph after you click Apply or OK. For example:



After creation, the quadrants can be dragged to any position. When data is scanned, the percentage of data in each quadrant will appear in the graph.



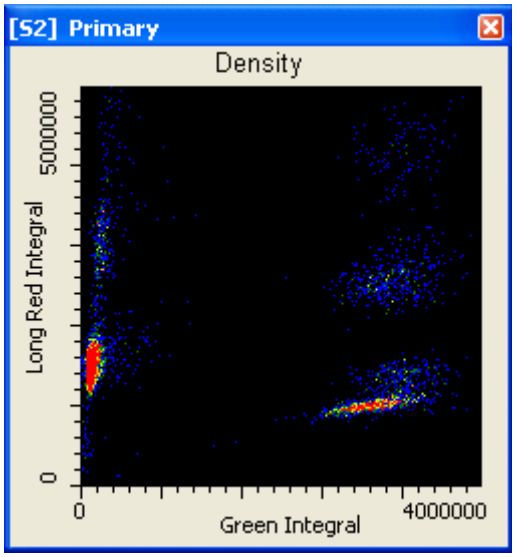
- **2-Parameter Histogram**—2-parameter histograms, provide a method displaying a third (Z) parameter in the scattergram using color to represent the Z axis. If you select 2-Parameter Histogram, you can choose one of the following options:
  - **Density**—Color codes the data according to how many events have been assigned to a given pixel.
  - **Expression**—Color codes the data according to the value of the expression you choose in the accompanying pulldown menus.

  2-parameter histograms are described in greater detail in “About 2-Parameter Histograms,” below.

About 2-Parameter Histograms

2-parameter histograms display aggregated data based on two parameters. iCys uses a color scale to show the value of a particular bin.

A 2-parameter histogram displays in iCys, as shown below.



iCys generates a 2-parameter histogram file for every scan area of every scattergram for which expression or density histograms have been requested. 2-parameter histogram files can also be viewed from iBrowser.

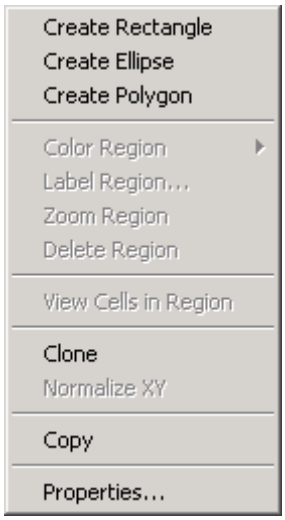
➢ To generate 2-parameter histogram, check 2-Parameter Histogram. You can display your data in color in one of two ways, **Density** or **Expression:**

- ❑ **Density**—Color codes the data according to how many events have been assigned to a given pixel. The more events accumulated, the higher the color of that pixel will be on the scale. This allows you to visually assess the distribution of the data.
- ❑ **Expression**—Color codes the data according to the value of the expression you choose in the accompanying pulldown menus. You may choose, for example, Integral of Green fluorescence as an expression on a graph of scatter vs. long red fluorescence. Although the graph does not display green fluorescence as a parameter, those events with the most green fluorescence will show as a higher color on the scale.

For more information about 2-parameter histograms in iBrowser, see the *iBrowser Data Integration Software User's Guide*.

Setting Scattergram Regions

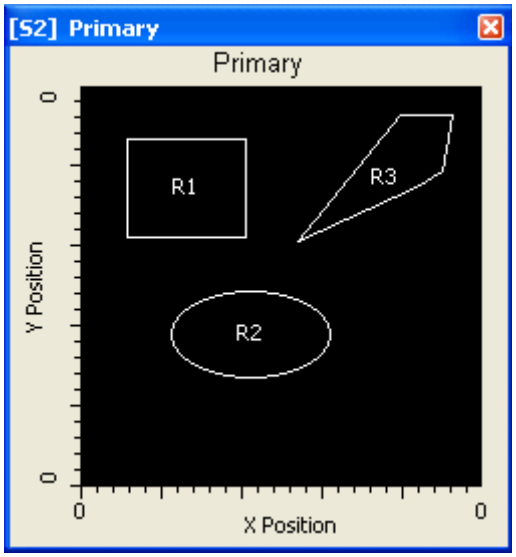
➢ To create a scattergram region, right click in any part of a scattergram to display the Scattergram menu. Note that several of the options are grayed out at this point.



Select the type of region you want to create**:**

- **Create Rectangle**—Allows you to define a rectangular scan area using the mouse. The mouse pointer changes from an arrow to a cross hair to indicate this mode. Note that you cannot change direction when creating the rectangle. That is, if you begin at the top left corner of the rectangle and move the cursor down and to the right, you can not then move up and to the left past the initial vertex.
- **Create Polygon**—Allows you to define a polygonal scan area using the mouse. The mouse pointer changes from an arrow to a cross hair to indicate this mode. Click to create the first vertex. Create additional vertices by moving the cursor to the next position (no need to hold the mouse button down) and left clicking. Finish the polygon with a right mouse click.
- **Create Ellipse**—Allows you to define a circular or elliptical scan area using the mouse. The mouse pointer changes from an arrow to a cross hair to indicate this mode. Note that there is a similar constraint on reversing direction as described above in Rectangle.

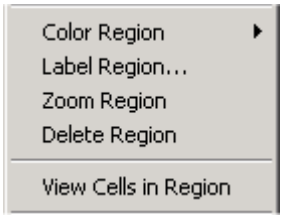
  The figure below illustrates the three different region types: Rectangle (R1), Polygon (R2), and Ellipse (R3).



Setting Region Attributes

Once you have created a region, you can set a number of attributes, including the size, color, and name.

➢ To activate an existing region, click anywhere inside the region. Then, when you right-click, many of the previously grayed-out selections on the scattergram menu are activated:



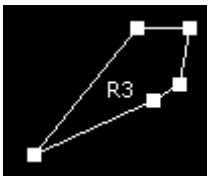
This section discusses the region attributes.

Adjusting Region Size

To adjust the size and position of an existing region, click anywhere inside a region to activate it:

- Click and drag anywhere inside an activated region to reposition it in the graph.
- Use the handles of an activated region to resize and reshape it. Drag on the handles in the corners to resize the entire region. Drag the handles between the corners to stretch the region vertically or horizontally.

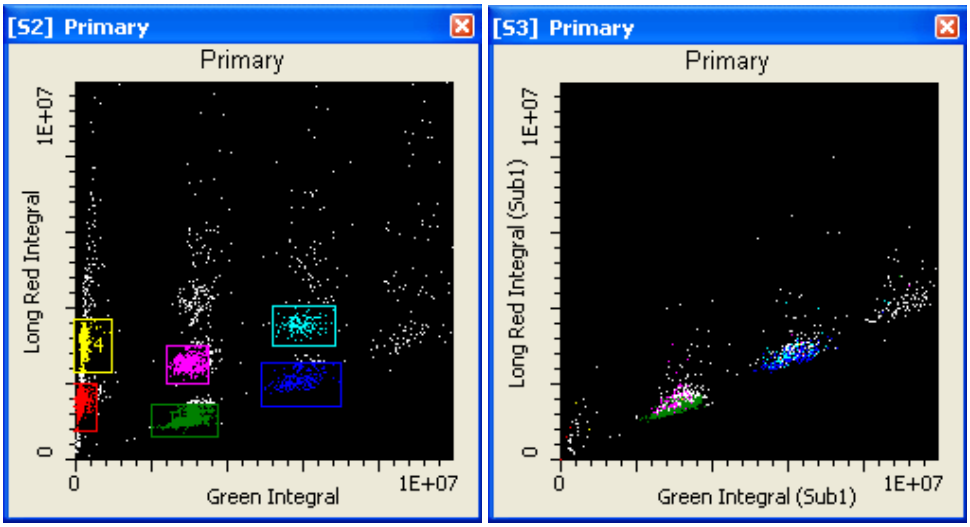
The figure below is an activated polygonal region. You can reposition any of the vertices by dragging the appropriate handle.



Once you have created a region, you can set attributes for it.

Changing Region Color

Assigning color to a region allows you to identify the same population in several graphs.



➢ To set the color for a region:

1. Select the region for which you want to set the color.
2. Right-click to display the Region Attributes menu, and click on Color Region.
3. Select the color you want for that region.
4. Click on the color. The color will change and be reflected in all graphs.

About Using Colors

The colors in the Color Region menu are shown in hierarchical order, from red to cyan.

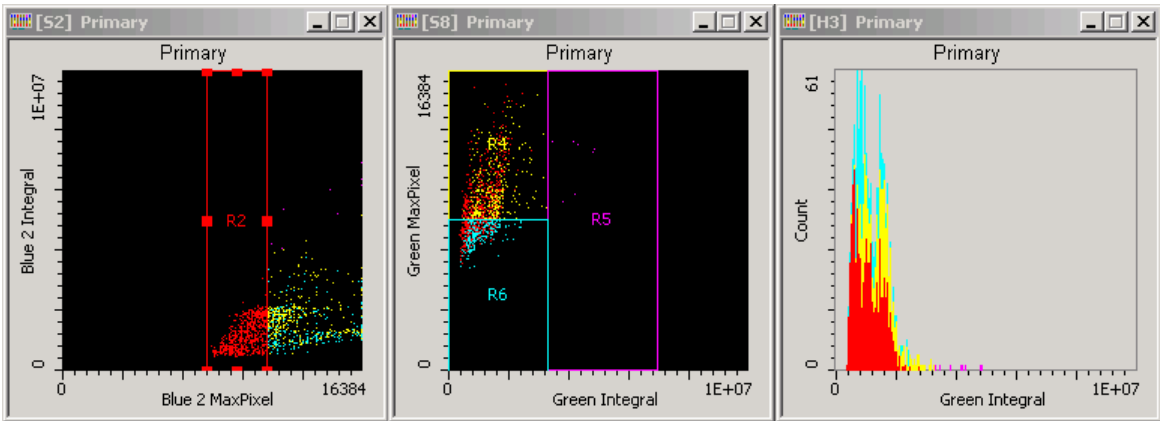
For example, let's say you create two scattergrams using different criteria: one with a red region and the second with a yellow region. Where the datapoints do not overlap, the red and yellow regions are colored accordingly. However, if any datapoint does overlap, it takes on the color of highest precedence; which in this case, is red.

In the following example, the following regions are shown:

- ❑ R2—Red
- ❑ R4—Yellow

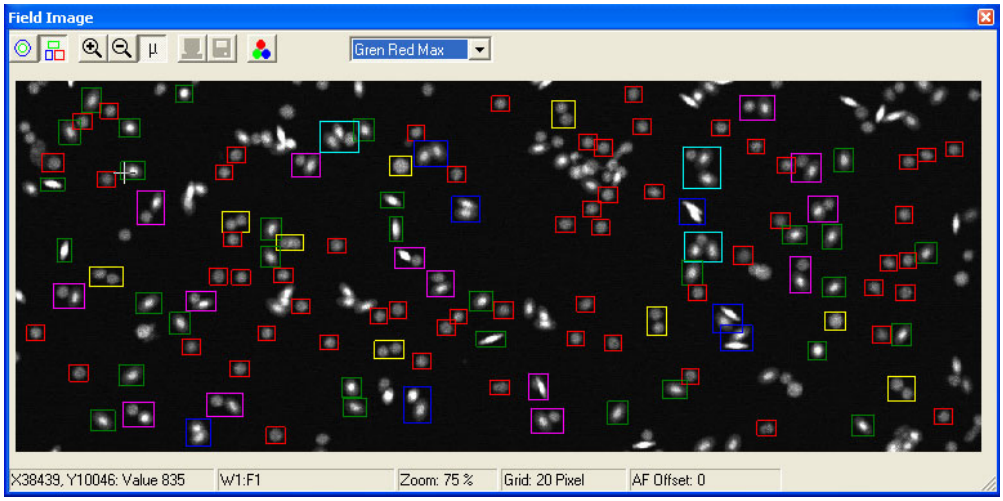
- R5—Magenta
- R6—Cyan

Figure 4-2: Region Color Example



Note the following:

- In the first scattergram (S2), the datapoints that do not fall within the red region take their colors from the second scattergram (S8).
- In the second scattergram, the datapoints that coincide with R2 in the first scattergram are also red.
- In addition to coloring the regions within the scattergrams, any color added to a region is reflected in the histograms, as shown in Figure 4-2. Conversely, colored histogram regions, as discussed in “Changing Region Color” on page 4-42, will be reflected in the scattergrams.
- You can use colored regions to highlight events within the Field Image window. For example, using the colored regions described above, and with the Highlight feature ( ) selected, the Field Image window shows the region in which each event falls:



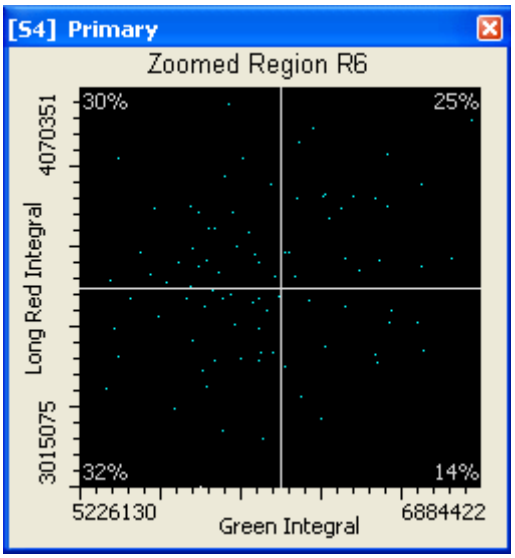
- If you do not add color to a scattergram region, the histogram bars default to black.

Labeling Regions

Select **Label Region** to add a name to the region. The label displays in the Region Statistics window, as described in “Displaying Region Statistics” on page 4-18.

Using the Zoom Function

If you select **Zoom Region**, iCys generates a new scattergram that contains just the area within the region.Wherever possible, the new scattergram uses the same parameters as the original scattergram. In the following example, the original scattergram had **Quadrant statistics** set (as described in “Scattergram General Tab” on page 4-27). The new scattergram, therefore, also has them:



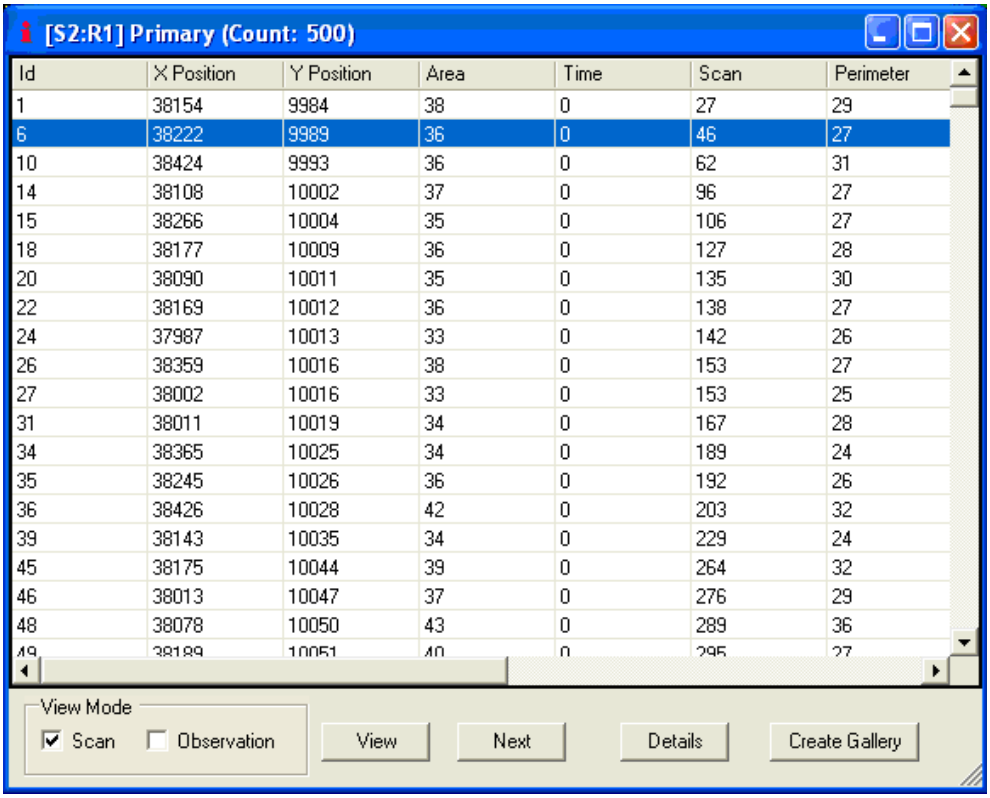
The zoom scattergram is a standard scattergram. You can create regions, zoom, and make any other changes to it.

Deleting a Region

To delete a region from a scattergram, click **Delete Region**.

Viewing Cell Details

To find detailed information about each event within the region, click **View Cells in Region**. The View Events window displays:



Id	X Position	Y Position	Area	Time	Scan	Perimeter
1	38154	9984	38	0	27	29
6	38222	9989	36	0	46	27
10	38424	9993	36	0	62	31
14	38108	10002	37	0	96	27
15	38266	10004	35	0	106	27
18	38177	10009	36	0	127	28
20	38090	10011	35	0	135	30
22	38169	10012	36	0	138	27
24	37987	10013	33	0	142	26
26	38359	10016	38	0	153	27
27	38002	10016	33	0	153	25
31	38011	10019	34	0	167	28
34	38365	10025	34	0	189	24
35	38245	10026	36	0	192	26
36	38426	10028	42	0	203	32
39	38143	10035	34	0	229	24
45	38175	10044	39	0	264	32
46	38013	10047	37	0	276	29
48	38078	10050	43	0	289	36
49	38189	10051	40	0	295	27

From this window you can:

- ❑ View information about a single event.
- ❑ Locate and display an event in the Field Image View window.
- ❑ Locate and display another event in the Field Image window.

The View Events window is described in this section. Locating events is described in detail in “Locating and Viewing Events in the Field Image Window” on page 4-45.

Title Bar Information

- ❑ Region number (in this case Region 1).
- ❑ Type of event (in this case Primary; the type of count depends on the scattergram Event Type—If the Event Type is Sub contour, the type of count will be the Sub *contourname*, where *contourname* is a subcountour specified as described in “Generating the Threshold Contour” on page 4-3).
- ❑ The count of events (in this case, 500).

Other Actions

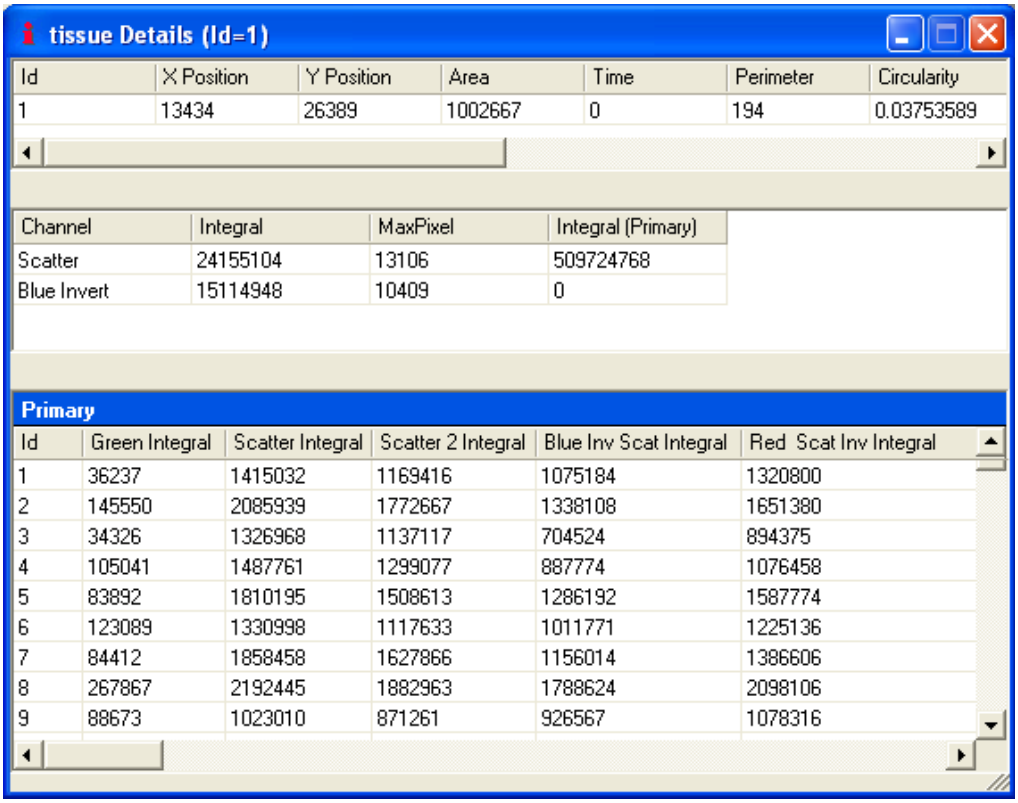
The View Events window offers the following options:

- ❑ **View Mode**—Allows you to determine whether you are viewing events in the Field Image window in Scan mode, Observation mode or both. For more details

about modes for locating events, see “Locating and Viewing Events in the Field Image Window” on page 4-45.

- ❍ **Scan—**When you move to events in Scan mode, the scan area may be rescanned and the event is displayed in the Field Image window.
- ❍ **Observation**—When you move to events in Observation mode, the scan area may be rescanned, and you can use the eyepieces to view the event, but the event will not display in the Field Image window. If you are using the camera, the event will be displayed in the camera monitor. See “Locating and Viewing Events in the Field Image Window” on page 4-45 for more information.

- ❑ **View—**Opens the Field Image window (if it is not already open) and draws a red box around the selected event. For detailed information about locating events, see “Locating and Viewing Events in the Field Image Window” on page 4-45.
- ❑ **Next**—Moves to the next event in the window and locates that event in the Field Image window.
- ❑ **Details**—Opens the **Event** window, which displays detailed information about a single event:



Id	X Position	Y Position	Area	Time	Perimeter	Circularity
1	13434	26389	1002667	0	194	0.03753589

Channel	Integral	MaxPixel	Integral (Primary)
Scatter	24155104	13106	509724768
Blue Invert	15114948	10409	0

Id	Green Integral	Scatter Integral	Scatter 2 Integral	Blue Inv Scat Integral	Red Scat Inv Integral
1	36237	1415032	1169416	1075184	1320800
2	145550	2085939	1772667	1338108	1651380
3	34326	1326968	1137117	704524	894375
4	105041	1487761	1299077	887774	1076458
5	83892	1810195	1508613	1286192	1587774
6	123089	1330998	1117633	1011771	1225136
7	84412	1858458	1627866	1156014	1386606
8	267867	2192445	1882963	1788624	2098106
9	88673	1023010	871261	926567	1078316

The details of the Event window are described in “Event Window Details” below.

- ❑ **Create Gallery**—Creates a gallery of event images. For detailed information, see “Displaying Run Statistics” on page 4-16.

Event Window Details

The event window is divided into two or three sections, as described below:

- The first section contains event features.
- The second section contains channel-specific event features:
- If you have generated any sub events, a third section displays a list of child events and their features for each sub component.

Other Scattergram Information

In addition to creating and manipulating regions, use the Scattergram menu to:

- **Clone**—If you display the Scattergram menu when you do not have the cursor within a region, iCys will create a scattergram that is an exact copy of the selected scattergram.
- **Clone For R*x***—If you display the Scattergram menu when you **do** have the cursor within a region, iCys will create a scattergram that contains only the data from the selected region and is gated to the selected region, as discussed in "Scattergram and Histogram Event Type Tab" on page 4-26.
- **Normalize XY**—For XY graphs only—Tracks the XY coordinates for each active well. During a scan, if checked, causes iCys to normalize to the scanning well.
- **Copy**—Creates a snapshot image of the selected scattergram (in bitmap format) and saves it to the clipboard. You can then paste the image to another application.

Creating and Changing Histograms

This section contains the following topics:

- ❑ Using the Graph Property Window, page 4-39
- ❑ Setting Histogram Regions, page 4-41
- ❑ Setting Region Attributes, page 4-41
- ❑ Viewing Cell Details, page 4-44
- ❑ Other Histogram Information, page 4-44

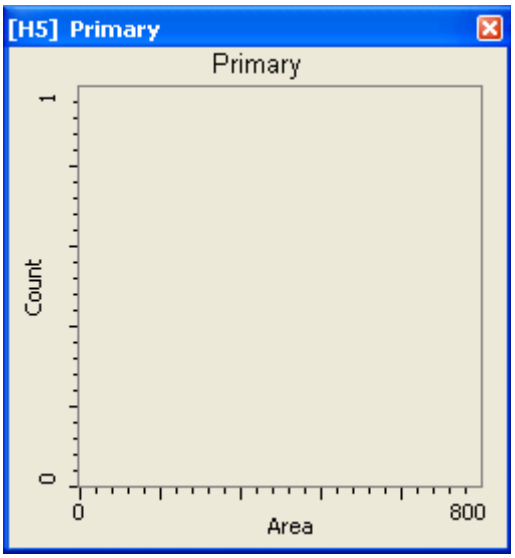
The default Workspace contains threes histograms. Histograms are graphs that allow you to view and save a wide variety of data during an iCys scan. Each line on a histogram represents a set of events that correspond to the attributes you have selected.

You can make changes to the existing histograms or add more histograms.

➢ To create a new histogram, select **View** ⇒ **New Histogram** from the menu bar or right-click on the Workspace and select **New Histogram**.

Note: Scattergram and Histogram windows are numbered consecutively. Scattergrams have an S prefix, histograms have an H prefix.

A new histogram is added to your Workspace.



Using the Graph Property Window

➢ To adjust the histogram, right-click on the histogram to open the **Graph Property** window. The **Graph Property** window contains three tabs:

- ❑ Axis Tab
- ❑ Event Type Tab
- ❑ General Tab

This section discusses the tabs in the **Graph Property** window.

Histogram Axis Tab

The Histogram Axis Tab allows to you define the X-Axis.The Histogram Axis Tab is exactly like the Scattergram Axis Tab with the exception that the Y-Axis is always Count and cannot be changed. For information about setting the Histogram Axis Tab, see “Scattergram and Histogram Axis Tab” on page 4-23.

Histogram Event Type Tab

This tab allows you to specify the events that will trigger a spot on the histogram.

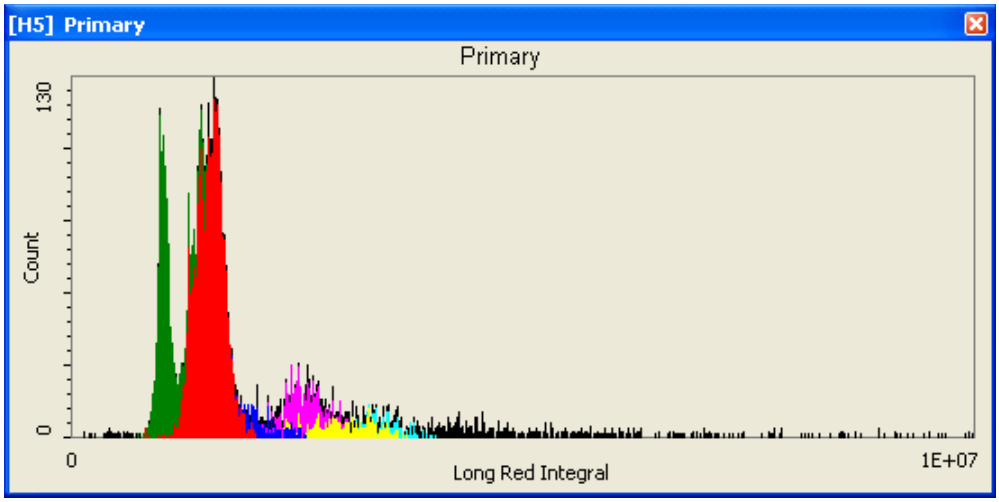
The Histogram Event Type Tab is exactly like the Scattergram Event Type Tab. For information about setting the Histogram Event Type Tab, see “Scattergram and Histogram Event Type Tab” on page 4-26.

Histogram General Tab

The General tab allows you to set a number of miscellaneous parameters. The parameters available from this tab are:

- ❑ **Title**—Enter a title for this graph. The title appears at the top of the graph.
- ❑ **# of channels displayed**—Specify the resolution of the X-axis. You can specify either 200 or 500 channels. A “channel,” in this instance, is the number of divisions along the X-axis. The default is 200.

  When you specify 500, iCys displays the histogram in wide mode, as follows:

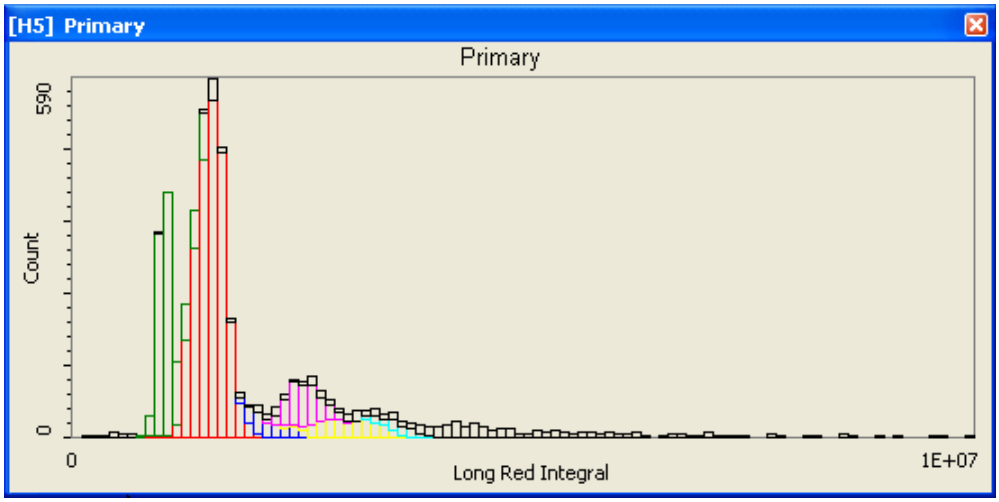


- ❑ **Bin count**—Specify the width of each datapoint in the bar graph. By default, the number of bins is equal to the number of channels, and each datapoint is one pixel

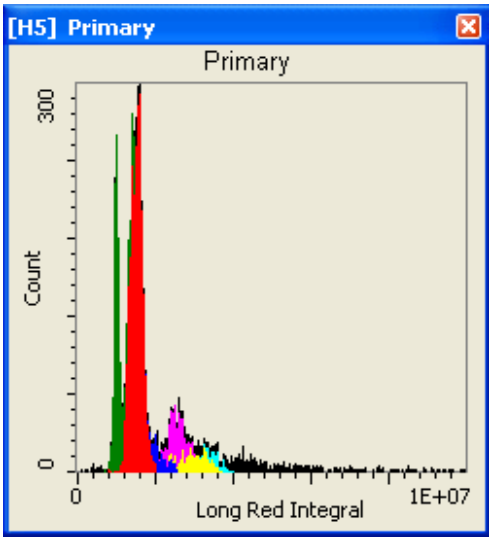
wide. When you change the number of bins, the bar width of each datapoint reflects the number of bins that will fit in each channel (that is, when both channel number and bin count are 200, the bar width is 1 pixel wide [200/200]).

To change the number of bins, select a new bin count from the Bin count pulldown menu. For example, in a 500 channel histogram with a bin count of 100, the bar width for each datapoint is 5 pixels (500/100=5).

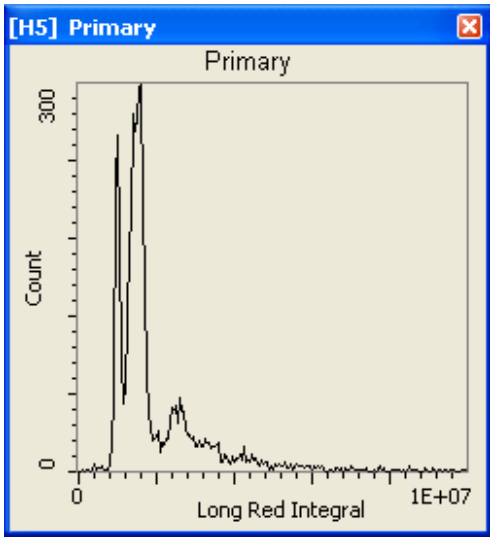
The histogram appears as follows:



- **Plot Style**—Specify whether you want the histogram to show as a bar graph or as an outline graph. For example:



Bar Graph



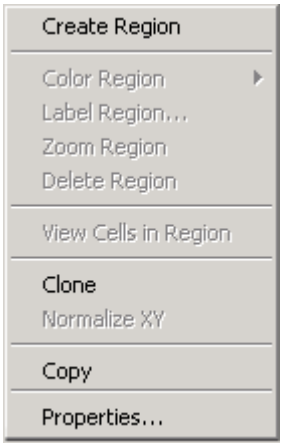
Outline Graph

- **Auto Y Scale**—If selected, Auto Y Scale sets the top of the Y scale to the maximum number of counts per channel registered for this histogram during the scan.

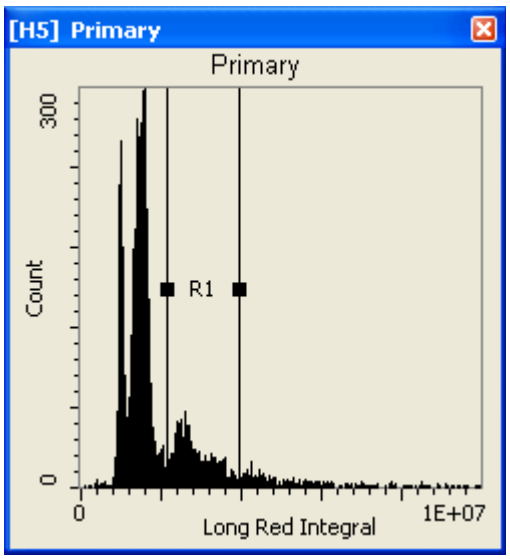
Setting Histogram Regions

➢ To create a histogram region:

1. Right click in any part of a histogram to display the Histogram menu. Note that several of the options are grayed out at this point.

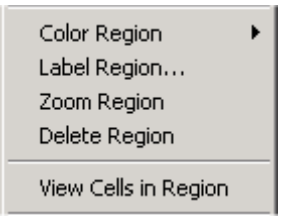


2. Select Create Region and place your cursor in the histogram. Left-click and hold your mouse while pulling the cursor to the left. As you pull, the region is created. For histograms, the region always encompasses a section of the X axis, as shown:



Setting Region Attributes

Once you have created a region, you can set a number of attributes, including the width, color, and name. To activate an existing region, click anywhere inside the region. Then, when you right-click, many of the previously grayed-out selections on the histogram menu are activated:



This section discusses the region attributes.

Adjusting Region Size

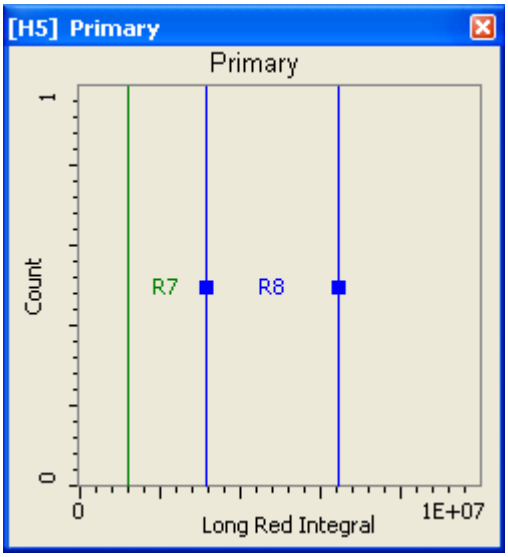
➢ To adjust the size and position of an existing region, click anywhere inside a region to activate it:

- ❍ Click and drag anywhere inside an activated region to reposition it in the graph.
- ❍ Use the handles of an activated region to resize it. Drag on the handles to resize the entire region.

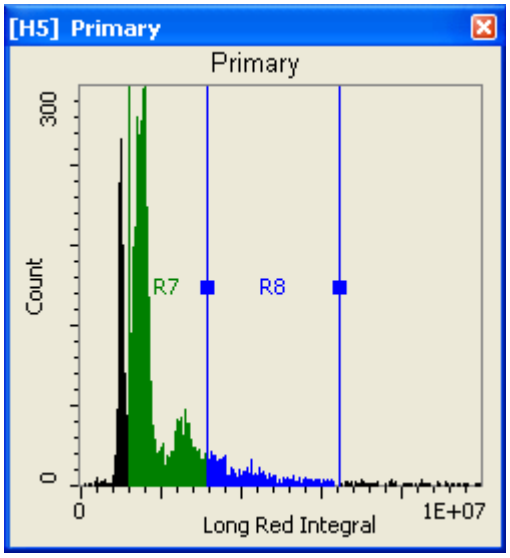
Once you have created a region, you can set attributes for it.

Changing Region Color

If you have multiple regions, you may want to add color to make it easier to differentiate. For example:



When you color the regions within the histograms, the color is reflected in the histograms. For example, the following histogram reflects data in Region 7 (green) and Region 8 (blue):



If you do not add color to a histogram region, the histogram bars default to black.

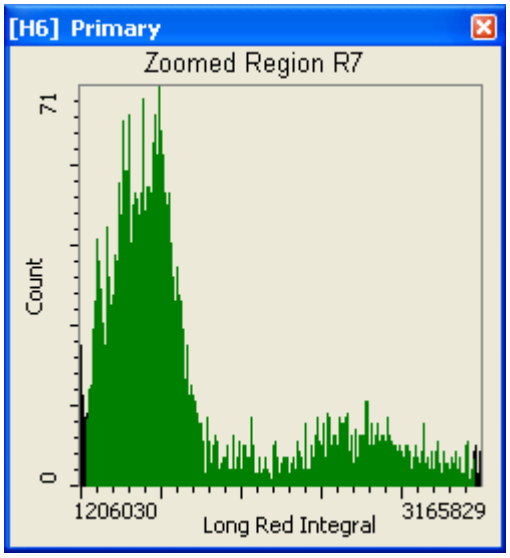
For more information about using colors within histograms and scattergrams, see “About Using Colors” on page 4-32.

Labeling Regions

Select **Label Region** to add a name to the region. The label displays in the Region Statistics window, as described in “Displaying Region Statistics” on page 4-18.

Using the Zoom Function

If you select **Zoom Region**, iCys generates a new histogram that contains just the area within the region.Wherever possible, the new histogram uses the same parameters as the original histogram:



The zoom histogram is a standard histogram. You can create regions, zoom, and make any other changes to it.

Deleting a Region

To delete a region from a histogram, click **Delete Region**.

Viewing Cell Details

As with scattergrams, click **View Cells in Region** to display the View Events window. For information about the View Events window, see "Viewing Cell Details" on page 4-35.

Locating events is described in detail in "Locating and Viewing Events in the Field Image Window" on page 4-45.

Other Histogram Information

In addition to creating and manipulating regions, you can use the Histogram menu to:

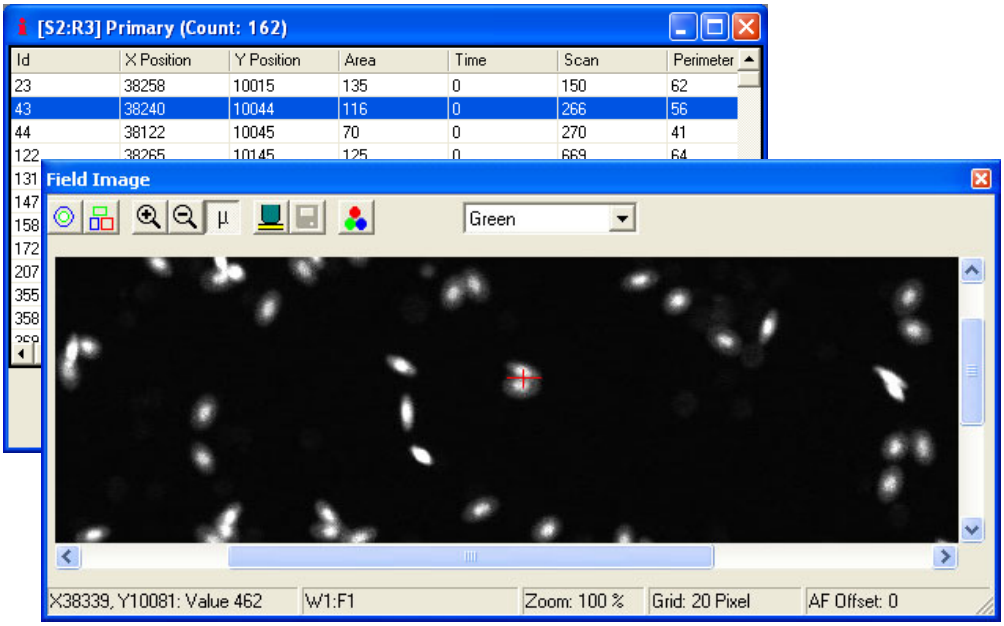
- **Clone**—If you display the Histogram menu when you do not have the cursor within a region, iCys will create a histogram that is an exact copy of the selected histogram.
- **Clone For R*x***—If you display the Histogram menu when you **do** have the cursor within a region, iCys will create a histogram that contains only the data from the selected region and is gated to the selected region, as discussed in "Scattergram and Histogram Event Type Tab" on page 4-26.
- **Normalize XY**—For XY graphs only—Tracks the X coordinates for each well.
- **Copy**—Creates a snapshot image of the selected histogram (in bitmap format) and saves it to the clipboard. You can then paste the image to another application.

Locating and Viewing Events in the Field Image Window

You can use the View Events window to locate and display events, by number, in the Field Image window or in an Observation Port (either the eyepieces or Color Camera Monitor) after data is acquired using any of the following scanning methods:

- ❑ Scan & Save
- ❑ Test Scan
- ❑ Play or Next in the Field Image window during a scan

When you locate an event in the View Events window, iCys highlights the event in the Field Image window by drawing a cross at its center.



When you select a new event from the View Events window, iCys moves the stage to the new event (if appropriate), and changes the display in the Field Image window.

There are a number of scenarios for locating and viewing events, depending on whether you are working with live data or existing run data. This section describes each scenario.

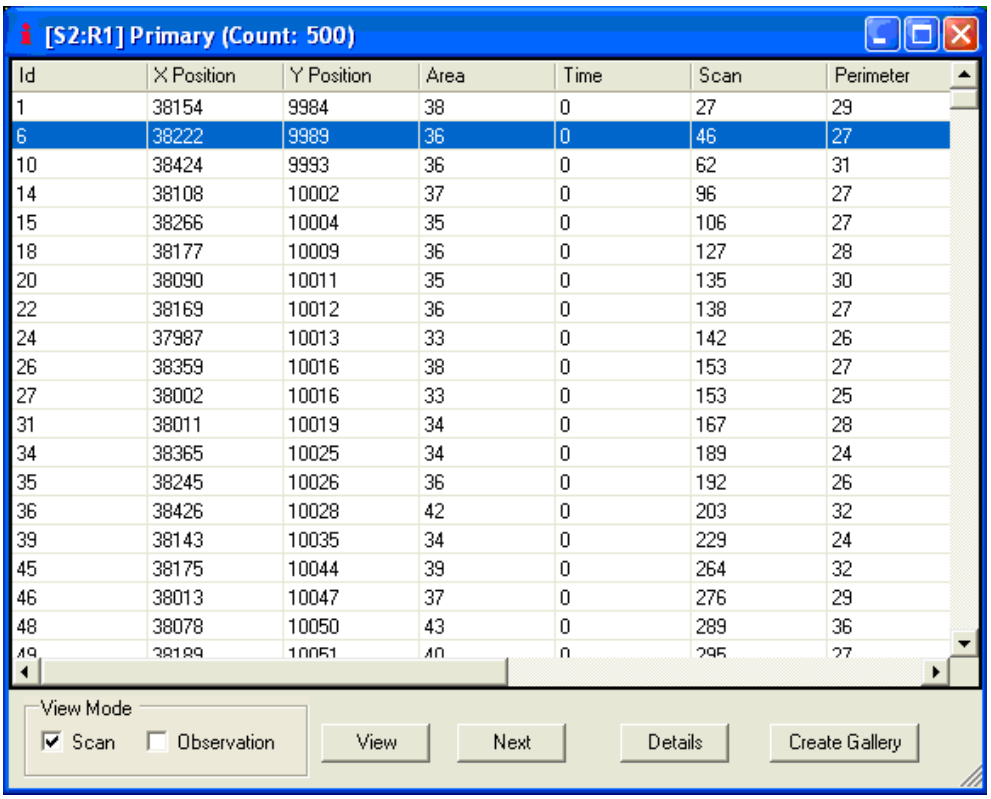
Locating and Viewing Events using Live Data

Immediately after a scan, but before you have saved your data into a Raw file, you can locate (and rescan) events using “Live Data,” that is, the samples in the carrier currently on the microscope stage. You can locate events using live data either immediately after iCys has acquired data during either a Test Scan or Scan & Save run or when you have the Event data available and the sample on the microscope stage.

When using live data, you can locate and view scan images of event directly from the data or from an Event file.

➢ To locate events immediately after a scan:

1. If you have a run open, iCys uses the event data of that run. If you do not have a run open, iCys uses the data that is currently stored in memory.
2. Use Select Active Well in the Carrier window, as described in Table 3-1, "Microplate Scan Area Setup Window," on page 3-18 or Table 3-2, "Freeform Scan Area Setup Window," on page 3-24, to determine which set(s) of data you want to activate.
3. Right-click on the region of interest and select View Cells in Region to open the View Events window and select the event you want to view, as described in "Viewing Cell Details" on page 4-35.



Id	X Position	Y Position	Area	Time	Scan	Perimeter
1	38154	9984	38	0	27	29
6	38222	9989	36	0	46	27
10	38424	9993	36	0	62	31
14	38108	10002	37	0	96	27
15	38266	10004	35	0	106	27
18	38177	10009	36	0	127	28
20	38090	10011	35	0	135	30
22	38169	10012	36	0	138	27
24	37987	10013	33	0	142	26
26	38359	10016	38	0	153	27
27	38002	10016	33	0	153	25
31	38011	10019	34	0	167	28
34	38365	10025	34	0	189	24
35	38245	10026	36	0	192	26
36	38426	10028	42	0	203	32
39	38143	10035	34	0	229	24
45	38175	10044	39	0	264	32
46	38013	10047	37	0	276	29
48	38078	10050	43	0	289	36
49	38189	10051	40	0	295	27

4. You can choose to view the selected event in the Field Image window, directly with the eyepieces, with the camera, or in any combination of the three.
   - ❍ To view the event in the Field Image window, select Scan from the View Mode box in the View Events window.
   - ❍ To view the event using the eyepieces, camera, or both, select Observation.
   - ❍ To view the event in the Field Image window and with the eyepieces, camera, or both, select both Scan and Observation.

Tip: If the View Mode is set to Scan, the microscope Light Path Selector lever must be in the Side Port position (), as described in "iCys Microscope Components" on page 2-13.

5. Click on the row of the event of interest (or default to the first event), then click View to acquire the image of the highlighted event. iCys locates the image as follows:
   - If Field Image is selected:
     - The stage moves to the start of the scan field that contains the event of interest.
     - iCys moves the microscope objective to the focus position used during the original scan.
     - The scan field is rescanned using the setup from the original scan (number of lasers, PMTs, and so on).
     - The event is displayed and highlighted by a red box in the Field Image window.
   - If both Scan and Observation are selected:
     - After the appropriate scan field is rescanned, the stage moves so the event of interests is in the middle of the field of view through the eyepieces or color camera monitor.
     - iCys moves the microscope objective to the focus position used during the original scan.
     - To view the event through the eyepieces or camera monitor, turn the Light Path Selector Lever to the Observation position ( ).
   - If Observation is selected, the event is not rescanned.The following steps occur:
     - The stage moves so the event of interests is in the middle of the field of view through the eyepieces or color camera monitor.
     - iCys moves the microscope objective to the focus position used during the original scan.

Note: When Observation is selected, iCys will turn the Brightfield on for viewing. Brightfield will be turned off when viewing an event with observation unchecked, or when closing the View Events window.

In Observation-only mode, the event is not displayed in iCys.

If Observation-only is selected, make sure the Light Path Selector lever is also in the Observation ( ) position.

Note: To locate an event for which the Time value (in the View Events window) is different from the previous event's value, and Scan is selected (with or without Observation), make sure the Light Path Selector lever is set to the Side Port position before selecting Next or View. This is required because the newly selected event is in a different scan area and will be rescanned.

If your system does not have AutoFocus, you will need to adjust the focus by the appropriate offsets for the current action (Scan and/or Observation).

Locating and Viewing Events using Saved Data

When you are locating events in runs that have already been saved, you can use the previously saved data.

1. Open a run, selecting “Analyze Events.”
2. When the run is open, use Select Active Well in the Carrier window, as described in Table 3-1, “Microplate Scan Area Setup Window,” on page 3-18 or Table 3-2, “Freeform Scan Area Setup Window,” on page 3-24, to determine which set(s) of data you want to activate.
3. Right-click on the region of interest and select View Cells in Region to open the View Events window and select the event you want to view, as described in “Viewing Cell Details” on page 4-35.
4. You can choose to view the selected event in the Field Image window, directly with the eyepieces, with the camera, or in any combination of the three.
   - To view the event in the Field Image window, select Scan from the View Mode box in the View Events window.
   - To view the event using eyepieces, camera, or both select Observation.
   - To view the event in the Field Image window and with the eyepieces, camera, or both, select both Scan and Observation.
5. Click on the row of the event of interest (or default to the first event), then click View to acquire the image of the highlighted event. iCys locates the image as follows:
   - If Field Image is selected, the .jpg image of field that contains the event of interest is displayed in the Field Image window. The live sample is not used (no stage or microscope objective movement required).
   - If both Scan and Observation are selected:
     - The stage moves so the event of interests is in the middle of the field of view through the eyepieces or color camera monitor.
     - If your system has AutoFocus, iCys moves the microscope objective to the focus position used during the original scan. If your system does not have AutoFocus, you will need to manually focus the objective on the event of interest.
     - To view the event through the eyepieces or camera monitor, turn the Light Path Selector Lever to the Observation position ( ).
   - If Observation is selected:

- The stage moves so the event of interests is in the middle of the field of view through the eyepieces or color camera monitor.
- If your system has AutoFocus, iCys moves the microscope objective to the focus position used during the original scan. If your system does not have AutoFocus, you will need to manually focus the objective on the event of interest.

Note: In Observation-only mode, the event is not displayed in iCys.

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Appendix *A*

Installing iCys™ Research Imaging Cytometer

iCys™ RESEARCH IMAGING CYTOMETER

General Installation Information

Installing iCys™ Research Imaging Cytometer requires the following:

- ❑ Sufficient space to accommodate the physical iCys equipment
- ❑ Proper electrical power configuration and receptacles
- ❑ Acceptable environmental conditions

Note: iCys needs a vibration-free bench top. (Shock mounting is not required.)

Unpacking the iCys System

The iCys should be unpacked and installed only by a representative from CompuCyte or an authorized agent. If the system is to be stored temporarily before installation, it should be in a secure, heated, and dry area.

Upon receipt of the iCys, examine the shipping materials for exterior damage or evidence of mishandling. If they appear damaged, notify the carrier and CompuCyte or an authorized agent and request an inspection from the carrier.

Following initial installation, a representative from CompuCyte Corporation or an authorized agent will instruct designated personnel on the basic operation and care of the iCys.

Physical Space Requirements

The following table specifies the iCys's space requirements:

Table A-1: iCys Physical Space Requirements

Analysis Unit	❑ Width 44 in. (112 cm) ❑ Depth 32 in. (82 cm) ❑ Height 31 in. (79 cm) To allow for servicing, the space above the table surface should be at least 44 inches or (112 cm) ❑ Weight 275 lbs. (125 kg.)
Computer	❑ Width 8 in. (21 cm) ❑ Depth 19 in. (49 cm) ❑ Height 17 in. (44 cm)
Monitor	❑ 20" Flat panel display monitor 20" (51cm) wide, 12" (31cm) depth, 20" (51cm) height ❑ Dual monitors are optional
Autofocus monitor (if installed)	❑ Width 9 in. (23 cm) ❑ Depth 10 in. (26 cm) ❑ Height 9 in. (23 cm)

Table A-1: iCys Physical Space Requirements (Continued)

Autofocus Control unit (if AutoFocus installed)	❑ Width 14 in. (36 cm) ❑ Depth 11 in. (27 cm) ❑ Height 4 in. (10 cm)
Laser cooling fan	❑ Width 9 in. (23 cm) ❑ Depth 9 in. (23 cm) ❑ Height 9 in. (23 cm) Laser cooling fan can be placed up to 6 ft. (1.85m) from the Analysis Unit. For isolation from vibration, do not place laser cooling fan on the same table as the Analysis Unit.)
Laser power supply	❑ Width 9 in. (23 cm) ❑ Depth 12 in. (31 cm) ❑ Height 6 in. (16 cm) Laser power supply can be placed up to 6 ft. (1.85m) from the Analysis Unit. For isolation from vibration, do not place laser power supply on the same table as the Analysis Unit.)
Color camera	❑ Width 2.5 in. (6 cm) ❑ Depth 2 in. (5 cm) ❑ Height 7 in. (18 cm)
Camera monitor	❑ Width 17 in. (43 cm) ❑ Depth 17 in. (42 cm) ❑ Height 21 in. (53 cm)
Brightfield power supply	❑ Width 3 in. (8 cm) ❑ Depth 8.5 in. (22 cm) ❑ Height 5 in. (13 cm)
EpiFluorescence power supply (if installed)	❑ Width 5 in. (13 cm) ❑ Depth 11.5 in (30 cm) ❑ Height 7 in. (18 cm)

Electrical Requirements

Specifications

The table below lists the power requirements for the iCys system.

Table A-2: iCys Power Requirements

Component	Voltage	Current	Frequency
Cytometer	120VAC or 230VAC	16 A 10 A	60 Hz 50 Hz

Table A-2: iCys Power Requirements (Continued)

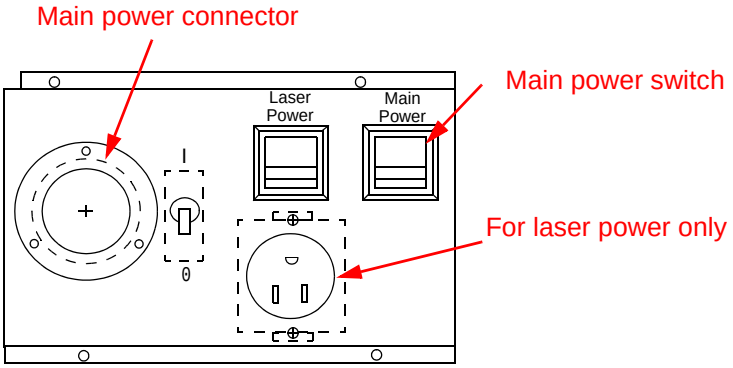
Computer	115/220 VAC	10/5 A	50/60 Hz
Flat screen monitor	110-120/220-240 VAC	0.7/0.3 A	50/60 Hz
Remote Control unit (if AutoFocus installed)	110/240 VAC	2 A	50/60 Hz
Autofocus monitor (if AutoFocus installed)	120 VAC or 230VAC	.3 A .2 A	50/60 Hz 50/60 Hz

Note: External protective conductor must be at least equal to supply wires in ampacity.

The system must be connected to a dedicated circuit with an adequate circuit breaker.

Input Power Controls

The main power switch for the iCys cytometer is located on the left side panel of the instrument. In the “I” position this switch applies AC input power to the instrument and provides AC power to the auxiliary outlets located on the left and right side of the instrument. The figure illustrates the iCys power entry panel



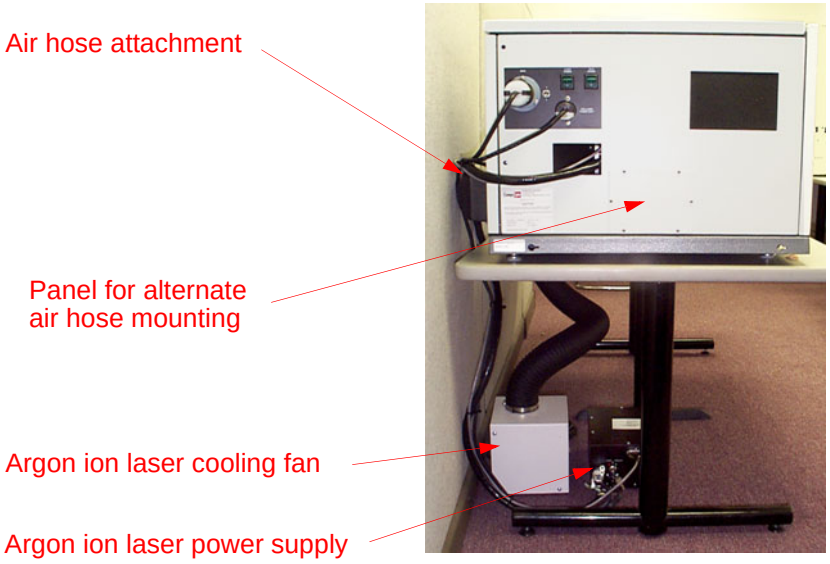
Power to the laser power supply, which is located external to the instrument, is activated through a keyed switch located on the laser power supply. Power for the computer and monitor is located on the front of the computer and monitor.

Figure A-1: iCys Selected Hardware Components

Optics/Electronics	Microscope, brightfield source, EpiFluorescence, camera, and automated stage	Camera and focus monitors, Remote Control unit	Monitor

Typical Installation Setups

Figure A-2: Installation Setup, Rear View



- ❑ Standard tower case computer may be placed on or beneath the table.
- ❑ Air hose attaches at either the back (shown in figure) or the left side of the instrument.
- ❑ If your system uses the optional AutoFocus feature, the AutoFocus monitor and controller may be installed on the table or within ~6 ft. (2m) of the cytometer.

Appendix *B*

Using the iNovator Toolkit

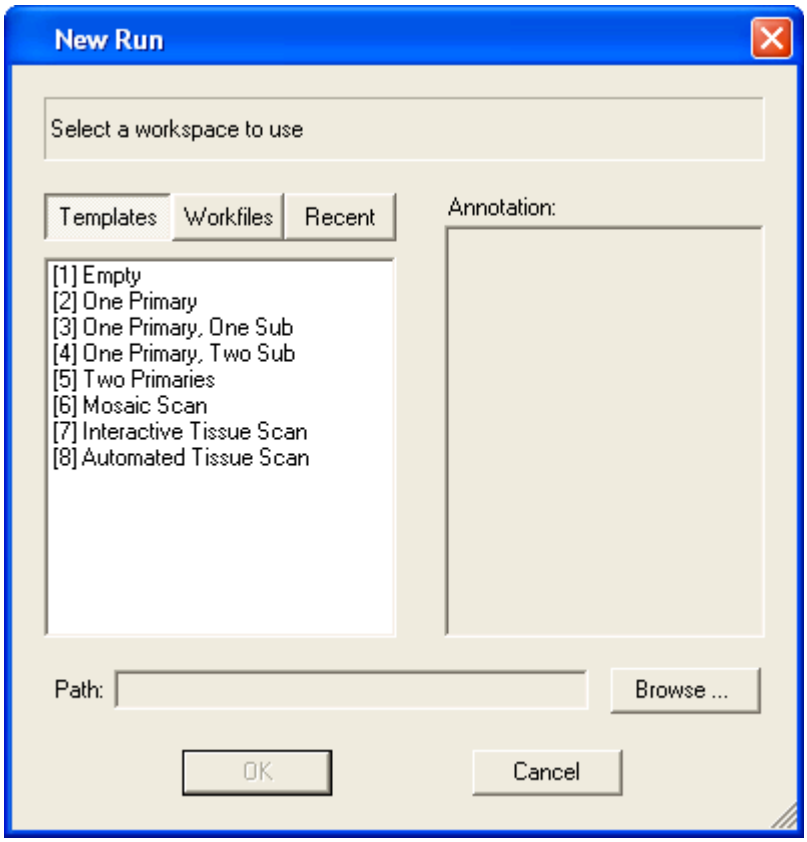
The iNovator Application Development Toolkit allows you to extend the capabilities of the basic iCys. The iNovator extends the module-based, graphical protocol development tools found in the basic iCys to allow the user considerable more flexibility and power. The iNovator provides additional modules to allow image filtering, Boolean and arithmetic operations on images, Mosaic scanning and multi-scale scanning.

Included in this chapter:

- iNovator-specific Templates, B-2
- References to Using the Protocol Menu Bar and to Building and Modifying Protocols, B-7
- iNovator-specific Protocol Modules, B-8
- Using the Protocol Menu View Image Feature with iNovator, B-28
- Multi-scale Scanning, B-30

iNovator-specific Templates

The iNovator provides additional templates not available in the basic iCys as shown in the New Run dialogue below.



These iNovator specific templates are:

[5] Two Primaries,

[6] Mosaic Scan,

[7] Interactive Tissue Scan and

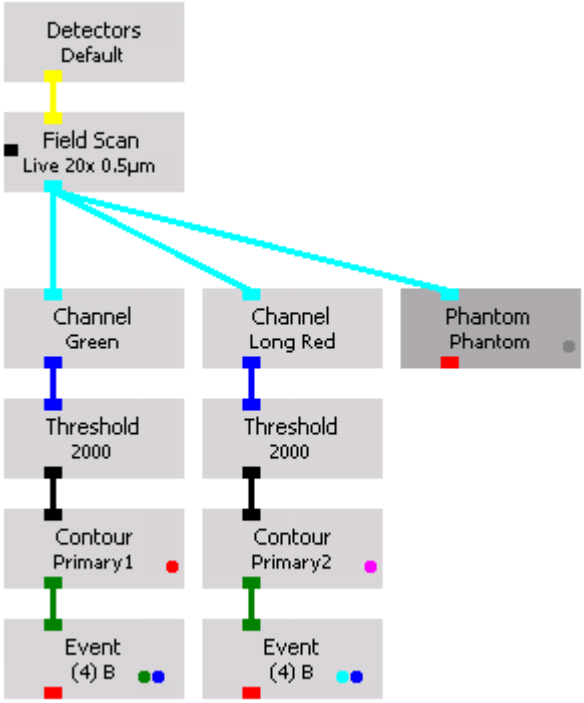
[8] Automated Tissue Scan

These templates are described below.

Note: Templates cannot be overwritten. If you wish to make changes to them, you must use the *Save as* option to create a new file.

[5] Two Primaries

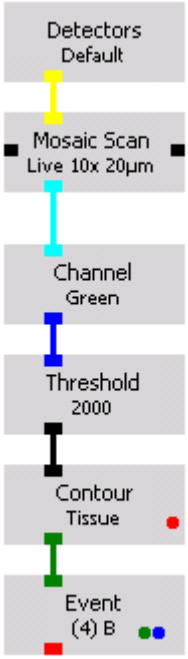
The Two Primaries template contains a *Protocol* tab and a blank *Data* tab. The default carrier is a 96 well plate. The protocol is shown below.



This protocol defines two primary event components that are generated based on independent contouring criteria. This is similar to the Primary-Sub protocol templates (see pages 3-12 to 3-14) but the two components are not associated. This template may be expanded to multiple independent primary event components.

[6] Mosaic Scan

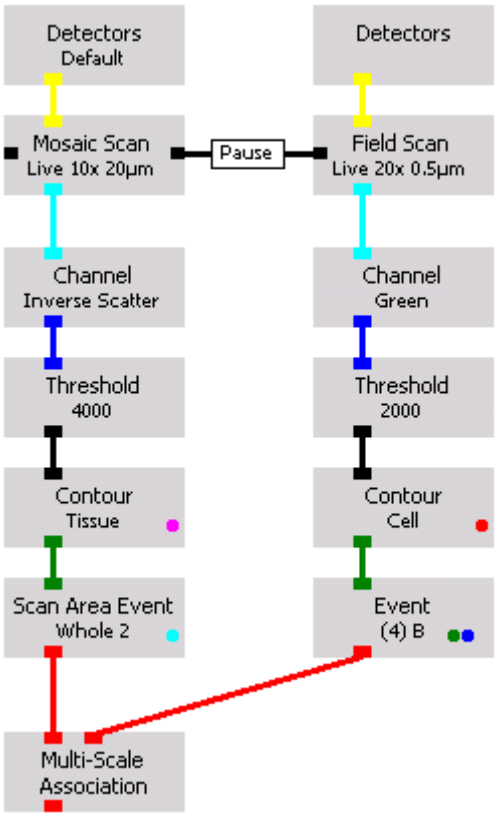
The Mosaic Scan template contains a *Protocol* tab; an *Image* tab with the carrier window and Well, Region and Field image windows; and a *Data* tab with the carrier window. The default carrier is a 4 slide carrier. The protocol is shown below.



This protocol is similar to the One Primary protocol except that it uses a Mosaic Scan instead of a Field Scan. Use this template if you want to contour on large events that cross scan fields.

[7] Interactive Tissue Scan

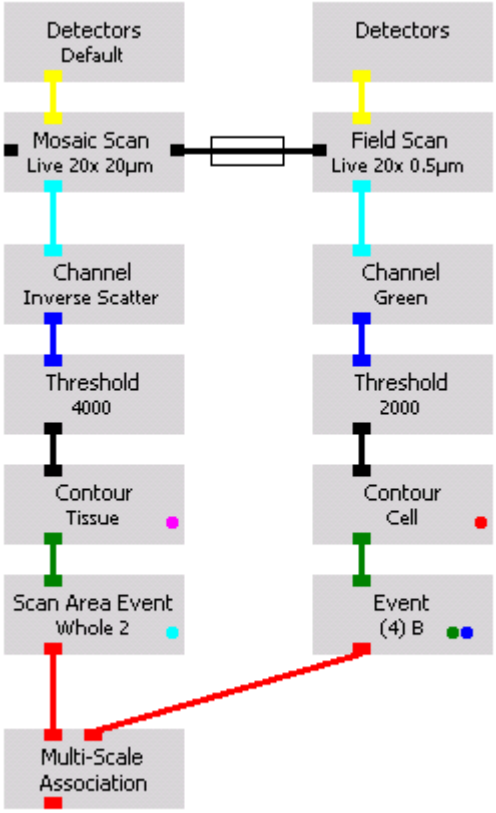
The Interactive Tissue Scan template contains a *Protocol* tab; an *Image* tab with the carrier window and Well, Region and Field image windows; and a *Data* tab with the carrier window. The default carrier is a 4 slide carrier. The protocol is shown below.



This protocol is designed for multi-scale scans. The 1st scale scan on the left is a Mosaic Scan that is set to run at low resolution to scout for tissue sections or other large events. The Scan Area Event module generates event data as well as *Dynamic Scan Areas* that are used as the scan areas for the subsequent Field Scan (on the right.) The bridge between the two Scan modules is set to *Pause*, indicating that the iCys will pause to allow the user to review the Dynamic Scan Areas prior to initiating the 2nd scale Field Scan. For more information on multi-scale scans see the section entitled "Multi-scale Scanning" on page B-30.

[8] Automated Tissue Scan

The Automated Tissue Scan template is identical to the Interactive Tissue Scan template except that the bridge between the two Scan modules is *not* set to *Pause*. In this mode the 2nd scale Field Scan will proceed automatically directly after the completion of the 1st scale Mosaic Scan. Use this template for fully automated analyses when the first and second scales use the same objective lens. For a more information on multi-scale scans see the section entitled "Multi-scale Scanning" on page B-30.


Detectors
Default
Detectors
Mosaic Scan
Live 20x 20µm
Field Scan
Live 20x 0.5µm
Channel
Inverse Scatter
Channel
Green
Threshold
4000
Threshold
2000
Contour
Tissue
Contour
Cell
Scan Area Event
Whole 2
Event
(4) B
Multi-Scale
Association

References to Using the Protocol Menu Bar and to Building and Modifying Protocols

Using the Protocol Menu Bar

Information on utilizing the Protocol menu tool bar and its features may be found in the section entitled “Protocol Menu Bar” beginning on page 3-30. Unique iNovator features of the View Image tool in the Protocol menu are described below in the section entitled “Using the Protocol Menu View Image Feature with iNovator” on page B-28.

Building and Modifying Protocols

Information on creating and modifying Protocols may be found in the sections entitled “Creating a New Protocol” beginning on page 3-35 and “Connecting Modules” beginning on page 3-59.

iNovator-specific Protocol Modules

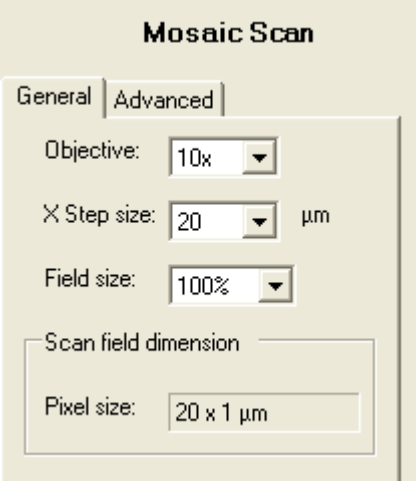
The iNovator toolkit provides you with a variety of protocol modules not found in the basic iCys. Each module's function and available parameters are explained below. The module descriptions are organized by the category in which they are found in the protocol tool box.

Scan

Mosaic Scan Module

The MosaicScan module is similar to the Field Scan module (see page 3-49) with some exceptions as described here. The MosaicScan module stitches together all of the scan fields within a given Scan Area prior to segmenting events, developing event features and displaying data for that scan field. This allows the MosaicScan to segment events (or draw phantom contours) that cross scan field boundaries. MosaicScan also reformats the data to provide a 1:1 aspect ratio of the X:Y resolution.

- **General Tab**—Under the General tab you can specify the objective you are using, the X-step size for the scan, and the field size as shown below:

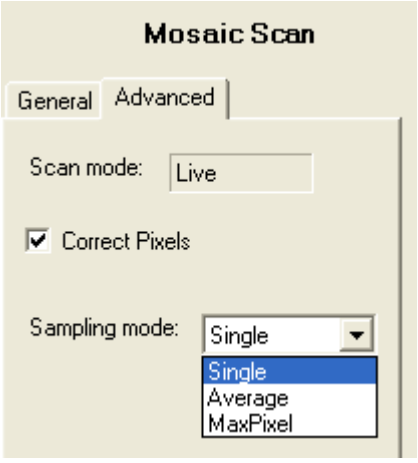


  - **Objective**—Allows you to select which microscope objective lens you will use for this scale of the run. When not actively scanning, the objective is moved out of the way of the motorized stage to prevent a carrier from being bumped by the objective.
  - **X Step size**—Determines the size of a single stage step in the X direction. From the X Step pull-down, select any value from .5µm to 20µm (in .5µm steps). For a low-resolution scan, select 20µm, the default value.
  - **Field size**—Determines the number of X steps that iCys will take when scanning. You can choose a percentage (100%, 50%, or 25%) or choose to generate a square scan field. The scan field and pixel size are determined by the dimensions you select and are displayed in the Scan field dimension window below. A 100% scan field is 1000 scan lines wide, a 50 % one is 500

scan lines wide, and so on. A square scan field is 768 scan lines wide (because the Y dimension is always 768 pixels tall.)

- **Scan field dimension**—Displays the size of each pixel based on the parameters you have selected.

- **Advanced Tab**—When the Advanced tab is selected the following options appear.



- **Scan mode**—Describes the current scan mode (Virtual, Live, Bypass)
  - **Live**—In this mode the Mosaic Scan acquires new data by scanning the sample.
  - **Virtual**—In this mode the Mosaic Scan re-contours the saved data.
  - **Bypass**—In this mode the Mosaic Scan in the first scale is by-passed and the user may perform a virtual second scale scan.

  For more information on scan modes see the section entitled “Analysis Modes for Multi-scale Scans” on page B-34.

- **Correct Pixels**—Checking this option enables a correction that accounts for variations in collection efficiency along the scan line. This is performed on each of the images as they are acquired. See also “Correct Pixels” on page 3-50.

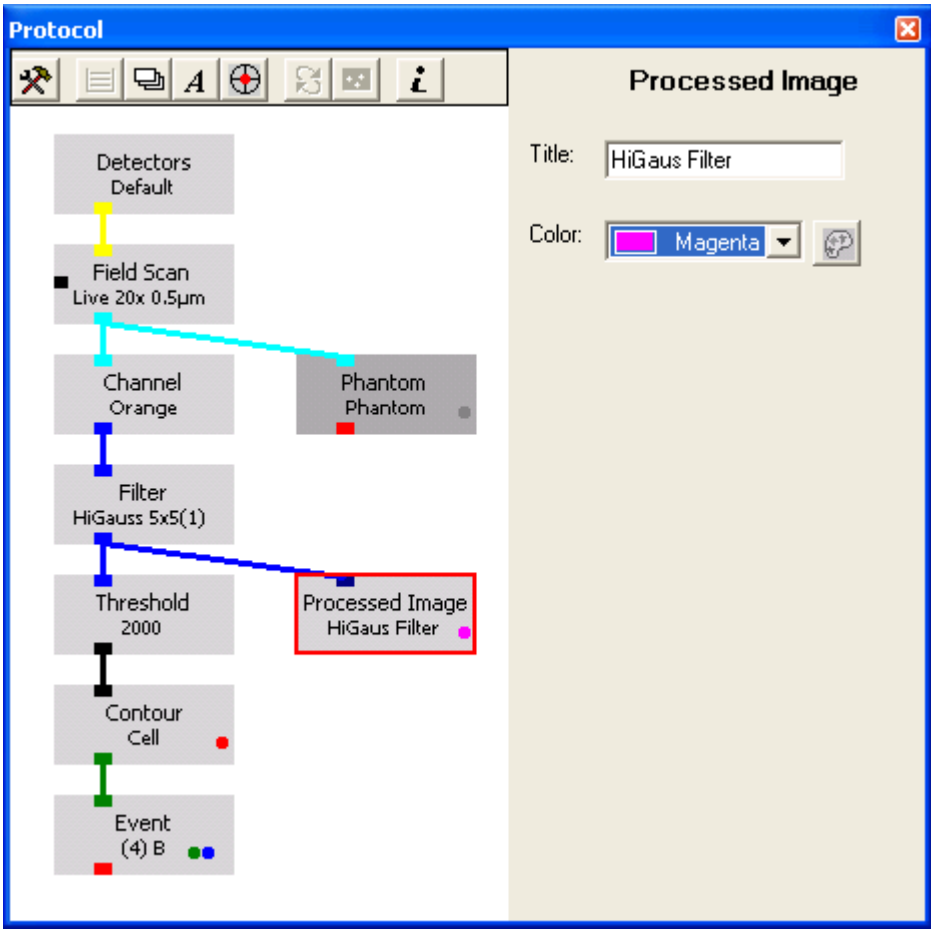
- **Sampling mode**—When generating the Mosaic images, iCys combines pixels from the individual Scan Fields to generate the 1:1 aspect ratio needed in the Mosaic image. There are three selections for the algorithm that iCys may use to calculate the resultant pixel value in the Mosaic image. These are:
  - **Single**—iCys uses the first pixel in the sampling bin.
  - **Average**—iCys uses the average value of the pixels in the sampling bin.
  - **MaxPixel**—iCys uses the pixel in the sampling bin with the maximum value.

Output

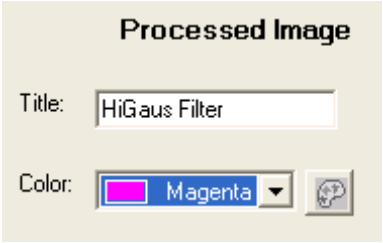
Processed Image

The Processed Image module allows you to view an image at some intermediate stage of processing in the protocol. In the Protocol below, a Processed Image is

defined to display the output image from the Filter module. This image is viewable in the View Image window (see “Using the Protocol Menu View Image Feature with iNovator” on page B-28) as well as the Field, Well and Region image windows, and in Galleries.



When the **Processed Image** module is selected the following options appear.



- **Title**—Specify a name for this image. In the example above the Processed Images have been named to reflect their respective input modules.

- **Color**—Set CompuColor for the Processed Image. Note that the CompuColor processed images do not appear in Galleries. Also, they are not saved with the Well, Region and Gallery images.

Image View

The Image View module is similar in function to the Processed Image module except that its output image is displayed in a separate window. This window opens at the start of the scan if an Image View module is part of the protocol. A separate CompuColor cannot be assigned to this image.

Filter Modules

Filter modules allow you to apply filtering to images. You can use the filter modules to enhance the quality of an image or to emphasize characteristics of interest within one or more images.

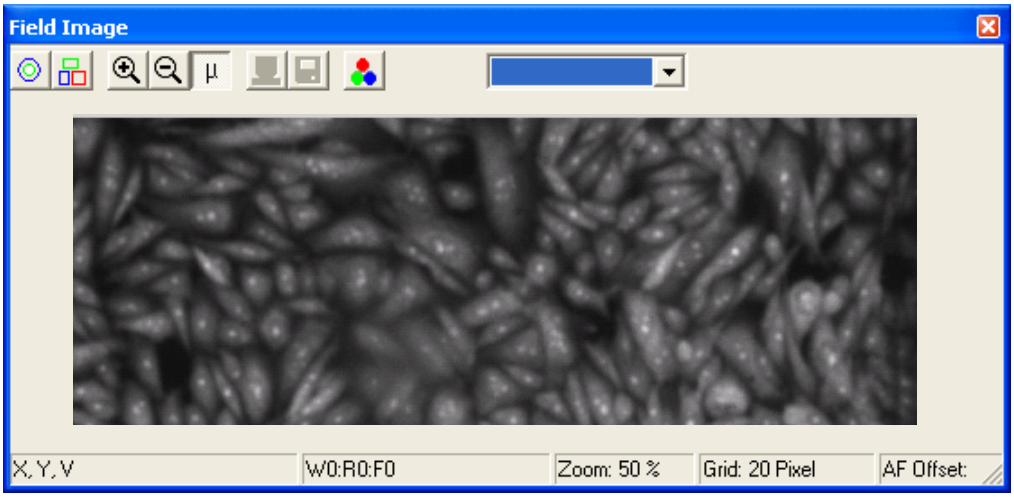
Tip: For detailed information about image processing and filtering, you may want to use a resource such as *The Image Processing Handbook, 2nd Edition*; Russ, John C.; CRC Press; 1995.

Image Filtering—the Filter Module

Performs filter operations on the input image.

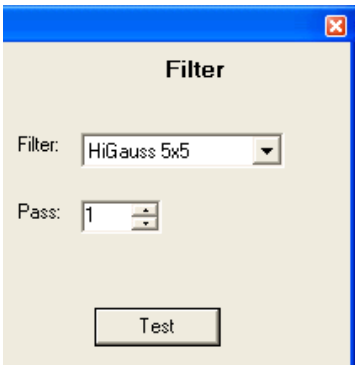
For comparison purposes, the image shown in Figure B-1 has no filtering.

Figure B-1: Unfiltered Image



➢ To set a filter:

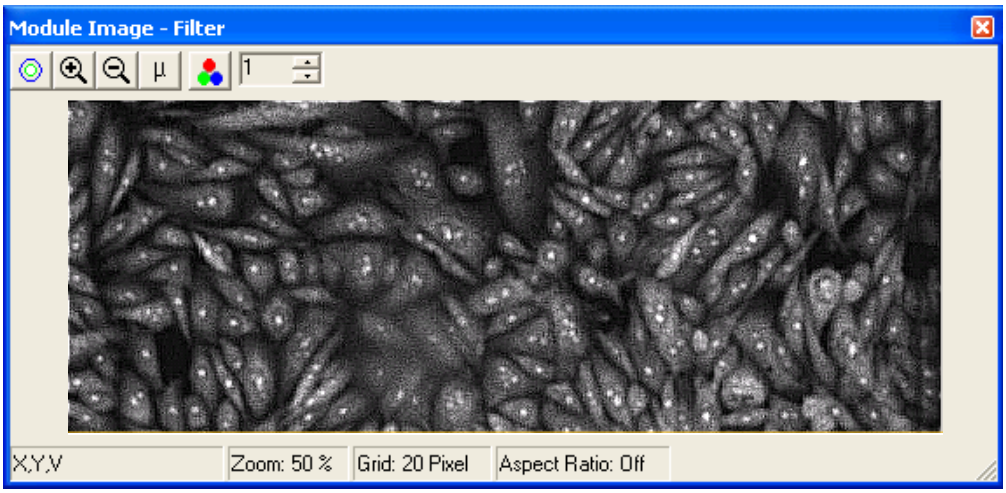
1. Click on the Filter module. The Filter dialog box displays:



2. Select the filter you want to use and for most filters, the resolution. The filters are:

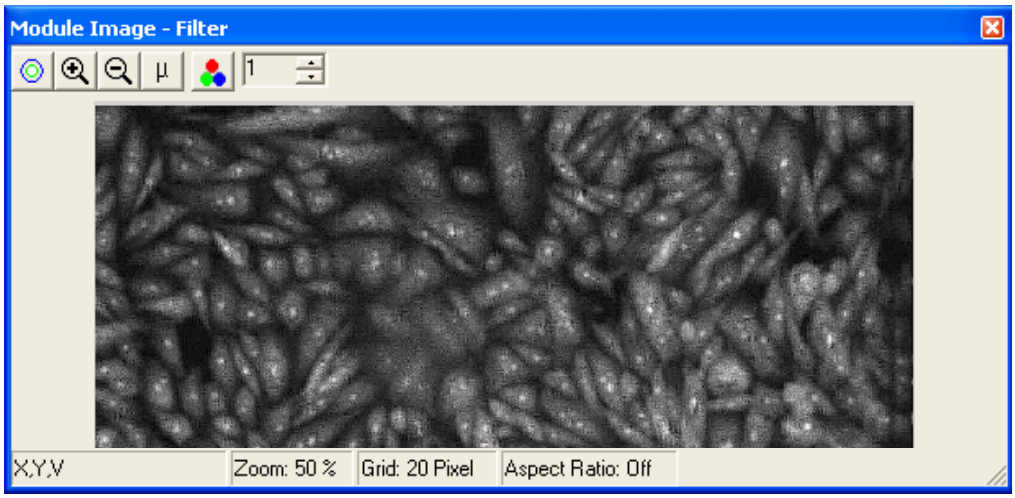
   ❍ **HiGauss 5x5, 7x7, or 9x9**—The Gauss filter performs a weighted sum (center pixel = highest weight). The result is normalized by the total kernel weight.

Figure B-2: HiGauss 5x5 Image



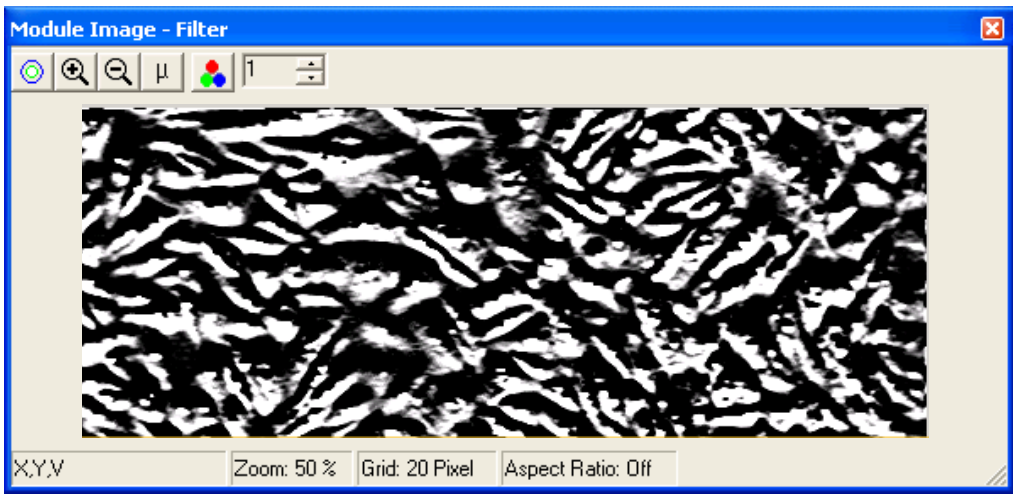
   ❍ **HiPass 3x3, 5x5, or 7x7**—This filter sharpens edges and enhances detail. This filter magnifies gradients along edges in any direction. The higher numbers produce more extreme magnification.

Figure B-3: HiPass 3x3 Image



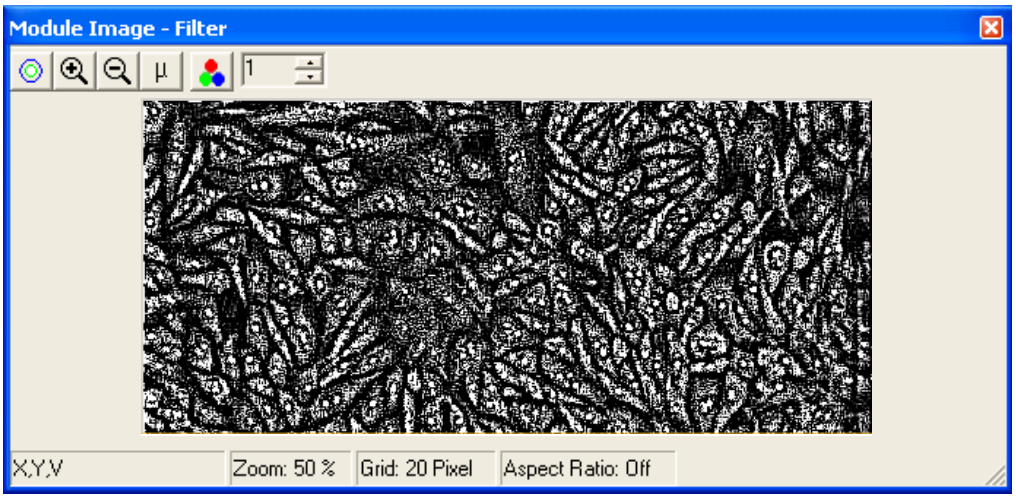
- **HorizontalEdge 3x3, 5x5, or 7x7**—This filter highlights horizontal edges (gradients) within the image. All pixels not part of horizontal gradients are changed to black; all edges become white.

Figure B-4: HorizontalEdge 7x7 Image



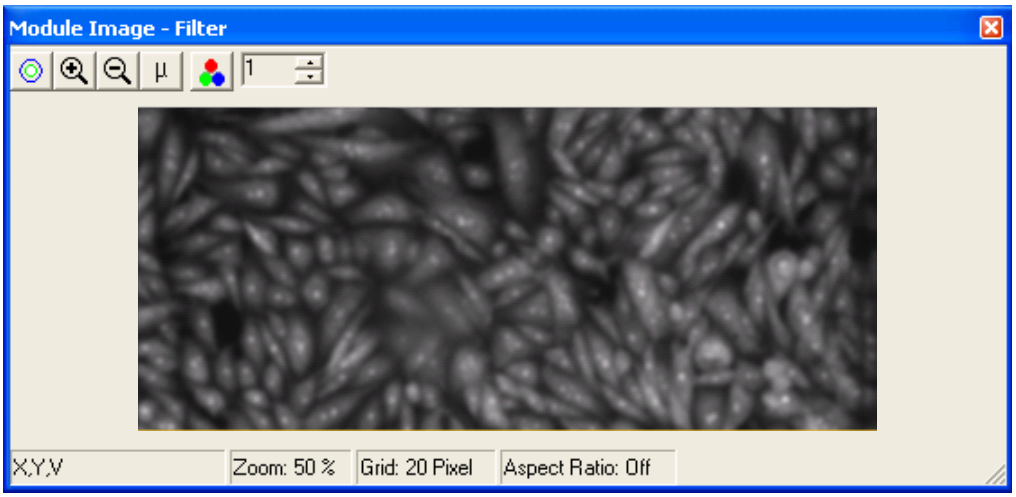
- **LaPlace 3x3, 5x5, or 7x7**—This filter weights the difference between the center pixel and its neighbors.

Figure B-5: LaPlace 7x7 Image



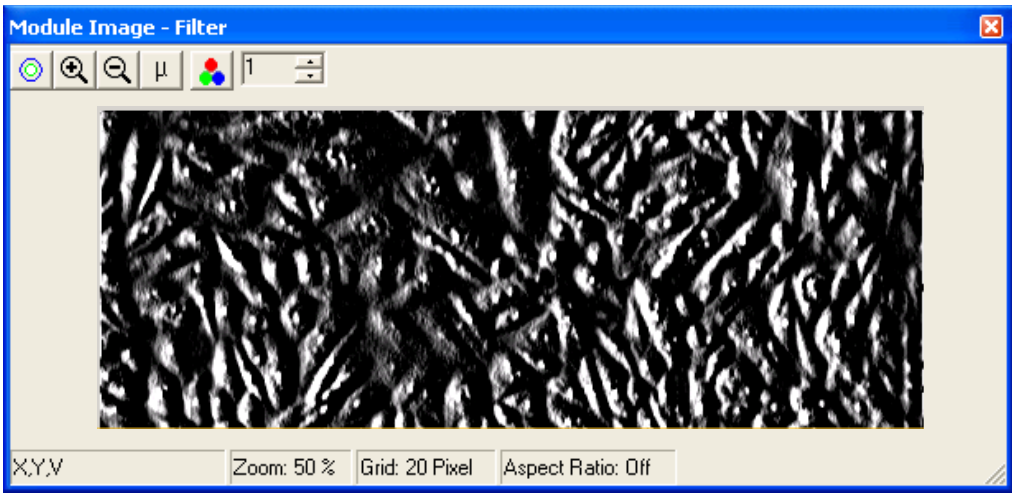
- **LoPass 3x3, 5x5, or 7x7**—For the LoPass filter, all pixels included in the filter's size are added up, and the result is divided by the number of pixels.

Figure B-6: LoPass 7x7 Image



- **VerticalEdge 3x3, 5x5, or 7x7**—This filter highlights vertical edges (gradients) within the image. Pixels that are not identified as part of a gradient are set to black.

Figure B-7: VerticalEdge 5x5 Image



- ❍ **Fish**—The Fish filter is not currently functional in iCys.

3. Specify the number of operations you want to perform. You can select between 1 and 5 passes.

Binary Image Filtering—Erode, Dilate, Open, Close Modules

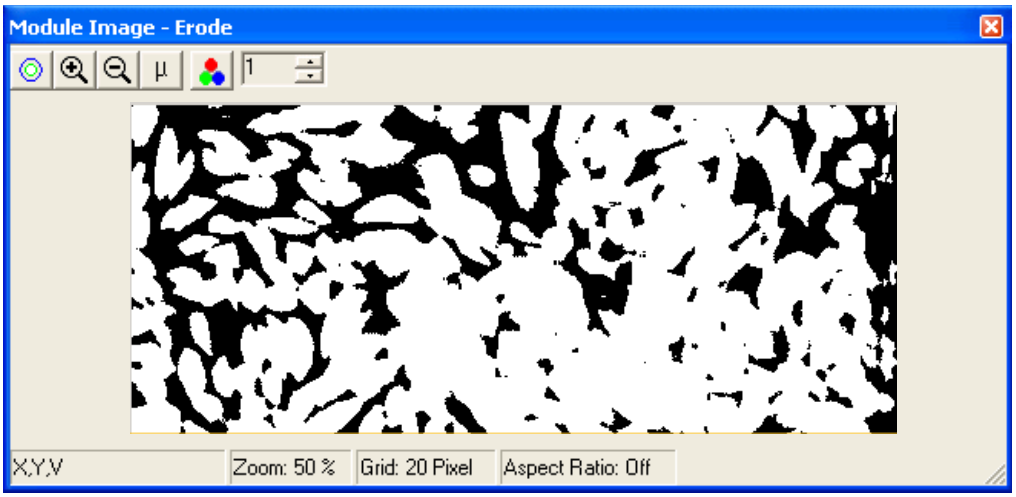
The Erode, Dilate, Open, and Close modules all morphologically change the image. The input image must be binary.

Tip:	To create a binary image, call the Threshold module before calling a morphological filtering module. Threshold produces binary output.

The morphological change modules are:

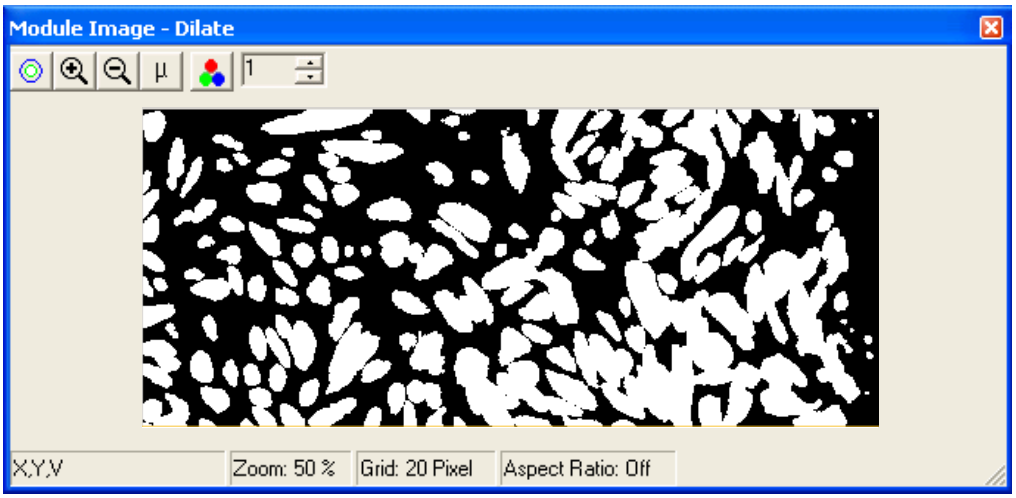
- ❑ **Erode**—Performs morphological erosion of the image. Erosion is the process of turning off (setting to zero) pixels at the perimeter of a cluster of pixels, thus reducing the size of each pixel cluster. This eliminates very small clusters and can separate multiple clusters that are barely "touching."

Figure B-8: Erode



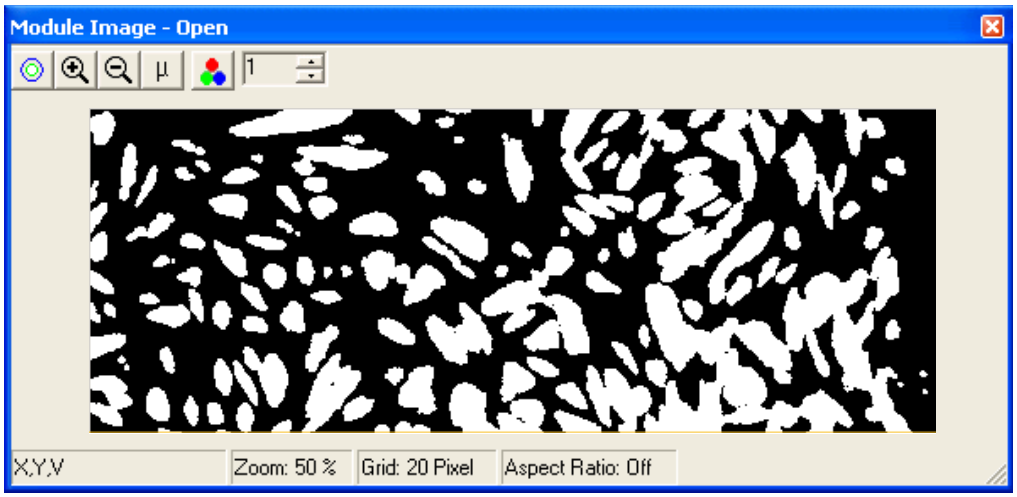
- **Dilate**—Performs morphological dilation on the image. Dilation is the opposite of erosion. It turns on pixels at the perimeter of a cluster of pixels thus increasing the size of each pixel cluster. It may be used to close "holes" within a pixel cluster.

Figure B-9: Dilate



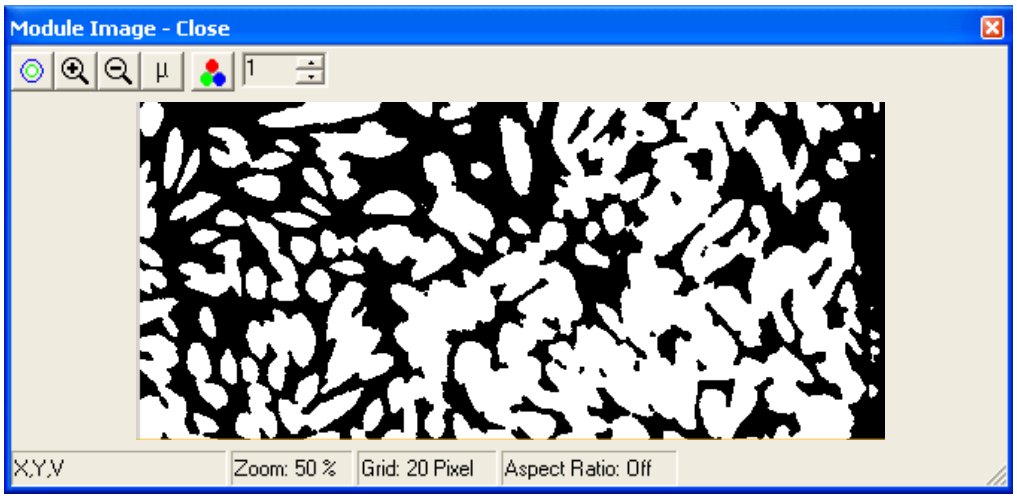
- **Open**—Performs morphological open on the image. Open is erosion followed by dilation. The Open operation may be used to open the spaces between pixel clusters or eliminate small clusters and then return the remaining clusters back to their original size.

Figure B-10: Open



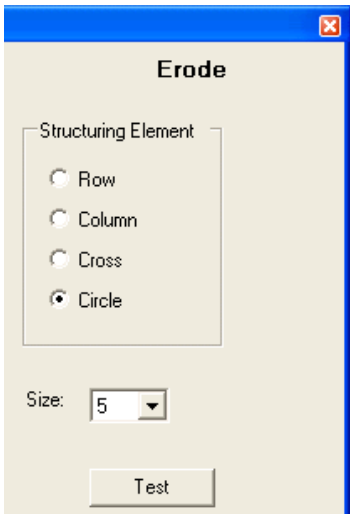
- **Close**—Performs morphological close on the image. Close is dilation followed by erosion.Closing, as the opposite process of opening, tends to close up the spaces between closely spaced events.

Figure B-11: Close



➢ To perform a morphological erode, dilate, open or close:

1. Click to open the module. For all of these functions, a similar dialog box displays.



2. In the Structuring Element panel, specify the shape and size of the kernel you want to morph.

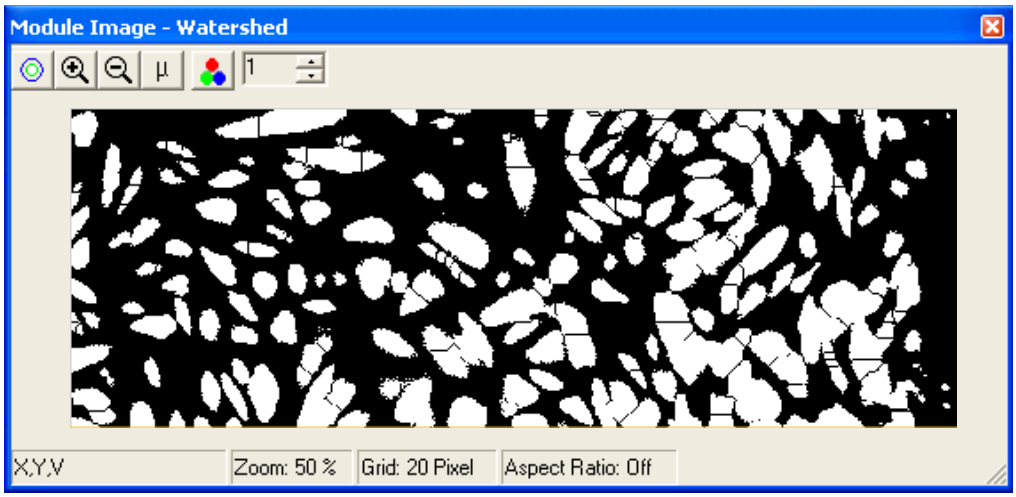
Watershed Module

The Watershed module performs watershed segmentation. Watershed segmentation is a process of using erosion and dilation (discussed in “Binary Image Filtering—Erode, Dilate, Open, Close Modules” on page B-15) on a Euclidean distance map to separate closely spaced or overlapping events. A grayscale image is generated from the binary image using a Euclidean distance map technique. A grayscale pixel value is generated for the positive pixels based on the distance of that pixel from the nearest background pixel. Localized peaks (called Ultimate Eroded Points or UEPs) are identified and the image eroded back to these UEPs. The image is then dilated until the positive pixels meet the original background boundary or until a second, dilating UEP is encountered. The dilation is stopped short of joining the UEPs, thus developing the intended separation. Various parameters may be set by the user to affect how this process proceeds.

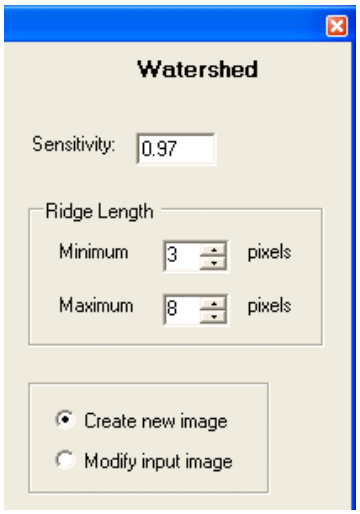
Tip: To create a binary image for input to the Watershed module, call the Threshold module before calling the filtering module. The Threshold module produces binary output.

Figure B-12 shows a different image after watershed segmentation.

Figure B-12: Watershed



1. Click to open the module. The Watershed dialog box displays.



2. Set the Sensitivity, which determines the aggressiveness of the segmentation by affecting the size of minimum allowable peak value of the UEP. Higher sensitivity causes more aggressive splitting. The maximum value is 1, a typical value is 0.95. The iCys default value is 0.97.

3. Set the Ridge Length. The ridge length determines how to treat the space between two Ultimate Eroded Points (UEPs). A UEP is a point at the center of an object.

   - **Minimum**—If the distance between two UEPs is less than the specified value, two points are combined into one object.

     A higher minimum ridge length causes more aggressive splitting.

   - **Maximum**—If the distance between two UEPs is larger than the specified value, two points are considered to be separate objects.

     A smaller maximum ridge length causes more aggressive splitting.

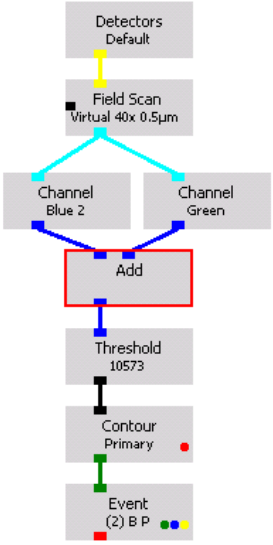
- **Create new image**—If **Create new image** is selected, a new image is created and the original input images are left intact.
- **Modify input image**—If **Modify input image** is selected, the input image is updated with the new image data.

Operation

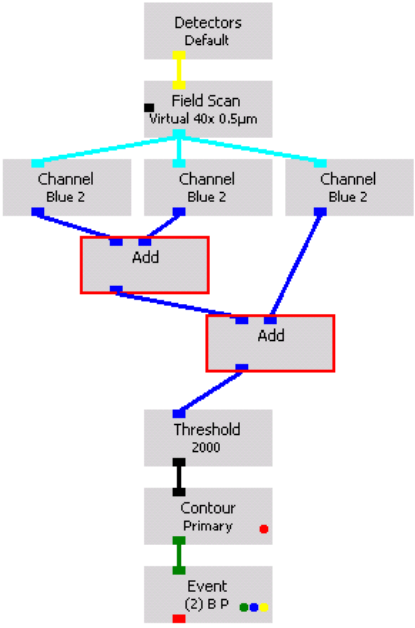
Arithmetic operators

Add and Subtract Modules

The Add and Subtract modules perform these operations on two input images to generate a third image. The resultant image is constructed by either adding or subtracting the values of each of the corresponding pixel values from the input images. In the case of the Subtract module the right image is subtracted from the left image. A protocol using the add module is shown below.



The Add and Subtract modules can be "nested" as shown below.



iCys only reports pixel values between 0 and 16383. The number 16383 is the 14-bit saturation level of any measured signal. Addition of pixel values that result in values higher that 16383 are reported as 16383. Subtractions that result in values lower that 0 are reported as 0.

Invert Module

The Invert module inverts the pixel values of the input image according to the following formula: 16383 – input value = output value. Thus saturated values of 16383 become zero and zero values become 16383. An intermediate value of 12383 becomes inverted to 4000. Note that the connector color of the Invert module is dark blue meaning that the input image may be *either* an Analog or a Binary image. An analog input produces an analog output; a binary input produces a binary output.

The parameters available to these modules are described under Boolean operator modules below.

Boolean operators

The Boolean operator modules, AND, OR and XOR (exclusive OR) allow you to combine two *binary* images to result in a third binary image. A binary image has been *thresholded*. When the threshold function is applied to images in the protocol, all of the pixels below the threshold value are set to zero. All of the pixels at or above the threshold value are set to 16383. The result is a purely black and white image with no gray.

The result of each Boolean operation is explained below.

And

The **And** module performs a Boolean AND on the two input images. A Boolean AND will yield the results shown below.

Input 1 pixel value	Input 2 pixel value	Output pixel value
0	0	0
0	16383	0
16383	0	0
16383	16383	16383

Or

The **Or** module performs a Boolean OR on the two input images. A Boolean OR will yield the results shown below..

Input 1 pixel value	Input 2 pixel value	Output pixel value
0	0	0
0	16383	16383
16383	0	16383
16383	16383	16383

Xor

The **Xor** module performs a Boolean XOR *(Exclusive Or)* on the two input images. A Boolean XOR *(Exclusive Or)* will yield the results shown below..

Input 1 pixel value	Input 2 pixel value	Output pixel value
0	0	0
0	16383	16383
16383	0	16383
16383	16383	0

When any Boolean or arithmetic operator module is selected you are given a choice between creating a new image and modifying the left image. For example, when the Xor operator module is selected, the following options appear.

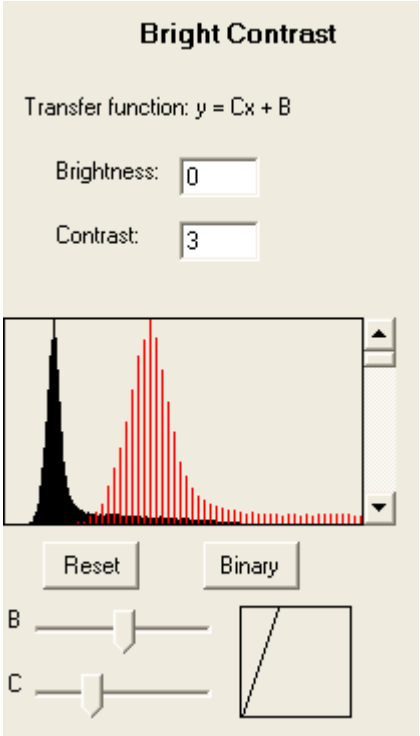
- **Create new image**—If **Create new image** is selected, a new image is created and the original input images are left intact.
- **Modify left image**—If **Modify left image** is selected, the left input image is updated with the new image data.

Bright Contrast Module

The Bright Contrast module allows you to manipulate the Brightness and Contrast of the image. Its function is identical to that of the BrightContrast feature of the image right-click menu described on page 3-72.

Whereas the BrightContrast feature of the image right-click menu only affects the displayed image, the Bright Contrast module may be used to alter an image in the protocol and aid in contouring. Altering the image contrast may make it easier in some images to better determine the optimum threshold value. Although selecting the **Binary** button makes the Bright Contrast module behave like the Threshold module, the **Binary** mode should only be used for visualization purposes. Once a threshold value has been selected, input this value into the Threshold module and deselect **Binary** in the Bright Contrast module.

Selecting the Bright Contrast module displays the following options, similar to the BrightContrast feature dialogue in the image view (see page 3-72).



Event

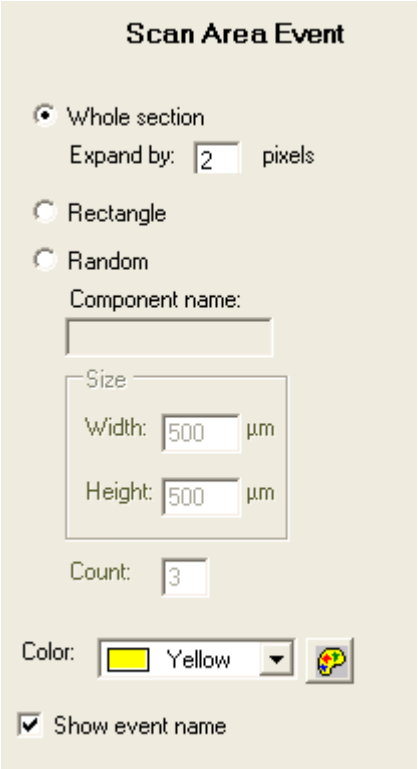
Scan Area Event

The Scan Area Event module is used only in the first scale of a two-scale scan. It generates two important outputs: events and Dynamic Scan Areas.

The event output is similar to that of the event module. The output of a contour module provides input and this module produces events for that component. Those events may be treated as any other event.

The second output, Dynamic Scan Areas, are scan areas that are subsequently scanned in the second scale scan. For a description of two-scale scanning and how this module is utilized as a part of that process see the section entitled “Multi-scale Scanning” on page B-30.

When the **Scan Area Event** module is selected the following options appear.



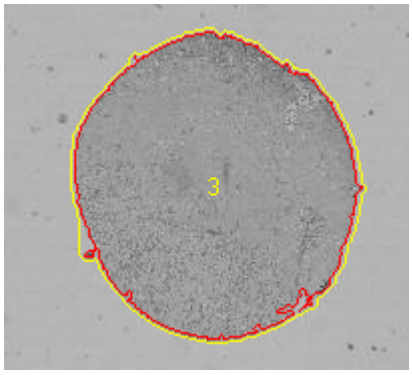
There are three options for generating dynamic scan areas and events: **Whole section**, **Rectangle** and **Random**.

- **Whole section**—generates a contour that meets the segmentation criteria in the protocol around the entire event. This contour becomes the event boundary and the dynamic scan area boundary.
- **Rectangle**—generates a contour around the entire event as above. iCys then generates a rectangular scan area within this Whole section contour, centered on the centroid of the **Whole section** contour.
- **Random**—generates a contour around the entire event as above. iCys then generates a specified number rectangular scan areas placed randomly within the **Whole section** contour. This is pictured below for a Tissue Micro-Array element.

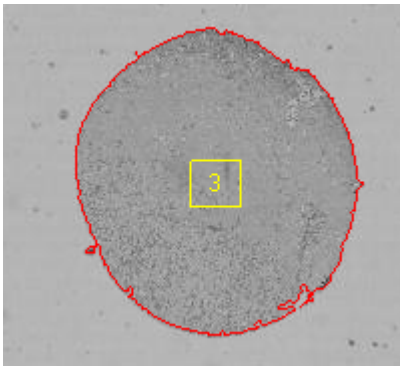
Additional Parameters are:

- **Component name**—Specify the name of the component (as in the Event module, see page 3-56.)
- **Size**—If either **Rectangle** or **Random** is selected, the **Height** and **Width** of the rectangle must be specified in microns.
- **Count**—If **Random** is selected the number of scan areas to be generated within each threshold contour is specified.
- **Color**—The color of the contour line is specified.
- **Show event name**—Checking this option displays the event ID number (or ordered pair if the Array Alignment module is used.)

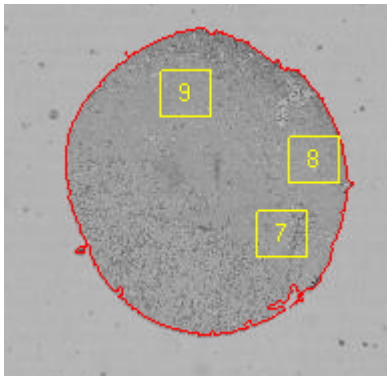
Pictured below, on the same Tissue Micro-Array element, are the three types of dynamically generated scan areas (outlined in yellow). The numbers shown are the event IDs generated by the **Show event name** option.



Whole section dynamic scan area



Rectangular dynamic scan area —placed at centroid of element



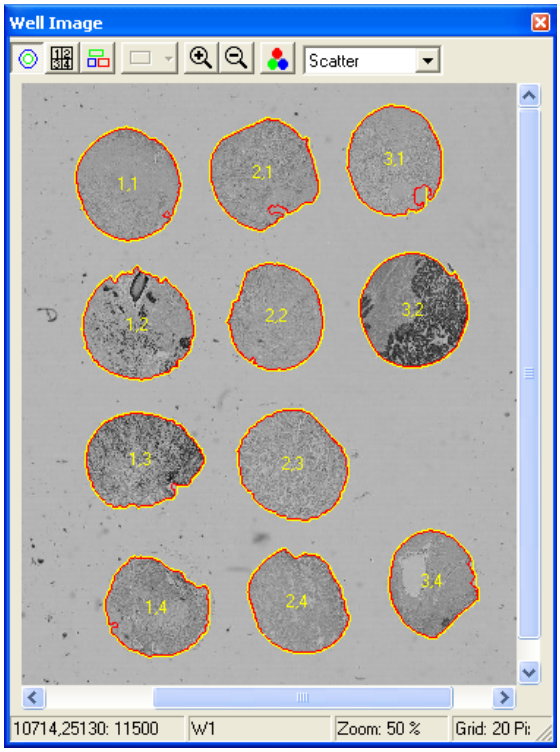
Random dynamic scan area —three scan areas specified

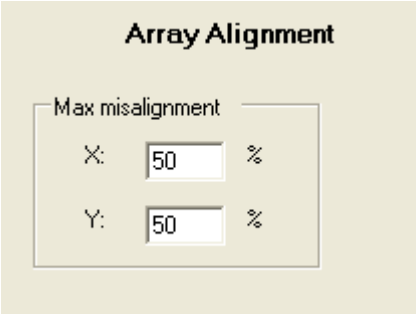
Multi-Scale Association

The Multi-Scale Association module is analogous to the Association module as described on page 3-58 except that it operates *across* first and second scale scans whereas the Association module operates *within* first or second scale scans (or single scale scans.) This is illustrated in Tutorial Exercise 4 where the second scale *event* component is associated with the first scale *Tissue* component. There are no parameters for the Multi-Scale Association module.

Array Alignment

The Array Alignment module is typically used when doing a low resolution scout scan to identify the elements of a Tissue Micro-Array. The module identifies the contoured events for the component to which it is attached, as an ordered pair of numbers. This ordered pair represents the Column and Row for the element within the larger array. This is pictured below.





Parameters available are:

- **Max misalignment X & Y**—The distance between centers of elements for which adjacent elements are considered to be in the same row or column (relative to the average X or Y dimension.) For example: a 50% X value means that adjacent tissue elements of 2 mm average size are considered to be in the same column if the X value of their centers are no more than 1 mm apart.

  Use a higher value if elements are not parallel to the X-Y axes and are far apart. Use a lower value if elements are close together and parallel to the X-Y axes.

Using the Protocol Menu View Image Feature with iNovator

In the basic iCys, the View Image feature (accessible by the icon in the Protocol main menu) allows the user to interactively set the Threshold. It may also be used in the iNovator to interactively set any of these image processing tools:

All Filter modules

- ❑ Filter
- ❑ Erode
- ❑ Dilate
- ❑ Open
- ❑ Close
- ❑ Watershed

All Operation modules

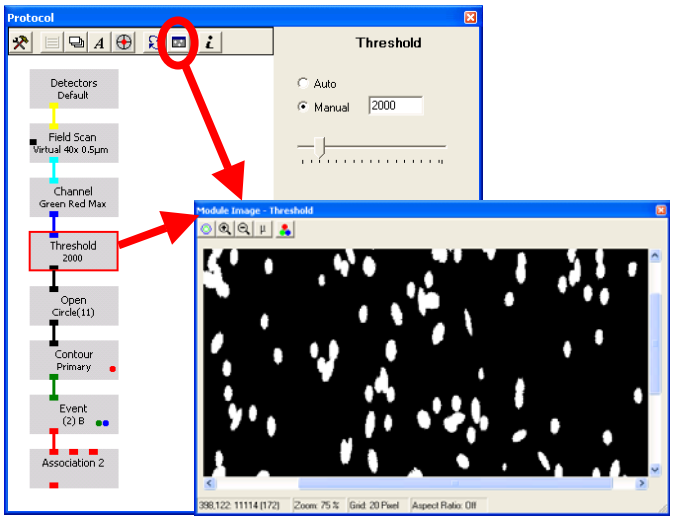
- ❑ Add and Subtract
- ❑ Invert
- ❑ And
- ❑ Or
- ❑ Xor
- ❑ Bright Contrast

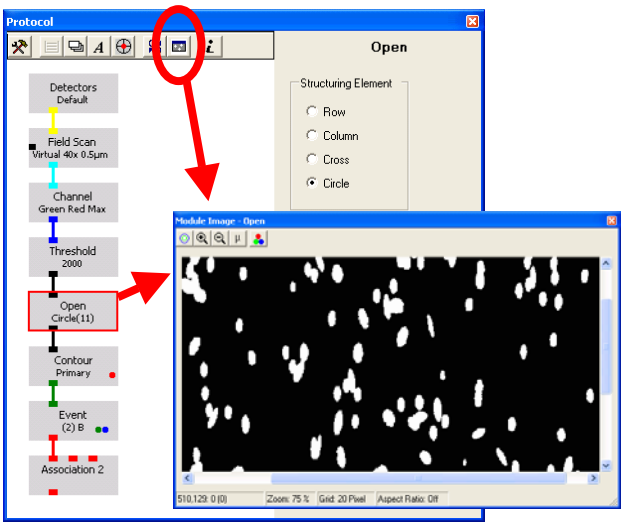
Selected Contour modules

- ❑ Channel
- ❑ Threshold

To utilize the View Image window: With an image or set of images in memory, such as during or after a Test Scan, select one of the image processing modules listed above, that is in your defined protocol. Then select the **View Image** icon from the Protocol main menu bar. The output of the selected module will display in the View Image window. To interactively adjust the module, select a different setting and, if available, select the **Test** button. To view the result of a modification in one module, on the output of a downstream module, select the downstream module of interest and then select the **Refresh** icon from the Protocol main menu bar.

The figures below show View image window displaying the results of the threshold module and of the Open module.

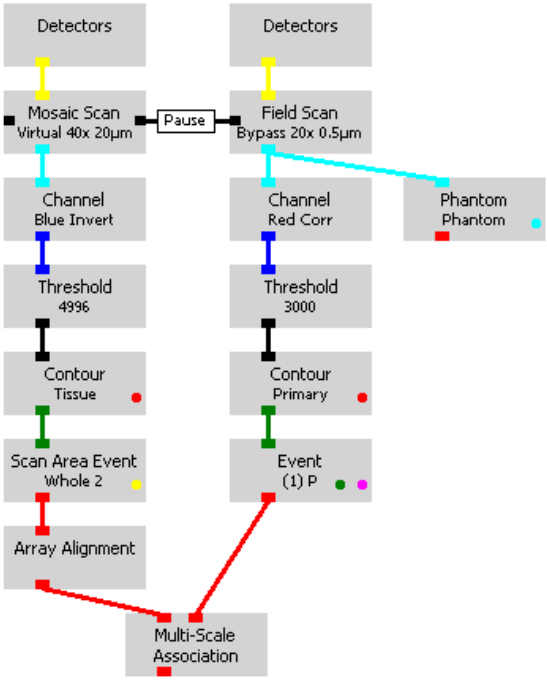

Protocol
Threshold
Auto
Manual
2000
Detectors
Default
Field Scan
Virtual 40x 0.5µm
Channel
Green Red Max
Threshold
2000
Open
Circle(11)
Contour
Primary
Event
(2) B
Association 2
Module Image - Threshold


Protocol
Open
Structuring Element
Row
Column
Cross
Circle
Detectors
Default
Field Scan
Virtual 40x 0.5µm
Channel
Green Red Max
Threshold
2000
Open
Circle(11)
Contour
Primary
Event
(2) B
Association 2
Module Image - Open

Multi-scale Scanning

Introduction

Multi-scale scanning involves defining a protocol for two sequential scans referred to as *scales* (because they are usually done at different resolutions). Typically, the first scale is done using the Mosaic Scan module at low resolution. This is referred to as a *scout* scan because it is scouting for relatively large structures such as tissue microarray elements, tissue sections, structures within tissue sections or cell colonies. Events found in this scout scan may be designated Scan Area Events. These scan area events become the defined scan areas for the second scale, typically higher resolution scan. Such a scan is defined in Tutorial 4, the protocol of which is shown below.



Unique Features

Different Detector Module Settings

The two scales' Detector module settings are independent of one another. Data channels, virtual channels, detector gain and offset, laser selection and (if applicable) laser power may be independently selected and set for each scale. This allows the user to set up the system for the very different scan requirements of scout vs. high resolution scans.

Different Scan Module Settings

The scan module settings are at the heart of defining the operation of each of the two scales. As was mentioned, the first scale is a Mosaic Scan, typically at low resolution

to find areas of interest for more detailed analysis. For example, a slide will be scouted to identify the locations of tissue samples. When identified, each tissue can then be analyzed in detail using a higher resolution Field Scan or Mosaic Scan.

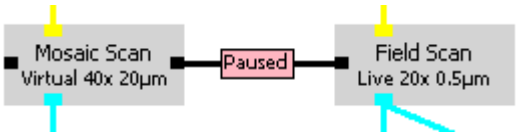
The settings required to locate tissue on a slide may be quite different from the settings for analyzing the structure within those tissues. Therefore, one might define a Mosaic Scan with a 10X objective and 20 micron steps to be followed by a Field Scan with a 40X objective and .5 micron steps.

Scan Area Events and Other Events

Multiple event components may be defined in either the first or second scale scan. Such a protocol is shown below. One of these event components must be designated a Scan Area Event. The Scan Area Event module generates event data as any other event component but additionally the Scan Area Event contours are used as scan areas for the second scale scan. These scan areas, generated from Scan Area Events, are called Dynamic Scan Areas.

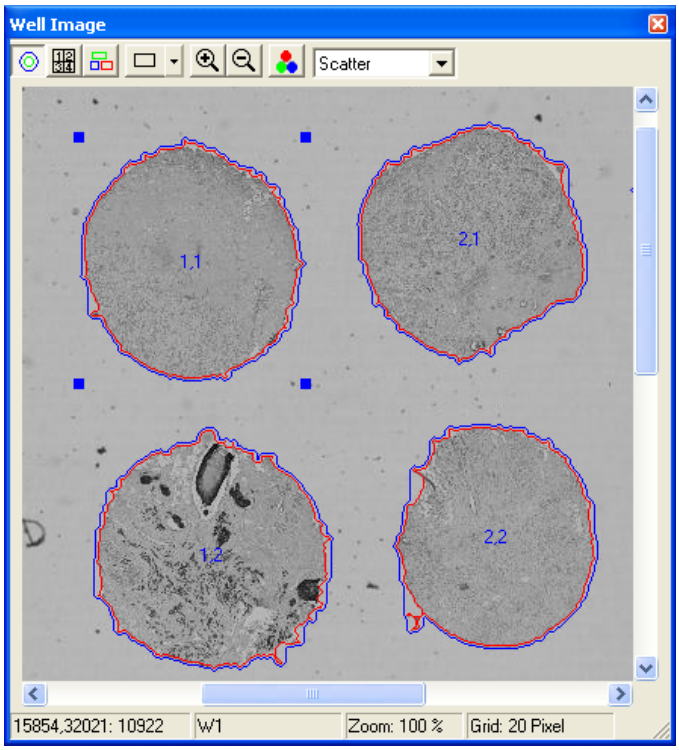
Manipulating Dynamic Scan Areas

While in the Pause state between scans, you may manipulate the Dynamic Scan Areas (DSAs) generated by the 1st scale scan or create new DSAs manually. When the system is *Paused* between the 1st and 2nd scale scan, the bridge between Scan modules will appear as shown below, pink and with the word *Paused* visible.



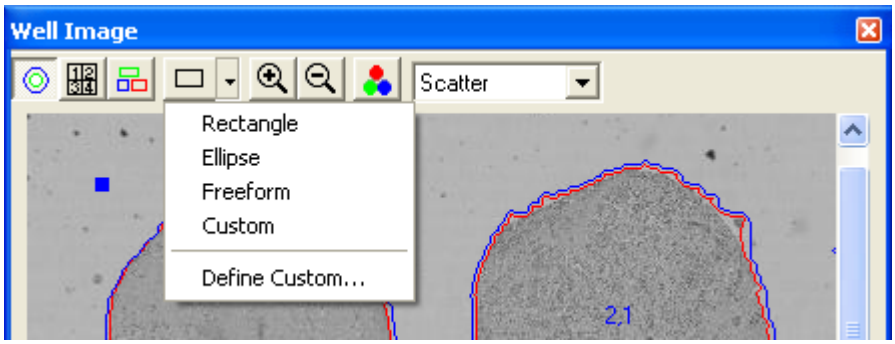
For more information about getting to a paused state, see “Analysis Modes for Multi-scale Scans” on page B-34.

To manipulate an existing DSA, click on the DSA of interest. "Handles" will appear at the four corners of the DSA as shown below for the TMA element in the upper left of the image.

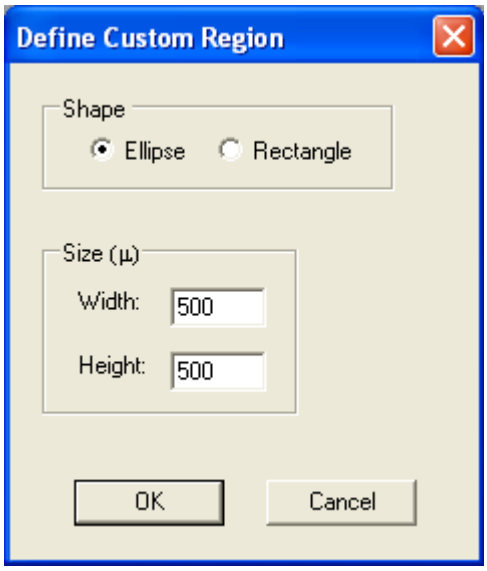


The DSA may now be moved by dragging it with the mouse, or it may be deleted using the delete key.

To manually add a DSA, use the create region tool ( ) in the Well Image tool bar.



Rectangle and *Ellipse* allow you to draw DSAs of those shapes anywhere in the Well image. *Freeform* let's draw a freeform shape by dragging the cursor to define the desired contour. *Custom* allows you to define a shape of a specified size and place copies of it wherever you would like. In this way, many DSAs of identical size and shape may be added to an image. Define the custom shape by selecting **Define Custom** and use the *Define Custom Region* dialogue box.



To manually add a DSA, select the desired shape from the pull-down menu and then select the tool, by clicking on the button. In the graphic below, the lower right DSA has been deleted and replaced with a manually added, elliptical DSA. The manually added DSAs may be resized by dragging the contour handles. Note that the DSA is numbered by the ArrayScan module based on its position relative to the other modules and the criteria defined in the ArrayScan module parameters. The entire array is renumbered, if necessary when new DSAs are added or deleted.

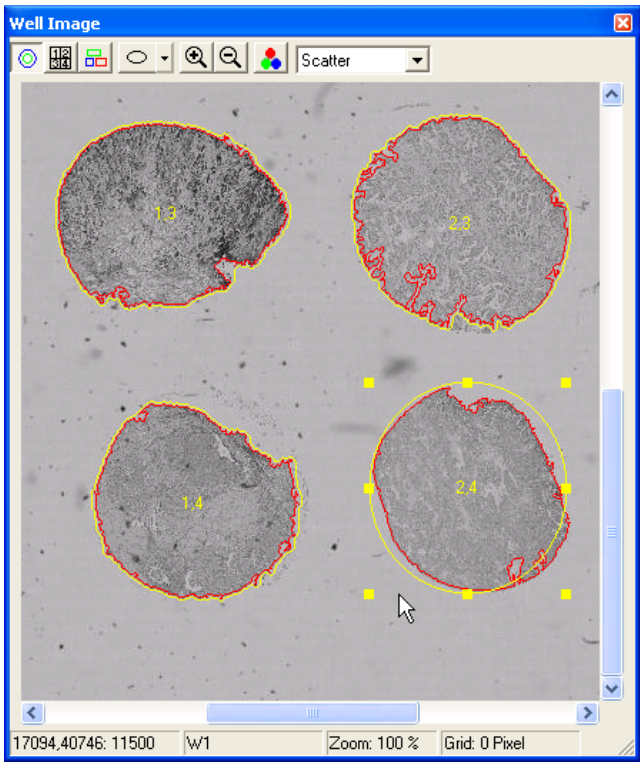


Image Hierarchy

The first scale Mosaic Scan image is displayed in the Well Image window. As the second scale Field Scan progresses a mosaic image of each Dynamic Scan Area is displayed in the Region Image window. Each scan field image is displayed in the Field Image window.

From the Well Image, clicking on a particular Scan Area Event will display that event in the Region image window. Similarly, clicking on a particular scan field location in the Region Image will display that scan field in the Field Image window.

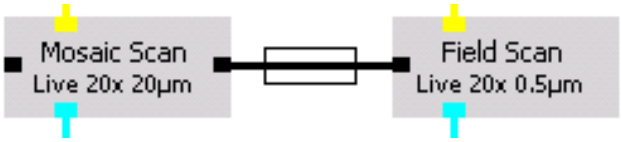
Analysis Modes for Multi-scale Scans

When setting up a new run or re-running an existing run, the user has several operating modes to choose from. What is meant by operating modes is this: Will the systems be acquiring new data by scanning a sample or will the system be reanalyzing previously acquired images by performing a virtual scan of those images? With multi-scale scanning, certain combinations of these two modes are possible. All of these options are described below.

New Runs

Live-Live

With a new run, data must be acquired with a *live scan*. If a fully automated run is chosen then data will be acquired for both scales by live scanning of the sample. To set up a fully automated 2 scale scan, make sure the bridge between the two Scan modules is NOT set to pause by clicking on the box on the bridge until it appears blank, as shown in the figure below.

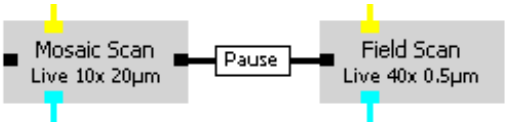


Test scans should be made to set the hardware and software protocol parameters for both scale scans. To set up the protocol for both scales create an abbreviated scout scan area, turn on the pause between scales and select the **Test Scan** button or the **Next** button (single field). During the scout scanning make the desired changes to the PMT settings. (NOTE: This may take several iterations of starting and stopping the test scan.) When the PMT settings are complete, stop the scan and then acquire another Scout scan using the correct PMT settings. During the *Pause*, use the View Image tool as described in “Using the Protocol Menu View Image Feature with iNovator” of this appendix. Once you are satisfied with the contouring protocol, select or create Dynamic Scan Areas in the Well Image window to use for setting up the 2nd scale hardware and contouring protocol. Delete any unnecessary dynamic scan areas. (NOTE: You may want to increase the size of the dynamic scan areas to be large enough to complete the 2nd scale setup.) Once you have selected the dynamic scan areas, press the **Paused** button to continue the test scan in the 2nd scale. During test scanning of the 2nd scale make the necessary hardware and contouring protocol changes. (Note: It may take several iterations of test scanning to accomplish the setup).

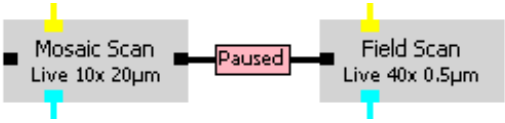
Once the protocol parameters have been set, select **Scan and Save** and the iCys will scan the 1st scale, automatically identify Dynamic Scan Areas and then scan these areas with the 2nd scale protocol.

Live (Scout only) and Live-Pause-Live

To scan only the first scale and then either save the data for later action or manipulate the Dynamic Scan Areas prior to continuing with the 2nd scale scan, set the bridge between the two Scan modules to *Pause* by clicking on the box on the bridge until *Pause* appears, as shown in the figure below.



When the first scale scan is complete, the *Pause* box will turn pink and *Pause* will turn to *Paused*:



In this paused state, you may review the Dynamic Scan Areas in the Well Image window. You have two possible options at this point:

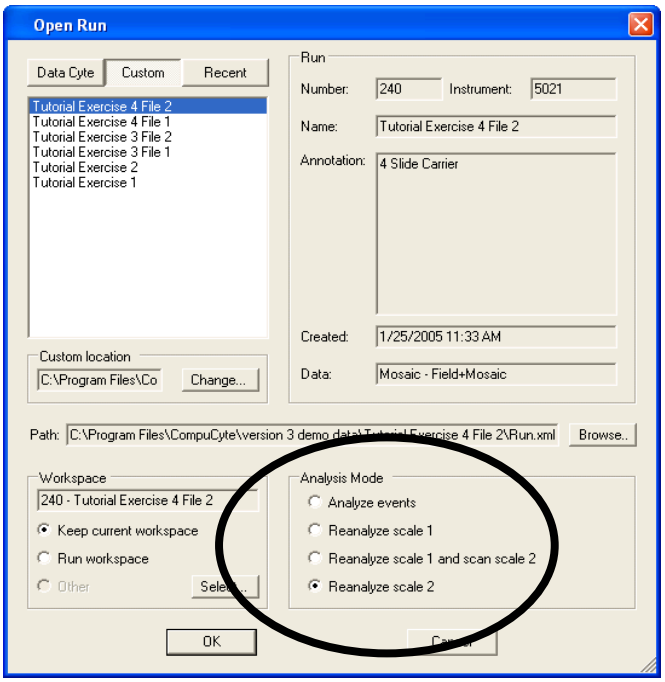
- ❑ Save the scout scan data for a later scanning of the 2nd scale scan,
- ❑ Continue with the 2nd scale scan now.

To save the scout scan data for later, click **Stop** and then yes to save the run. We will discuss opening and continuing with scout-only runs in the next section.

If you intend to continue with the 2nd scale scan, you may first review the Dynamic Scan Areas and if necessary, move or delete them, or create new ones. For more information on this process, see “Manipulating Dynamic Scan Areas” on page B-31. Once you are happy with the DSAs, you may resume scanning by clicking on the bridge; the box will become clear and scanning will resume.

Reanalyzing Existing Runs

When the user selects **Open Run**, several options are presented in the *Analysis Mode* area of the dialog box highlighted below.



Analyze events (bypass-bypass)

Select this option if you do not want to rescan any existing images or acquire new data, but want to work only with the existing event data from a previous scan.

Reanalyze scale 1 (virtual-bypass)

Select this option if you want to virtually rescan existing scale 1 data, that is, reprocess scale 1 image data after modifying the protocol.

Modify the protocol to optimize the analysis, then click **Scan and Save** to generate new event data and DSAs and save the data as a new run.

Reanalyze scale 1 and scan scale 2 (virtual-live)

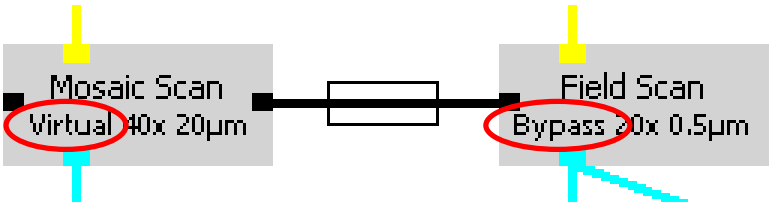
This option allows you to reprocess scale 1 image data as described above and then to acquire new data from a live scale 2 scan of your sample.

Modify the scale 1 protocol to optimize the analysis, then click **Scan and Save** to generate new scale 1 event data and DSAs and save the data as a new run. To continue with the 2nd scale scan, manipulate the DSAs if desired, and then click the *Paused* box on the bridge between the Scan modules to initiate the 2nd scale scan.

Reanalyze scale 2 (bypass-virtual)

Select this option if you want to use the saved 1st scale scan "as is" and virtually reanalyze the already acquired 2nd scale scan data. To initiate the 2nd scale scan, click **Scan and Save**.

Note that the Mode signature (such as virtual-bypass) serves as shorthand for the type of run that is being performed. This mode type is shown in the Scan Module graphics, as shown below.



Appendix C

Administrator Tasks

This chapter describes certain iCys™ Cytometric Analysis Software administrative tasks.

Included in this chapter:

- Displaying the Administration Menu, page C-3
- Using the Manage Carrier Types Window, page C-5
- Defining Custom Carriers, page C-7
- Using the Light Meter Utility, page C-24
- Forward Scatter Pixel Correction, page C-26

iCys™ RESEARCH IMAGING CYTOMETER

About Administrator Tasks

The iCys administrative tasks fall into two tiers:

- Tasks available to an administrative user at the customer site. These tasks, which can be accessed from the Administration menu with the correct password, include changing the administrator password, managing carrier types, scatter pixel correction and using the lightmeter utility.
- Tasks that should be used only by (or with the assistance of) CompuCyte personnel. These tasks include various calibration utilities that can be used in troubleshooting and repairing the iCys hardware.

The Administration password will only display the first level of tasks in the Administration menu. For information about more advanced tasks, contact your CompuCyte representative.

Displaying the Administration Menu

When you open the Administration menu in general administration mode, you will see four options:

- ❑ Manage Carrier Types
- ❑ Change Password
- ❑ Light Meter
- ❑ Scatter Pixel Correction

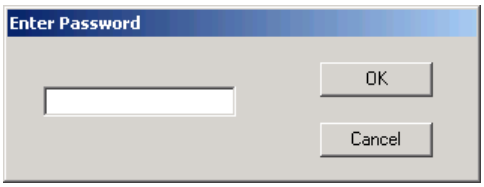
Managing carriers is discussed in “Using the Manage Carrier Types Window” on page C-5 and “Defining Custom Carriers” on page C-7.

Opening the Administration Menu

When you first open iCys, the Administration menu does not display on the task bar.

➢ To gain access to the Administration menu:

1. Click the “Show Administration Menu” option from the Setup menu.
2. The Enter Password window displays:



3. Type *Hello* to gain access to the user administration menu.

Tip: As the administrator, you may want to change the password, as described in “Changing the Password” on page C-4.

4. After you enter the password and click **OK**, the Administration menu displays in the iCys menu bar. From this menu you can:
   - ❍ Change the password, as described in “Changing the Password” on page C-4.
   - ❍ Make changes to the carrier display list, as described in “Using the Manage Carrier Types Window” on page C-5.
   - ❍ Add new carriers, as described in “Defining Custom Carriers” on page C-7.
   - ❍ Use the Light Meter utility as described on page C-24.
   - ❍ Calibrate the Pixel Correction Values for the Forward Scatter channels, as described on page C-26.

Changing the Password

To ensure that other users do not inadvertently make changes to the carrier type definitions, you may want to change the administrator password.

➢ To change the administration password:

1. Click **Change Password**. The Change Password window displays:

2. Enter the current password.
3. Enter the new password.
4. Confirm the new password.
5. Click **OK**. The new password will be required the next time you open the Administration menu.

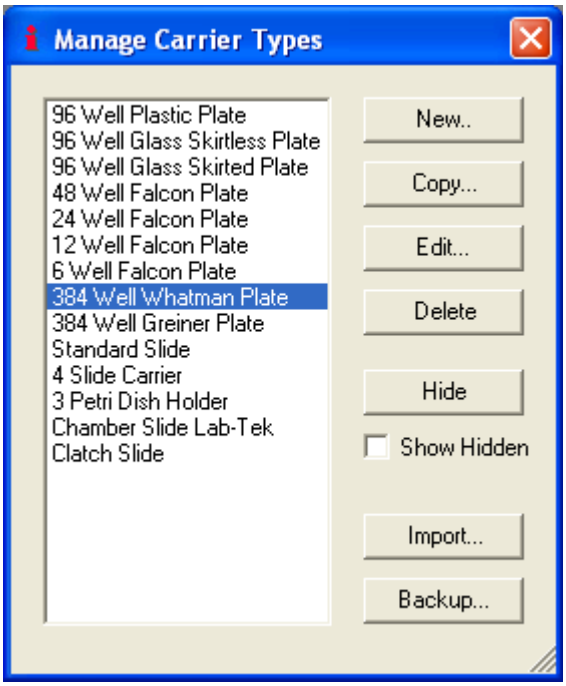
Hiding the Administration Menu

➢ To remove the Administration menu from the menu bar:

1. Select Setup ⇒ Show Administration Window. The Administration selection will have a check mark (✓) next to it indicating the Administration menu is active.
2. Highlight ✓ Administration to close menu.

Using the Manage Carrier Types Window

Select Manage Carrier Types from the Administration window to display the Manage Carrier Types window:



From the Manage Carrier Types window you can:

- ❑ Select which carriers you want your users to see in the General tab of the Instrument window, as described in “Hiding Unused Carriers” on page C-5.
- ❑ Make changes to carrier names, delete carriers from the Carrier Types list and make other changes to the supported carriers.
- ❑ Add custom carriers, as described in “Defining Custom Carriers” on page C-7.

Using the Hide Carrier Types Feature

If your site uses a subset of the carrier types supported by iCys, you can choose to hide the carrier types that you do not use. Hidden carrier types are not displayed in the Select Carrier Type window, accessed from the Carrier View.

Hiding Unused Carriers

➢ To hide carrier types:

1. From the Manage Carrier Types window, select a carrier type that you want to hide.
2. Click **Hide**.

The selected carrier type no longer appears in the Carrier Type list. More importantly, it will not display in the Select Carrier Type window, accessed from the Carrier View.

Note: CompuCyte recommends that you hide, rather than delete, unused standard carriers.

Displaying Hidden Carriers

If desired, you can show the hidden carriers in the Carrier Types list.

➢ To show hidden carriers, select Show Hidden. The hidden carriers will reappear in the list; but will not be available in the Select Carrier Type window.

Including Hidden Carriers

➢ To include hidden carriers:

1. Make sure that Show Hidden is checked.
2. Select a hidden carrier (you can tell because UnHide displays on the Hide/UnHide button).
3. Click **UnHide**. The carrier type is now included both in the Carrier Types list and from the Select Carrier Type window.

Defining Custom Carriers

CompuCyte now allows you to define custom carriers for your iCys system. Every carrier type has a unique name and ID number.

You can define a custom carrier by either copying an existing carrier type or by defining an entirely new carrier type. Either way, the new custom carrier is given a unique ID.

Tip: Before you define a new custom carrier, try to obtain the dimensions of your carrier from the supplier or from the manufacturer's website. Having the exact dimensions will greatly simplify the definition process.

➢ To define a custom carrier from the Carrier Types list:

- ❑ Select a similar carrier and click **Copy**. When you follow the directions in "Defining a Microplate Carrier" on page C-7 or "Defining a Freeform Carrier" on page C-16, keep in mind that you will be changing existing parameters rather than entering new parameters.

  CompuCyte recommends that, whenever possible, you copy and rename a similar existing carrier.

- ❑ Click **New**. In this case, follow the directions in "Defining a Microplate Carrier" on page C-7 or "Defining a Freeform Carrier" on page C-16.

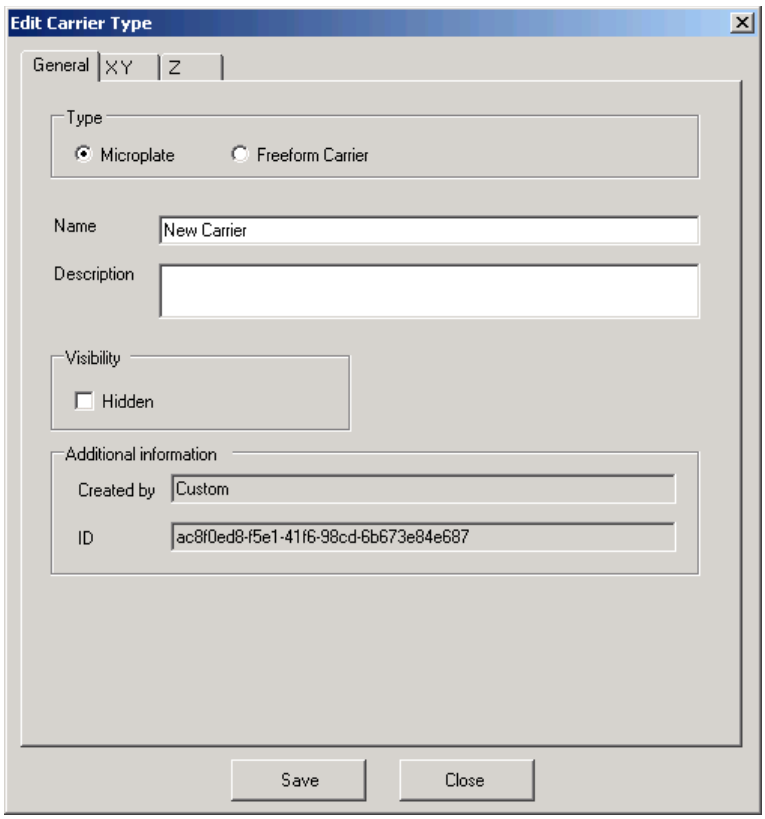
Defining a Microplate Carrier

A microplate carrier contains a specified number of wells, each of which is identical. When generating a microplate scan, iCys assumes that the scan areas are identical for every well.

Note: When defining a custom plate, the plate map (that displays when you click Carrier), may not be a precise representation of the plate. The plate wells will always appear to start in the same location in the upper left corner of the plate map. However, the distances between wells and other criteria should display correctly.

➢ To define a new microplate carrier:

1. From the Manage Carrier Types window, select **New**. The Edit Carrier Type window displays:



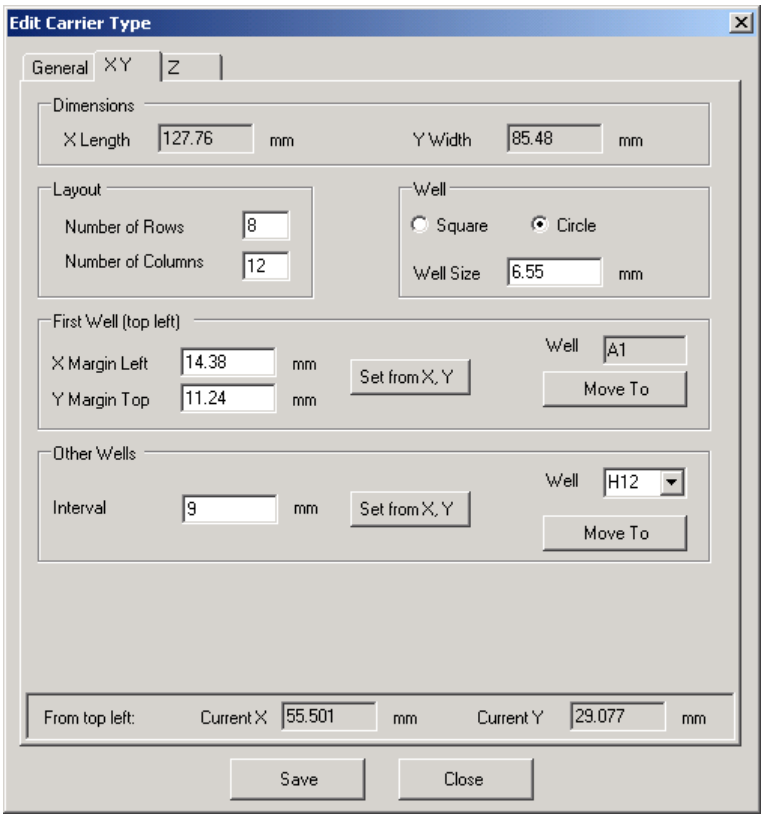
2. In the General tab, set the following:
   - **Type**—Microplate. To define a freeform carrier, see “Defining a Freeform Carrier” on page C-16.
   - **Name**—Enter a meaningful name for this carrier type. The carrier name will appear in the drop down list in the carrier window.
   - **Description**—Optional, but useful.
   - **Visibility**—Select **Hidden** if you want to make this carrier type unavailable from the Instrument Setup window. The visibility can be overwritten by the Hide/UnHide button on the Carrier Types list.
   - r

   CompuCyte automatically fills out the **Created by** and **ID** fields. The ID is unique ID for each carrier.

3. To view the carrier while you are defining it, click **Carrier** from the task bar. When you click **Save** on the Edit Carrier Type window, the Carrier window updates to reflect the changes.

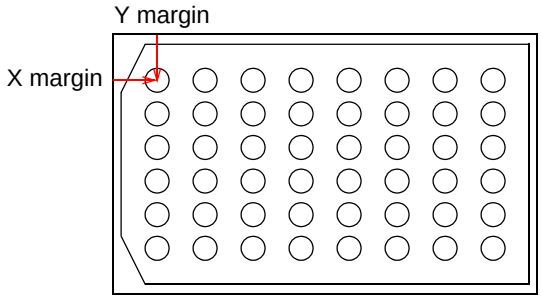
4. Before you start describing the dimensions, make sure that the microscope objective selected in the current Workspace matches the objective you are using. The objective is set in the Objective window in the task bar.

5. Open the XY tab to set the XY parameters:



When you click **New**, the default microplate descriptor is for a 96-well plate. The XY tab allows you to describe the wells in this carrier. The XY tab has the following options:

- **Dimensions**—The length and width (X and Y dimensions) are standard and cannot be changed.
- **Layout**—Allows you to specify the number of wells, as follows:
  - **Number of Rows**—The number of rows for this microplate.
  - **Number of Columns**—The number of columns for this microplate.
- **Well**—Specify the size and shape of each well:
  - Specify the well shape as either **Square** or **Circle**.
  - **Well Size**—Specify the width of the well. For a circular well, specify the diameter.
- **First well**—If you know the distance between the edge of the carrier and the A1 (top left) well, enter that distance as follows:
  - **X Margin Left**—The distance, in millimeters, from the left edge of the carrier to the center of the first well.
  - **Y Margin Top**—The distance, in millimeters, from the top edge of the carrier to the center of the first well.



If you do not know the distance, use one of the methods described in "Determining the A1 Parameters" on page C-13. When you have entered the measurements for the First well and for the Other wells (discussed below), return to Step 6.

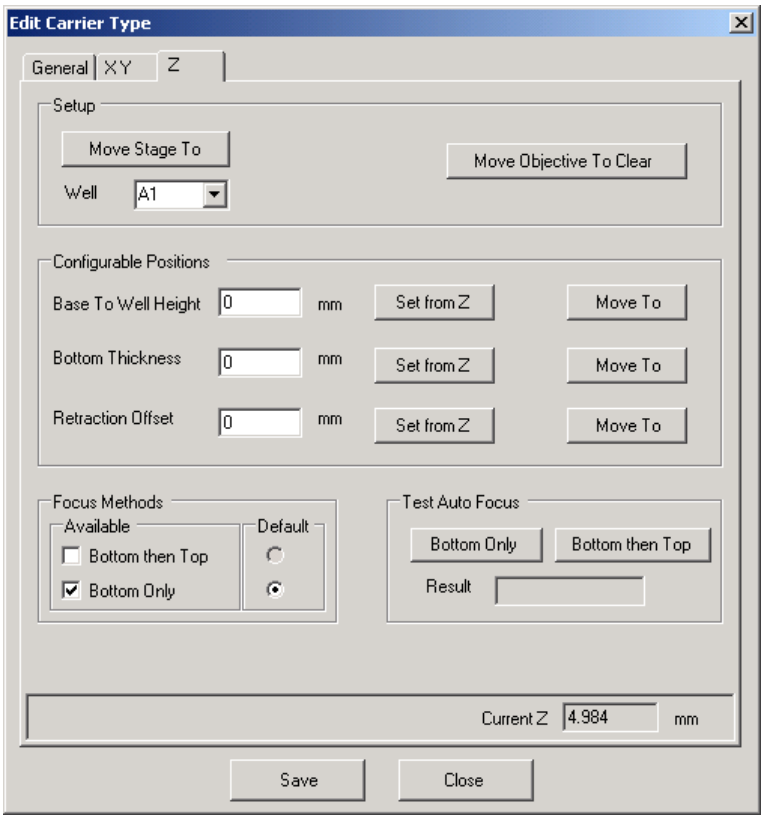
Note: The **Move To** (or **Move Stage To**) and **Set from XY** buttons are discussed in the context of "Determining the A1 Parameters" on page C-13.

- **Other Wells**—Specifies the distance between each well.

  Interval—Specify the distance from the center of one well to the center of the next well. For microplate carriers, the wells are spaced so the interval is the same from left to right and from up to down.

  If you do not know the distance, use one of the methods described in "Setting the Interval using iCys" on page C-14. When you have entered the measurements for the First well and for the Other wells, return to Step 6.

6. Open the Z tab to set the Z parameters:

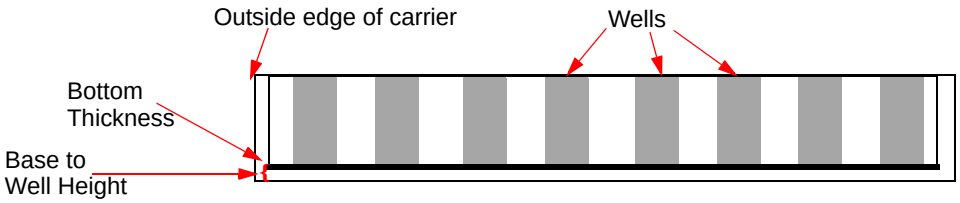


The Z tab allows you to specify, or determine, the appropriate start AutoFocus positions for the carrier. The parameters are:

- **Setup**—Allows you to move the stage to a well to calibrate or to the Clear position (near Home).
  - **Well**—Select which well you want to use to calibrate the Z parameter. Select the well in the Well pull-down and click **Move To**. The stage moves to the specified well.
  - **Move Objective to Clear**—Click to move the objective to the Clear position.
- **Configurable Positions**—Specifies the required values for the plate height (the Z parameter):
  - **Base to Well Height**—Specifies the distance from the base of the carrier to the bottom of the plate.
  - **Bottom Thickness**—Specifies the thickness of the glass or plastic through which the microscope needs to focus.

For example:

Carrier Side View



If you do not know the measurements for Base to Well Height or Bottom Thickness, use the method described in "Determining the Microplate Z Parameters" on page C-14. When you have entered these measurements, return to Focus Methods, below.

- ✧ **Retraction Offset**—Only used with some Freeform Carriers. For more information, see "Defining a Freeform Carrier" on page C-16.

❍ **Focus Methods**—Specify the preferred (or required) focus method.

- ✧ **Bottom then Top**—For thicker plastic-bottomed plates (greater than 400µ) especially; iCys will focus first on the plate bottom, then translate up by the bottom thickness to the plate top and refocus.
- ✧ **Bottom Only**—For glass-bottomed or thinner plastic-bottomed (less than 400µ) plates, iCys focuses directly on the bottom then moves up by the bottom thickness plus other offsets to bring the sample into focus.
- ✧ **Default**—To allow your users to select a focus method (as discussed in "Focus and Performance" on page 3-33), select both focus methods and then select the method that you want to use as a default setting.

Note: If the Bottom Thickness is less than 400µ, select Bottom Only as the only available focus method. iCys will set the default focus method to match.

❍ **Test Autofocus**—To test whether you have the values for your carrier set correctly, you can run Test Autofocus. To test under working conditions, CompuCyte suggests that you fill the test wells with water or another buffered solution (such as phosphate-buffered saline [PBS].)

Test Autofocus tries to focus the objective correctly on the carrier and produces a result number, as follows:

- ✧ If the Test Autofocus Result is greater than 800, the parameters are correctly set.
- ✧ If Autofocus did not work, an error message will display. Check the General tab of the Instrument Setup window (and the Focus parameter in the task bar) to ensure that Autofocus is selected. You may also need to check the values of the X,Y, and Z parameters for errors.
- ✧ If the Test Autofocus Result is between 0 and 800, try to refine the values for the carrier and retest.

7. When you are happy with the results, click Save to save your changes. Close the Edit Carrier Type window.

Determining the A1 Parameters

Finding the A1 Position by Measuring

➢ To determine the position of the top left (A1) well:

1. Measure the distance between the right edge of the microplate (closest to A1) and the center of the A1 well. Enter this value as the X Margin Left.
2. Measure the distance between the top edge of the plate (closest to A1) and the center of the A1 well. Enter this value as the Y Margin Top.
3. Measure the distance from the center of well A1 well to the center of the well A2 well. and Enter this value as the Interval in the Other Wells section.
4. If you cannot accurately measure the distance of the A1 well from the edges, see "Finding the A1 Position using iCys" on page C-13.

Finding the A1 Position using iCys

➢ To use iCys to find the A1 position:

1. Load your custom microplate onto the microscope stage, as described in "Loading a Microtiter Plate" on page 2-18.
2. Open the Oscilloscope window by selecting Instrument ⇒ Oscilloscope.

Tip: When determining measurements, the oscilloscope is only used to turn the galvanometer on and off. For more information about the oscilloscope, see "Using the Oscilloscope Feature" on page 3-90.

3. From the Oscilloscope window, turn on the oscilloscope by clicking the "Play or Stop" button (▶).
4. Place a piece of thin white paper over the A1 well of the microplate. From the XY tab of the Edit Carrier Type window, click the **Move To** button in the First well section.
5. Select the Argon Laser *488 from the task bar to open the shutter.

Warning: When using paper to find the well measurements, avoid direct eye exposure to the Class 3R radiation.

6. If the beam is not centered in well A1, use the touchpad buttons or the Remote Control unit X and Y knobs to drive the stage such that the beam is centered in the A1 well.
7. Drive the objective lens upwards until you see a line (or pair of lines) on the Autofocus (black and white) monitor. Adjust the focus until you are satisfied with the results. Recheck, and if needed, recenter the beam in A1.
8. When the beam is properly centered and focused, turn the oscilloscope trace off (by clicking on the "Play or Stop" arrow [▶]). Click **Set from X,Y** in the First

Well section of the XY tab to set the location. The X and Y margin parameters are automatically filled in by iCys.

Note: Always turn the oscilloscope off and wait for the Current X and Current Y values (at the bottom of the XY tab) to update before you click **Set from X,Y**.

The update may take a few moments, but will ensure that the measurement is correct.

Setting the Interval using iCys

➢ To set the interval, as described in Step 5, above:

1. Turn on the oscilloscope trace, and open the Argon Laser Shutter, then select B2 from the Well pull-down list in the Other Wells section of the XY tab.
2. View the beam, using a piece of thin white paper placed over well B2, and adjust the stage so the beam is centered in the well.
3. Turn off the oscilloscope and click the **Set from X,Y** button in the Other Wells section. The Interval parameter is automatically filled in by iCys.
4. To make sure that the interval is correct, select the last well H12 for a 96-well plate) in your microplate from the Other Wells Well pull-down list and click **Move To** to drive the stage to that well. Check that the beam is centered on the last well, again using thin white paper. If it is not centered, adjust the interval (but do not use the last well) and check again using the last well.

Determining the Microplate Z Parameters

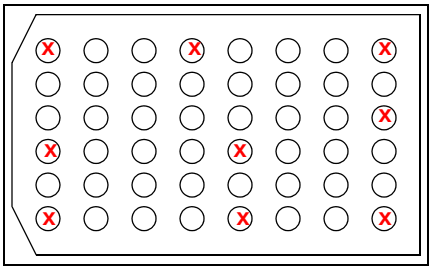
Finding the Base to Well Height

➢ To determine the Base to Well Height:

1. Click on the Z tab in the Edit Carrier window.
2. With the stage at well A1, the same objective in place (as displayed in the task bar), and the Oscilloscope window open and on, and the Argon Laser Shutter open, use the rocker switch on the Remote Control unit to drive the objective upwards slowly until you observe a reasonably well focused line or lines on the autofocus monitor. Make sure that you are focusing on the first line or pair of lines to be imaged on the display.
3. Use the Fine Focus knob on the Remote Control unit to create a well focused image.
4. Click **Set from Z** for the Base To Well Height field in the Z tab. Mark down this value, and then take the same measurement at a number of other points on the plate, such as:
   - ❍ The other three corner wells
   - ❍ A well near the center of the plate

- A well at or near the center top row
- A well at or near the center bottom row
- A well at or near the center left column
- A well at or near the center right column

For example:



5. Determine which of these 9 positions yields the lowest value, and enter that value as the Base To Well Height.

Determining the Bottom Thickness

➢ To determine the bottom thickness:

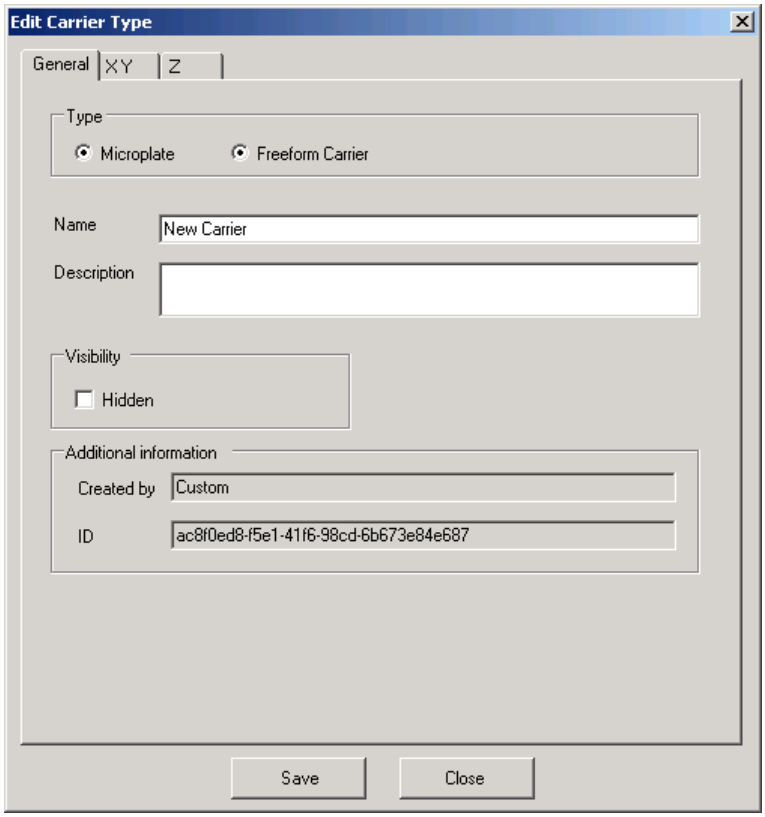
1. Move the stage so that the objective focuses on the well with the lowest Base to Well Height (which you determined in “Finding the Base to Well Height” on page C-14), and click on **Move To** from the Base to Well Height field.
2. Make sure the well you use for focusing is empty.
3. Slowly rotate the fine focus knob counter-clockwise until the second line or lines appear to be finely focused on the autofocus monitor. For thicker-bottomed plates, this may take 5 or more knob rotations.Click **Set from Z** for the Bottom Thickness field of the Z tab.

Defining a Freeform Carrier

Rather than consisting of uniform wells, a freeform carrier can contain one or more carrier display areas, which do not have to be identical (but usually are).

➢ To define a new freeform carrier:

1. From the Manage Carrier Types window, select **New**. The Edit Carrier Type window displays:

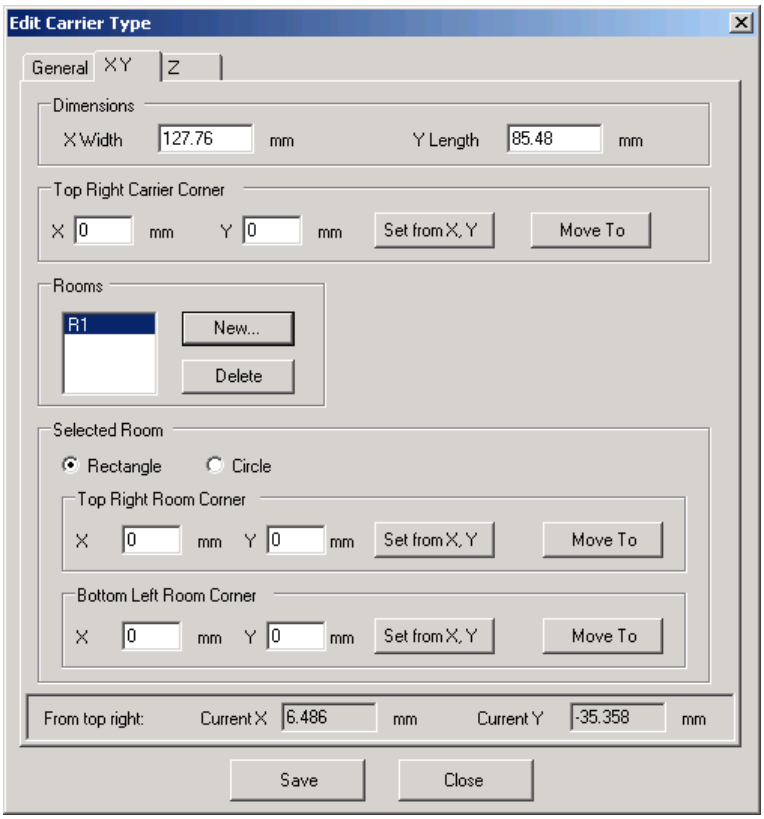


2. In the General tab, set the following:
   - **Type**—Freeform Carrier. To define a microplate carrier, see “Defining a Microplate Carrier” on page C-7.
   - **Name**—Enter a meaningful name for this carrier type. Keep in mind that your users will select this carrier type using the name specified here.
   - **Description**—Optional, but useful.
   - **Visibility**—Select **Hidden** if you want to make this carrier type unavailable from the Instrument Setup window. The visibility can be overwritten by the Hide/UnHide button on the Carrier Types list.
3. rTo view the carrier while you are defining it, click **Carrier** from the task bar. When you click **Save** on the Edit Carrier Type window, the Carrier window updates to reflect the changes.
4. Before you start describing the dimensions, make sure that the microscope objective selected in the current Workspace matches the objective you are using.

The objective is set in the General tab of the Instrument Setup window and listed in the task bar.

5. Open the XY tab to set the XY parameters.

6. In the Rooms section, select **New**. Room R1 will be created and the XY tab displays the parameters for that room:



7. In the XY tab, you can set the following general parameters:

   - **Dimension**—The dimensions of the display area. In general, CompuCyte suggests that you do not make changes to the Dimension values, and display the entire carrier.

     - **X Width**—The width of the display area, in millimeters.

     - **Y Length**—The length of the display area, in millimeters.

Note: If you specify the carrier display area (or copy a carrier that specifies the display area), keep in mind that the display area must be smaller than the standard carrier size, which is 127.76mm x 85.48mm. Additionally, when specifying room locations, all rooms must reside within the display area.

   - **Top Right Carrier Corner**—The distance between the outside edge of the carrier and the edge of the top right carrier display area. CompuCyte suggests that you set both the X and Y value to 0.

- **Rooms**—Click New (or Delete, if necessary) in the Rooms section to add rooms until the number of rooms in the display equals the number of rooms on the carrier.

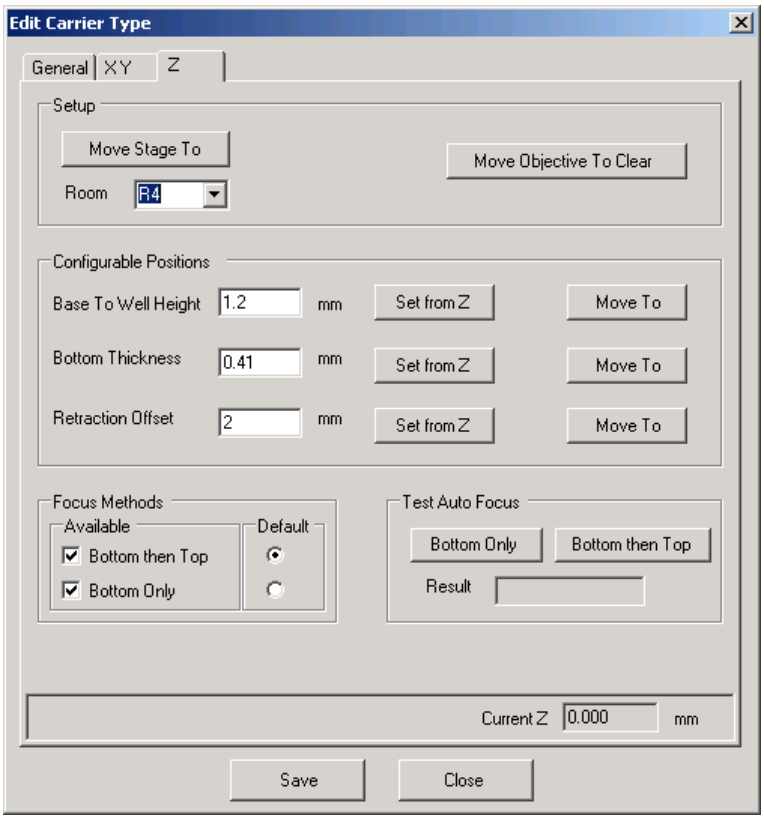
8. Define each room in the XY tab. You need to define the parameters separately for each room. Select a room from the Rooms pull-down.The Select Room section displays.

If you know the exact shape, location, and dimensions for the room, enter them as follows:

- **Rectangle** or **Circle**—Specify the shape of the room.
- For a Rectangle:
  - **Top Right Room Corner** (X and Y values)—The location of the top right corner of the room relative to the X and Y values of the **Dimension** field (described in Step 7).
  - **Bottom Left Room Corner** (X and Y values)—The location of the bottom left corner of the room relative to the X and Y values of the **Dimension** field.
- For a Circle:
  - **Center** (X and Y values)—The center of this room relative to the X and Y values of the **Dimension** field (described in Step 7).
  - **Diameter** (in millimeters)—The diameter of the room.

If you do not know these values, determine them as described in “Defining a Room Using iCys” on page C-21.

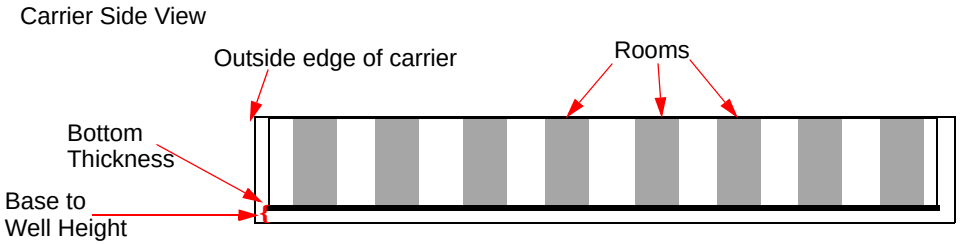
9. Open the Z tab to set the Z parameters:



The Z tab allows you to specify, or determine, the appropriate start AutoFocus positions for the carrier. The parameters are:

- **Setup**—Allows you to move the stage to a well to calibrate or to the Clear position.
  - **Room**—Allows you to specify which room you want to use to calibrate the Z parameter. Select the room in the Room pull-down and click Move Stage To. The stage moves to the specified room.
  - **Move Objective to Clear**—Click to move the objective to the Clear position.
- **Configurable Positions**—Specifies the required values for the carrier height (the Z parameter):
  - **Base to Well Height**—Specifies the distance from the base of the carrier to the bottom of the room.
  - **Bottom Thickness**—Specifies the thickness of the glass or plastic through which the microscope needs to focus. If you use #1 coverglass with this carrier, the value is *0.12*.

    For example:



If you do not know the measurements for Base to Well Height or Bottom Thickness, use the method described in "Determining the Freeform Z Parameters" on page C-22. When you have entered these measurements, return to Focus Methods, below.

✧ **Retraction Offset**—The Retraction Offset is only needed if you have a carrier that may be bumped by the microscope when moving from room to room (the only standard carrier for which this is an issue is the 3 Room Petri Dish). If a Retraction Offset is required, but you do not know it, you can find it as follows:

– Click on Instrument ⇒ Home Objective. Manually move the stage to a position *between* rooms using the touchpad or Remote Control unit knobs.

– As described in "Finding the Base to Well Height" on page C-22, use the Remote Control unit knobs and the autofocus monitor to focus on the top of the carrier.

– When you have a clear focus, click Set from Z for the Retraction Offset value.

❍ **Focus Methods**—Specify the preferred (or required) focus method.

✧ **Bottom then Top**—For thicker plastic-bottomed plates (greater than 400µ) especially; iCys will focus first on the plate bottom, then translate up by the bottom thickness to the plate top and refocus.

✧ **Bottom Only**—For glass-bottomed or thinner plastic-bottomed (less than 400µ) plates, iCys focuses directly on the bottom then moves up by the bottom thickness plus other offsets to bring the sample into focus.

✧ **Default**—If you want to allow your users to select a focus method (as discussed in "Focus and Performance" on page 3-33), select both focus methods and then select the method that you want to use as a default setting.

Note: If the Bottom Thickness is less than 400µ, select Bottom Only as the only available focus method. iCys will set the default focus method to match.

❍ **Test Autofocus**—To test whether you have the values for your carrier set correctly, you can run Test Autofocus. To test under working conditions, CompuCyte suggests that you fill the test wells with water or another buffered solution (such as phosphate-buffered saline [PBS].)

- ✧ If the Test Autofocus Result is greater than 800, the parameters are correctly set.
- ✧ If Autofocus did not work, an error message will display. Check the General tab of the Instrument Setup window (and the Focus parameter in the task bar) to ensure that Autofocus is selected. You may also need to check the values of the X,Y, and Z parameters for errors.
- ✧ If the Test Autofocus Result is between 0 and 800, try to refine the values for the carrier and retest.

10. After you set the parameters, test the offset: Cycle through the rooms, clicking on **Move To** under the Selected Room section to ensure that the parameters are correct.

11. When you are happy with the results, click Save to save your changes. Close the Edit Carrier Type window.

Defining a Room Using iCys

➢ To determine the values for a room:

1. Load your custom carrier onto the microscope stage, as described in “Loading Other Carrier Types” on page 2-19. You may want to pick the type of carrier closest to your custom carrier.

2. Open the Oscilloscope window by selecting Instrument ⇒ Oscilloscope.

Tip:	When determining measurements, the oscilloscope is only used to turn the galvanometer on and off. For more information about the oscilloscope, see “Using the Oscilloscope Feature” on page 3-90.

3. From the Oscilloscope window, turn on the oscilloscope by clicking the “Play or Stop” button (▶).

4. Place a piece of thin white paper over the selected room of your carrier.

5. Select the Argon Laser *488 from the task bar to open the shutter.

Warning:	When using paper to find the room measurements, avoid direct eye exposure to the Class 3R radiation.

6. In the Selected Room section of the XY tab, specify Rectangle or Circle for the room shape.

7. Use the touchpad buttons and Remote Control unit X and Y knobs to drive the stage until the room is over the microscope objective.

If the room is a **Rectangle**:

a. Adjust the stage until the top (back) right corner of the room is over the objective (use the white paper to view the projection of the beam).

b. Turn the oscilloscope off by clicking the “Play or Stop” button (▶|).

c. Press **Set from X,Y** for the Top Right Room Corner measurement.

d. Turn the oscilloscope on again, open shutter, and adjust the stage until the bottom (front) left corner of the room is over the objective.

e. Press **Set from X,Y** for the Bottom Left Room Corner.

If the room is a **Circle**:

a. Use the touchpad buttons and Remote Control unit X and Y knobs to drive the stage to a position such that the room is centered over the microscope beam.

b. Turn the oscilloscope off by clicking the “Play or Stop” button (▶|).

c. Press **Set from X,Y** for the **Center** measurement.

d. Turn the oscilloscope on again and open shutter.

e. For the Diameter measurement, you can measure the room diameter manually, look up the room size on the specification sheet for this carrier and enter it for the **Diameter** value, or use the Remote Control unit X and Y knobs to drive to the edge of the room and click on **Set from X, Y** for the Diameter field.

8. Repeat Step 8 for each room you need to define.

Determining the Freeform Z Parameters

Finding the Base to Well Height

➢ To determine the Base to Well Height:

1. With the stage at room R1, the same objective in place as displayed in the task bar, and the Oscilloscope window open and on, and the Argon Laser Shutter open, use the rocker switch on the Remote Control unit to drive the objective upwards slowly until you observe a reasonably well focused line or lines on the autofocus monitor. Make sure that you are focusing on the first line or pair of lines to be imaged on the display.

2. Use the Fine Focus knob on the Remote Control unit to create a well focused image.

1. Click **Set from Z** for the Base To Well Height field in the Z tab. Mark down this value, and then take the same measurement at least once each room on the carrier (so that you have measurements for a number of positions on the carrier.)

2. Enter the lowest value measured as the **Base to Well Height**.

Determining the Bottom Thickness

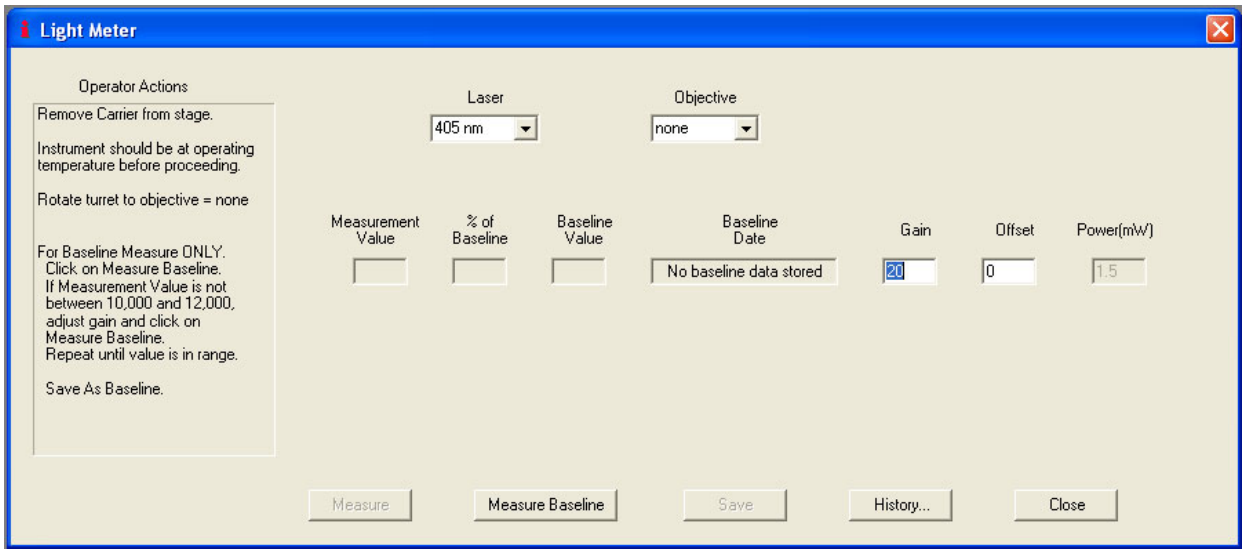
➢ To determine the bottom thickness:

1. Move the stage so that the objective focuses on the room with the lowest Base to Well Height (which you determined in “Finding the Base to Well Height” on page C-14), and click on **Move To** from the Base to Well Height field.
2. Make sure the room you use for focusing is empty.
3. Slowly rotate the fine focus knob counter-clockwise until the second line or lines appear to be finely focused on the autofocus monitor. For thicker-bottomed plates, this may take 5 or more knob rotations.Click **Set from Z** for the Bottom Thickness field of the Z tab.

Using the Light Meter Utility

The Light Meter utility allows you to measure the throughput of the system lasers, and determine if any action is needed to improve laser throughput.

➢ To use the Light Meter Utility, click on Light Meter. The following window displays:



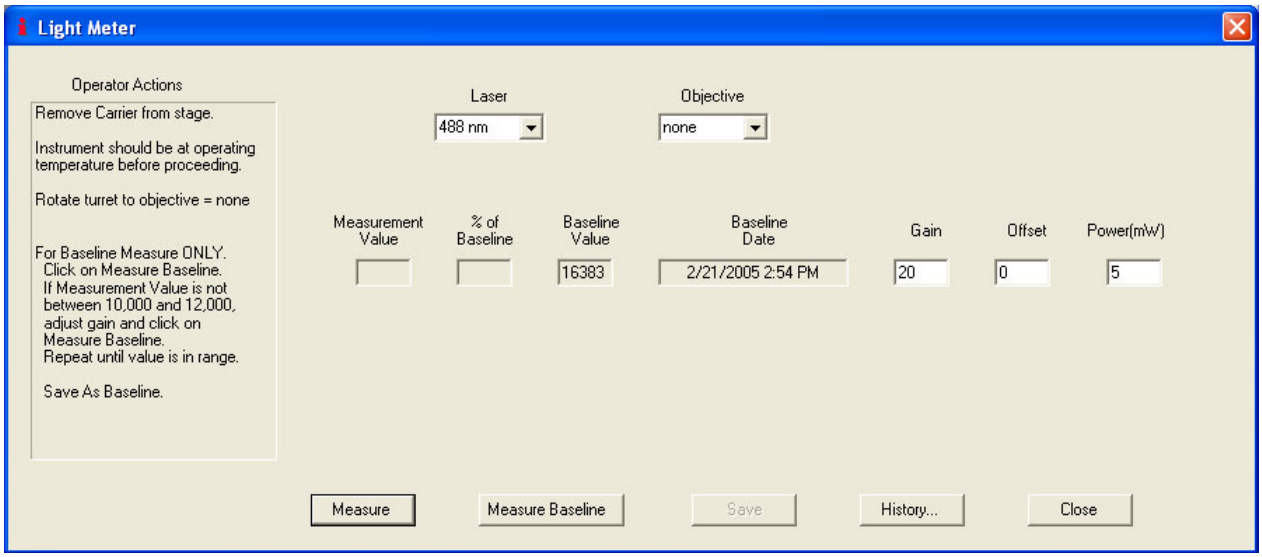
1. Follow the directions in the Operator Actions window, that is, make sure that:
   - ❍ The iCys and the lasers are warmed up.
   - ❍ There is no carrier on the stage.
   - ❍ For the primary Argon measurement, set the turret so no objective is selected. If necessary, unscrew the cap in the empty objective slot.

Note: If your system does not have AutoFocus, you will need to manual move the objective to be level with the stage. A message will display reminding you to do so.

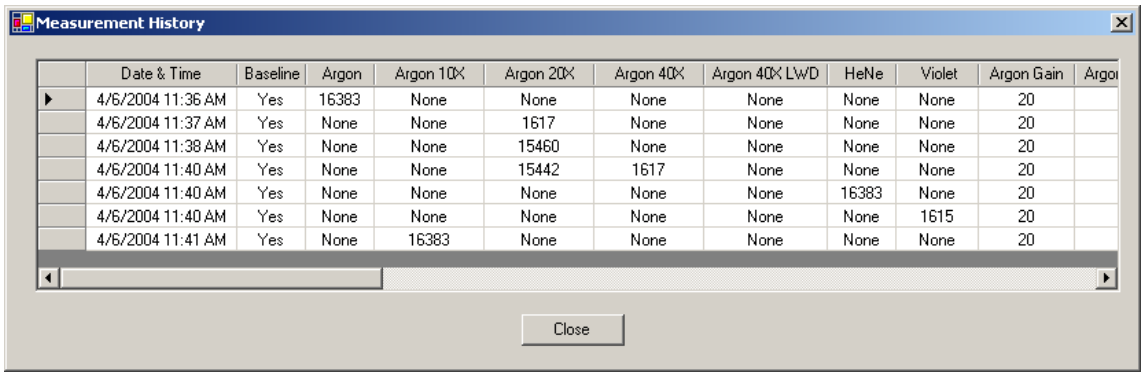
2. Once the instrument is correctly set up and warmed up, select Argon (488 nm.) and click the **Measure** or **Measure Baseline** button. (If no Baseline data is available, only the Measure Baseline button is available.) The measurement is recorded in the appropriate window.
3. iCys digitizes the scan line produced by the Argon laser. To save that measurement, click **Save**.
4. Ideally, you want the measurement value to be close to, but not at, the maximum signal intensity of 16383. To get good readings, you may need to adjust the Gain, Offset, and for the Argon measurements, the power. These settings are described in “Optimizing PMT settings” on page 3-39.

5. Measure and, if desired, save, measurements for any other objectives and lasers. Note that the objectives only need to measured once (with the Argon laser). If they are dirty, then it will affect the measurements for any laser.

6. Once the measurement has been saved as the baseline, you can remeasure it at any time. The new measurement is taken against the baseline, as shown:



7. To view the current measurements, click History to display the Measurement History file:



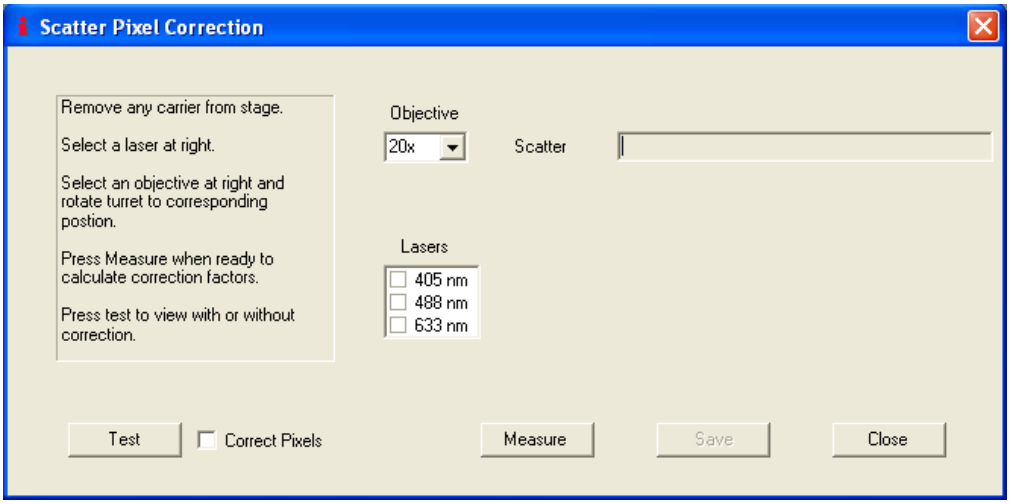
Date & Time	Baseline	Argon	Argon 10X	Argon 20X	Argon 40X	Argon 40X LWD	HeNe	Violet	Argon Gain	Argo
4/6/2004 11:36 AM	Yes	16383	None	None	None	None	None	None	20	
4/6/2004 11:37 AM	Yes	None	None	1617	None	None	None	None	20	
4/6/2004 11:38 AM	Yes	None	None	15460	None	None	None	None	20	
4/6/2004 11:40 AM	Yes	None	None	15442	1617	None	None	None	20	
4/6/2004 11:40 AM	Yes	None	None	None	None	None	16383	None	20	
4/6/2004 11:40 AM	Yes	None	None	None	None	None	None	1615	20	
4/6/2004 11:41 AM	Yes	None	16383	None	None	None	None	None	20	

The History file can also help you track any change in measurement. If the objectives need to be cleaned, for example, their measurements will degrade. In this case, contact your CompuCyte service representative.

Forward Scatter Pixel Correction

When images are collected using the forward scatter mechanism, variations in pixel intensity are produced because the laser beam strikes the sensor at various angles during its “sweep.” In order to produce the best quality images, these variations must be corrected.

The Administration menu offers a Forward Scatter Pixel Correction utility. The opening screen is shown below:



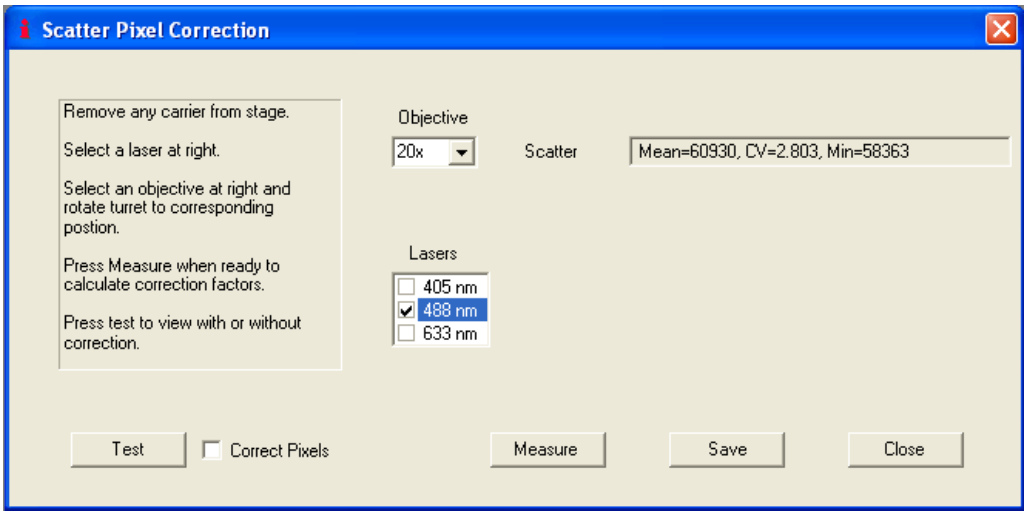
The textbox on the left contains instructions on how to proceed. List boxes allow selection of a laser and objective. Each combination of laser and objective must be corrected.

Once selections are made, press the Measure button. The utility will move the empty stage to the center and automatically adjust the gain and offset for the best exposure. The textbox on the left will display the steps of the measurement.

The minimum and maximum values of pixel brightness required to pass these adjustments are stored in the calibration file under “scatter-auto-exposure.” The factory settings should work in most circumstances.

As the adjustments progress, you can see the results in the image window. When the adjustments are complete, the textbox will display the message “Scan complete.” When the measurement is complete, a message box will display the message “Measurement complete.”

After clicking OK on the message box, you will note that the Save button has become available. You may elect to save the correction factors now or wait until you make another measurement. When the Measure button is pressed again, the current correction data will be replaced with new data.



Before saving, it is wise to review the results of the correction. This can be done by pressing the Test button with the Correct Pixels checkbox checked. The utility will collect and display one scan field of data, either corrected or uncorrected, depending on whether the checkbox is checked.

Once you are satisfied, the data can be saved. If the utility is exited with data unsaved a message will be displayed asking if the data should be saved or not.

Appendix *D*

Glossary

This chapter contains a glossary of terms used with iCys™ Research Imaging Cytometer, iCys™ Cytometric Analysis Software and iBrowser™ Data Integration Software.

To find a term quickly, click on one of the following letters:

A B C D E F G H I L M N 0 P Q R S T V W

Glossary of Terms

A

access key The key that corresponds to an underlined letter on a menu, command, or dialog box option.

active window An area of the screen where the next action will be performed. The active window appears in the foreground and the title bar is highlighted.

acquisition Consists of a sequence of processes some of which are: detecting the signals of interest, performing suitable analog signal processing, converting the data to a digital representation and then storing the data in a computer memory for analysis.

aggregate Particles or cells that have clustered or united in a mass.

algorithm A set of procedures by which a given result can be obtained. A computer can carry out the steps of many different types of algorithms.

amplifier An electronic device which increases the voltage or current of a signal to allow additional signal processing such as conversion to digital representation.

analog Representation of an electronic signal by a continuum of values.

Analog-to-Digital Converter (ADC) Converts an analog signal to a digital or “discreet value” representation to enable processing by a computer or other digital electronics systems.

analysis Analysis subsequent to data acquisition consists of any number of processes performed on the data including: segmenting and contouring the data to determine valid events, calculating a fixed set of features for each of the events, and producing correlated displays for selected features.

argon ion laser The principle light source in the iCys generating 5-20 mW of coherent blue light output at a single line wavelength of 488 mW.

B

background Measured signal content which is not generated by a cell or particle but by intrinsic properties of the reagent or substrate, by optical anomalies in the light path or by electronic noise of the signal detection system.

bar chart A graph consisting of parallel vertical bars or rectangles with lengths proportional to specific quantities.

bitmap An area in the computer’s memory organized so that each unit of memory in the bitmap represents the color of one pixel.

.BMP file A graphic file format which is the native bitmap format of Microsoft®Windows™ software.

C

carrier

The microtiter plate or other tray on which specimens are viewed. CompuCyte supports a number of different carrier types, including microplates, slide trays, 4-room slide suites, and petri dish carriers.

carrier display area

The area within a carrier shown in iCys. For example, for a slide carrier, the carrier display area is generally 125mm by 75mm (the dimensions of the slide), while the carrier itself is much larger.

channel

A channel refers to one of the six filter/detector data acquisition paths in the iCys. The six channels are four fluorescent, one scatter, and one for summed signals.

Channel is also used to refer to the assignment of ranges of light intensity that result from converting an electrical signal from a photodetector into a digital value. A histogram is the graphical representation of the channel assignment where the x axis represents increasing signal intensity.

check box

A square box associated with an option in a dialog box. When there is a check in the box, that option is selected. More than one check box can be selected.

circularity

The measurement of how close an event is to a perfect circle. The circularity is determined by:

$\text{perimeter}^2/\text{area}$

Therefore, the circularity of a perfect circle is 4π (12.566). The larger the circularity number, the less circular it is.

Note that, due to normalization, the circularity of an event may be less than 12.566.

click

An action that is performed by quickly pressing and releasing a button on the mouse. The action selects a menu, a command, moves an insertion point, or selects a graphical object so that you can work with it. It also places the insertion point in a text box.

clipboard

A portion of computer memory that is set aside for temporary storage of whatever was last cut or copied. Material moved to the clipboard can be pasted into another application.

coefficient of variation (CV)

A statistical quantity which describes the variation or spread of a probability distribution (or of a cell size distribution). Mathematically, it is calculated by dividing the mean of the distribution by the standard deviation of the distribution.

color depth

A measure of how much color information is available for each pixel in an image. A pixel with a color depth of 1 has two possible values: black and white. A color depth of 8 has 2^8, or 256 colors and a color depth of 24 has 2^{24}, or roughly 16 million colors.

component

A group of events generated from single contouring process.

composite image

An image generated during the first phase of a two-step scan. The image is comprised of multiple scan fields, each of which is 20 microns wide and runs the length of the scan area (which may be the width of a slide, a well, or a petri dish). The composite image is used by iCys to determine the meaningful events within the larger area. These events are then scanned using normal scanning techniques.

CompuColor A method of assigning colors to scanned images of samples.

context-sensitive Information located on the computer to help a user while in a program. It provides information when and where the user wants it.

contouring The event detection process in the iCys which results in a two-dimensional outline of the cell. There are separate contours for threshold, integral, background, or peripheral.

contour histogram Plots the values of two user-selected features such that the frequency of occurrence is displayed as a 3D bar graph with color defining frequency.

control A standard against which observations or conclusions of other samples may be checked for their validity.

CV *See* coefficient of variation

D

data file A file with an .EVT or .RAW extension that was saved to disk which has measured values from a sample.

desktop A window in which all the menus and tools to operate a specific application are located.

dichroic mirror An optical element which selectively reflects one bandpass in the optical spectrum while simultaneously transmitting a second bandpass. A dichroic mirror is usually positioned so that the incident beam is 45° to the mirror surface, the reflected light thus being 90° to the incident beam.

dimmed An unavailable menu item or option. It is unavailable to the user because it is inappropriate or not selected at that time.

diploid Cells in the initial cell cycle stage that have two sets of chromosomes.

directory A file containing the names and locations of all the files contained on a storage medium.

double-click A user action which quickly presses and releases the left mouse button twice to select an action based on the cursor location.

drag-and-drop A method of moving or copying text, graphics, or other items from one window to another.

drop-down arrow An arrow on a dialog box that points down indicating that when pressed more options will be displayed.

E

ellipsis points Ellipsis points appended to a menu item indicate that when selected a dialog box will be displayed with more options.

event A particle, cell, or aggregate that has features that can be measured by a cytometer.

EVT file An iCys file type which contains features for all the events that have been scanned.

F

features A set of physical and/or chemical characteristics of a event (particle, cell, etc.) as measured by the iCys. Such features include area, integral, max pixel, time, x position, y position, scan, distance, perimeter, perimeter^2, # of spots, perimeter integral, texture, and Fish integral.

filename A unique name used to identify a location where information is stored.

fluorescence A form of light emitted by atoms or molecules when electrons fall from excited energy levels to their lower less-energetic ground state.

fluorochrome A dye that absorbs light at a particular wavelength and then emits light at a higher wavelength.

folder A container for a group of files (usually related in some way). It is used to organize the files on your disk.

font A collection of alphanumeric characters and symbols that are in a consistent and unique typeface.

forward scatter Light that has been deviated at relatively small angles to the incident beam as it passes through a particle.

four-room suite A slide tray that contains four slides. Each slide, or room, generally contains 2 scan areas (“beds”). Within iCys and iBrowser, output from a slide is generally referred to by its Room/Bed location; the first scan area on the third slide has the WellID R3B1 (Room 3, Bed 1).

FWHM (Full Width Half Maximum) A width of a curve (generally assumed to be Gaussian) at half its maximum value. A method for calculating the CV of a histogram in which the histogram FWHM is divided by 2.345 times the standard deviation.

G

gain An electronic circuit which increases the current or voltage of the input signal to enable subsequent processing.

gallery A collection of cell images captured by iCys software.

galvanometer An electromechanical device which, in the iCys system, converts an electronic current to a rotational displacement. With a mirror attached, it enables a fixed laser beam incident on the mirror to be converted to a scanning laser beam.

gate A region defined on a histogram or scattergram which acts as a software filter for acquired events. Events within the window may be subsequently processed by looking at additional parameters.

grayscale An image type that contains more than just black and white, and includes actual shades of gray. In a grayscale image, each pixel has more bits of information

encoded in it, allowing more shades to be recorded and shown. 8 bits can reproduce a photo-realistic 256 shades of gray.

group box A standard control used to group a set of options in a dialog box.

H

hard disk A permanent storage device either mounted inside a computer or as an external device in its own case.

HeNe laser (Helium-neon) A light source emitting red light at 633 nm with a power output of approximately 5 mW. (This definition is specific for the iCys.)

histogram A two-dimensional graphical means of presenting data for measured features. The horizontal axis of the represents signal intensity of the feature while the vertical axis represents the number of events (counts).

I

icon A symbol on the screen that simplifies access to a program, command, or data file.

iCys Cytometric Analysis Software The software application that configures the iCys system, governs the scanning operations, and collects the scan data.

iCys Research Imaging Cytometer The laser scanning cytometer that includes the scanning mirrors, lenses, photomultipliers and all other components required to perform the laser scanning operations.

immunofluor-escence The use of fluorocarbon-labeled antibodies to detect or quantitate cell surface markers.

insertion point The position, indicated by a blinking vertical bar, at which text is entered into a report.

integration contour An event contour that is a fixed number of pixels outside the threshold contour. The event's total or integrated fluorescence is computed by adding all the pixel values within the contour.

isometric plot A plot mode in which two user-selected features are displayed with the frequency of occurrence being represented as a 3D surface.

L

laser (Light Amplification by Simulated Emission of Radiation) A device that converts various frequencies of light into one small and extremely intense beam of one wavelength radiation.

laser scan image A bit map image of the raw data that is detected by the iCys for a scanned area of the slide.

lens A transparent refracting device in a microscope, usually made of glass, that changes the shape of a beam of light.

linear amplifier An electronic device which produces an output current or voltage directly proportional to the input current or voltage.

log scale A scale used to increase the dynamic range of the displayed data.

M

Max Pixel One of the measured features of an event. It is the measurement of the pixel within the event contour having the largest value (highest intensity of fluorescence or scatter).

mean A value calculated by finding the sum of the channel numbers from all the cells in the sample and dividing it by the total number of cells in the sample.

median A value obtained by lining up the cells in order of increasing intensity and taking the channel number of the cell that is at the midpoint in that sequence.

mode *See* peak

multiplexing The process in computers and instrumentation of switching several different analog signals in succession to the input of a single analog-to-

digital converter and then storing the output data in sequence.

N

network A group of interconnected computers which are linked together for the purpose of sharing files, programs, or peripherals.

normalize A software process of automatically setting the scale and offset so that:

- An axis displaying Max Pixel makes a cell with the maximum possible Max Pixel appear in the last channel
- An axis displaying Scan makes a cell with the maximum possible scan number (767) appear in the last channel

0

objective A microscope lens which magnifies the specimen on the stage by a specified power (such as 10X, 20X, or 40X).

offset A shift in the position of the data in a histogram or scattergram relative to its original position. An offset results in a shift of the data along the selected axis.

operating system (OS) The software that controls the execution of computer programs of a specific computer system.

optical filter An optical component which either transmits or attenuates incident light of specific wavelength.

optical sectioning A series of scans of the same well or scan area at different focal points. The result is a series of scans that move through the sample from top to bottom by a specified distance. Optical sectioning is a form of repetitive scanning.

option button The round buttons in a dialog box that indicate a mutually exclusive choice (for example, a choice between Portrait or Landscape).

P

palette A grayscale palette containing color definitions for a laser scan image. The palette can be used to adjust the tonal range of the laser scan image.

parameter A channel that has been chosen for analysis in a protocol.

peak A value that is used to describe the most frequent fluorescence intensity of a population of cells.

"Peak mode" is the channel number that is used to describe the largest number of cells in a sample.

peripheral contour This contour, along with the integral contour, define a toroidal region of pixels used to calculate the peripheral value or the cytoplasmic fluorescence.

photobleaching The result of light altering fluorescent molecules so that they have decreased fluorescence or are no longer fluorescent.

photodetector A device that senses light and converts the energy from that light into an electrical signal.

photodiode A photodetector used for absorption, extinction, and forward scatter signals.

photomultiplier tube (PMT) A photodetector used to detect and measure and fluorescence signals.

pixel The smallest logical unit that can be used to build an image on the screen.

PMT *See* photomultiplier tube

protocol A description of settings saved from a successful run.

probe Any detectable marker that binds to a specific cellular component or molecular sequence.

properties Properties refer to the characteristics of a window in the iCys software application. The properties dialog box is used to change the information displayed in a window and how it is displayed.

Q

quadrant A scattergram that has been divided into four rectangular areas to identify subpopulations for analysis.

R

RAM (Random Access Memory) Computer memory into which the user can both read and write information.

region A user-defined bounded area on a scattergram or histogram. It is used to isolate a subpopulation of events.

relocation The process of the iCys software repositioning the microscope stage to the previously saved location of an event on a slide.

repetitive scan The process of scanning the same well or scan area multiple times. This allows you to see the change in a single sample over a period of time (which can be especially useful for live samples).

report A window which displays selected data windows with additional text and graphics. Acquired data can be displayed in the data windows and the report window can be printed.

S

sampling rate The frequency at which the laser beam scan occurs.

saturation When the detector or display device no longer responds to increase levels of signal.

saving The process of placing information in the permanent memory of the computer.

scan area The entire area to be scanned. For a well, this is usually the entire well. For samples that reside in other kinds of carriers, the scan area can be defined by the user to encompass all or part of the sample.

scan field A single pass of the iCys laser. A scan area is made up of multiple scan fields.

Scan View window The window in iCys in which scans are displayed. The source of the window's data appears in the title bar. When data is coming directly from the iCys cytometer, the window title is Scan View.

scanning The process of recording an image on a visual display screen.

scattergram A two-dimensional display of data from a scan run or from a cell data file displayed as a plot of two user-selected measured features.

scroll A computer term for moving the contents inside a window up, down, right, or left so that more of the contents of the window can be viewed.

scroll bar A bar that appears at the side and/or bottom of a window that controls scrolling movement with a mouse action. A rectangular box inside the scroll bar shows the current position relative to the entire contents.

segmenting The process of setting boundaries to define events for storage in the computer memory.

sensor A device that detects and measures light and converts the energy from that light into an electrical signal.

status bar The horizontal bar at the very bottom of the iCys desktop. It contains an assist line, the names of the active files, the cell count, and the power of the objective being used.

statistics The calculations that the iCys software makes for cells within regions of the active window of a histogram or scattergram. The data (region number, count, percentage of cells in the region, the mean, full width at half maximum, CV,

interquartile range, standard deviation) can be printed out in the Region Statistics window.

stepper controller A device that controls moving the microscope stage.

stochiometric Measurements that are proportional to the amount of substance present.

Stokes shift The wavelength difference between the light absorbed by a fluorochrome and the light emitted during fluorescence.

T

tab A tab is a way of moving within a dialog box. (Use the TAB key.)

A group of tabs along the top of a dialog box provide you with subcategories of options.

taskbar In Windows XP, a taskbar is a horizontal bar located at the bottom of your screen. A button on the taskbar indicates a program that is open.

In iCys, the Taskbar displays standard tasks, usually on the right-hand side of the iCys window.

text box An area in a dialog box where the user can enter text.

texture The measure of the variability of fluorescent intensity of a cell. Cells that have increasing pixel values from the cell's edge to the peak have a relatively low texture value. Cells that have undulating pixel values have relatively high texture measurements.

threshold An instrument setting that is used to determine a boundary based on the intensity of the photodetector signals. The threshold assists the user in making the optimum instrument settings. Once these settings are made, the measurements on those events can be saved for later analysis.

.TIF file (Tagged-Image File Format) A bitmap graphics file of an image.

tissue microarray A slide that contains many small pieces, or cores, of sample tissue. Using the iNovator Application Development Toolkit, you can use iCys to label and scan tissue array samples.

title bar The horizontal bar at the top of a iCys window that displays a label for the contents of the window.

toolbar An area on the desktop used to place icons. The icons placed in the toolbar can be customized by the user.

torus An area bounded by two user-defined contours. It is used to determine the amount of fluorescence around the perimeter of a cell and also for calculating the amount of background fluorescence.

2-parameter histogram A color plot of the values of two user-selected parameters with color representing either the density or an expression. 2-parameter histograms are set from the Scattergram menu in iCys.

V

voltage The force that causes the movement of electrons through an electrical circuit. In a computer circuit, everything is reduced to bits (binary digit of 0 or 1). High voltage is represented by the number 1 and low voltage is represented by the number 0.

W

wavelength Describes the color of light. The color of the light is related to amount of energy in the photons of that light. Blue light has wavelengths of approximately 400-540 nm and its photons have relatively large amounts of energy while red light has wavelengths of approximately 600-700 nm and photons with less energy than those in blue light.

Workspace The combined instrument and display settings for a given experiment.The Workspace can be saved as a file with the extension .ws.xml.