

iCyte/iCys Revision 3.0 User Tutorial

Exercise 3

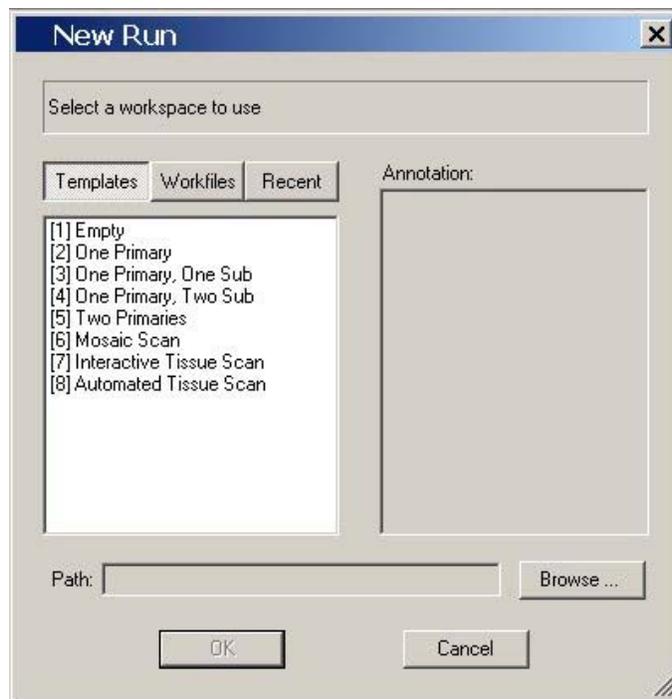
Exercise 3: Liver Tissue Scans

The purpose of this tutorial is to provide the experienced iCyte user with the basic operational steps to perform a single pass tissue scan using event and phantom contouring.

The specimen used in this analysis is a liver tissue section that has been stained with an antibody conjugate that is detectable in the green channel with the argon laser. The specimen has also been stained with DAPI for nuclear segmentation.

Start-up/Creating Workspaces

1. Turn on the iCyte main power and laser power.
2. Turn on the computer and all accessories.
3. Launch the iCyte application by double clicking on the iCyte desktop icon. The main iCyte screen will open.
4. From the side Task Bar select **New Run**. The New Run window will open:



It is from this window that you will begin the process of setting up a workspace for scanning.

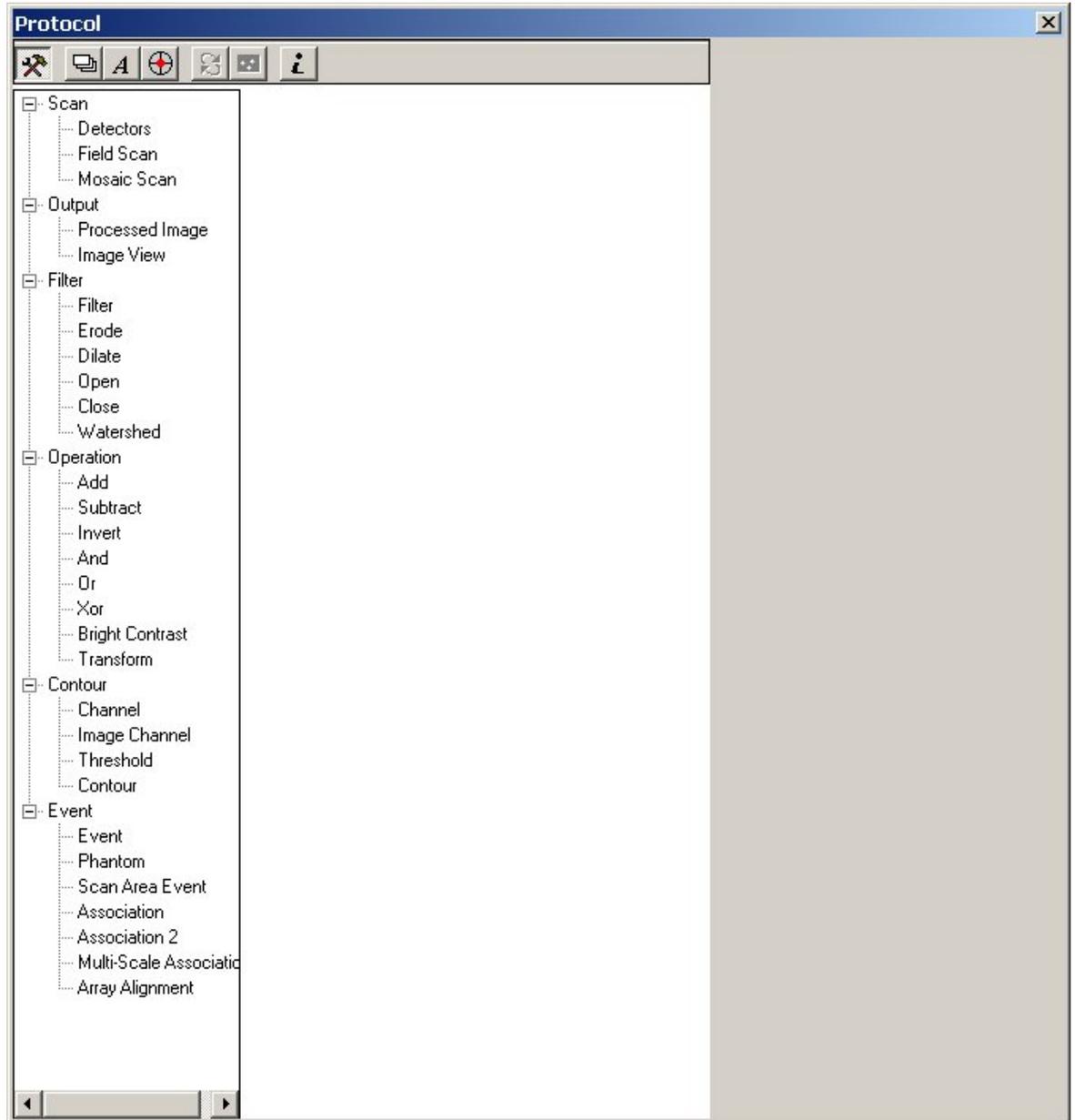
CompuCyte has provided a number of Templates that cover the most common scanning modes. These templates can be used as a starting point to build customized Workfiles

Note: The Templates listed in the graphic above is from an iCyte/iCys instrument equipped with the iNovator Application Development Tool Kit. Instruments without this option will have a slightly different Template list.

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5. For this exercise you will build a template from scratch. Highlight the Template box and select **Empty** from the Template list and click on **OK**. A single tab titled Protocol and a blank Protocol window will open.
6. Click on the Tool Box icon  in the Protocol window to display the list of Modules



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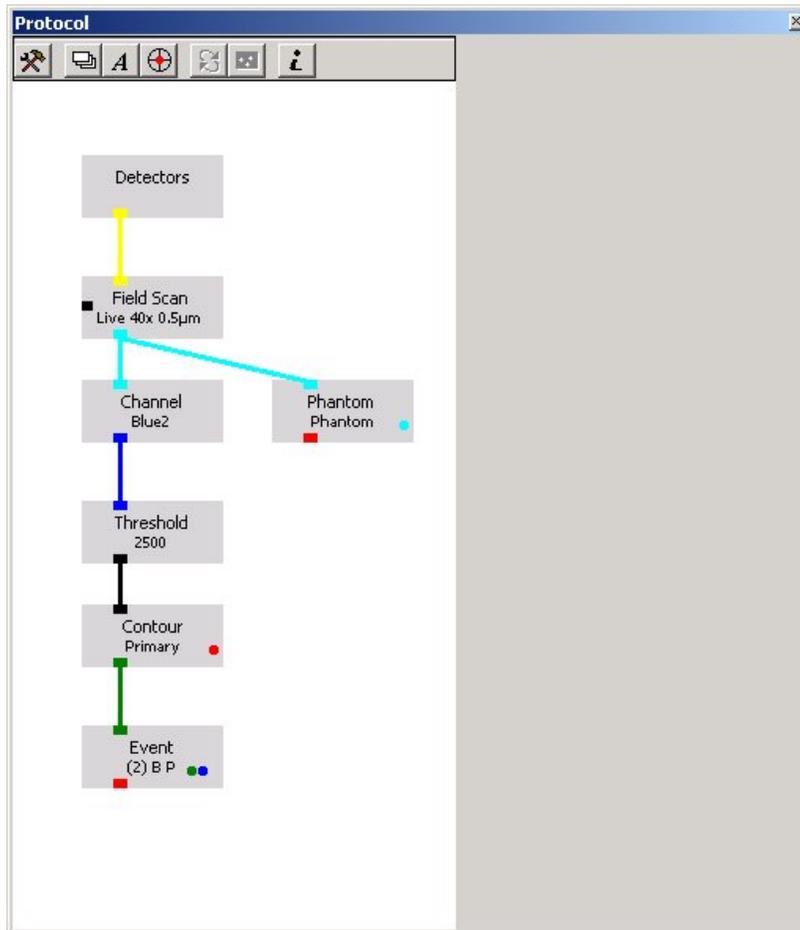
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Adding Modules to Protocol

7. To start building a workspace, click on the desired module in the Tool Box to highlight it, and then click on the open space in the Protocol window to add the module. For this exercise, add the following modules:

Detector
Field Scan
Channel
Threshold
Contour
Event
Phantom

Connect the colored connection nodes between the modules. Note that only similar colors can be connected. Connect the Phantom Module to the Field Scan Module. The Protocol window should appear as shown below.



8. Set up the Primary event contour settings as follows:

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Module	Field	Setting
Detectors	Laser-First Pass	488 nm
	Channels	PMT2 Green Scatter- Shaded Relief
	Laser Second Pass	405 nm
	Channels	PMT1 Blue2
CompuColor	Green Blue2 Scatter	Green Blue Gray
Field Scan	Objective	40x
	X Step size	0.5 microns
Channel		Blue2
Threshold		Manual - 5000
Contour	Component Name	Primary
	Min Area	5 μm^2
	Color	Red
Event	Expand by	2
	Color	Green
	Background	Dynamic 6-3, 30-70% applied only to Blue 2 channel
	Color	Blue
Peripheral Contour	Enabled	Range 4-20 pixels
	Color	Yellow
Phantom	Enabled	
	Pattern	Random
	Radius	5 microns
	Minimum Distance	1
	Count	1000

Refer to Exercise 1 for information on setting parameters within the modules.

9. Initialize the protocol by clicking on the  in the Protocol Window icon bar.

Data Display

10. Select **Set up** from the top menu bar and **New Tab**.
11. Give the new tab the title **Data** and select **OK**.
12. Select **View** from the top menu bar then New Scattergram, repeat this 3 more times to create a total of 4 scattergrams.
13. Configure the scattergrams as follows:

Scattergram	Component	X Axis	Y axis
1	Primary	Green Peripheral Integral	Blue2 Integral
2	Primary	X position	Y position

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3	Phantom	Green Integral	Blue2 Integral
4	Phantom	X position	Y position

14. Select View from the top menu bar the New histogram, repeat this one more time for a total of 2 histograms.
15. Configure the histograms as follows:

Histogram	Component	X Axis
5	Primary	Green Peripheral Integral
6	Phantom	Green Integral

16. Select File and Save Workspace as: The save window will open providing you with an opportunity to name this workspace and any notations about the workspace. Name the workspace Tutorial Exercise 3.

Test Scanning

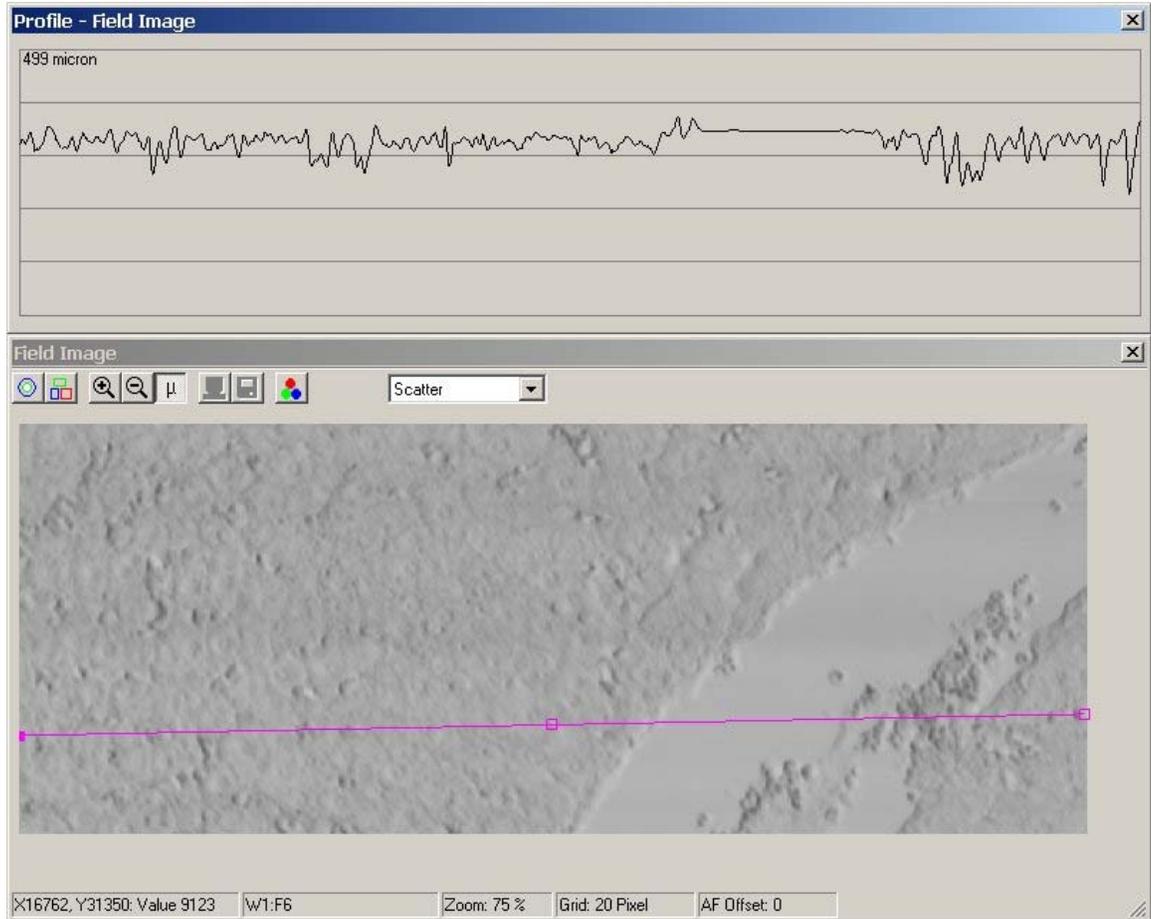
Note: Although this tutorial will use a saved data file, the steps used in setting up the scan are described below.

Locating the tissue and focusing

1. Select Field Image from the side Task bar, this will open the Field Image window This window is essentially the same as the Scan View window in software version 2.6, with one notable exception; the scan control keys have been removed from the Field Scan window and placed in the side Task Bar.
2. Start with a tissue that is positive for the antigen of interest.
3. Place the tissue in the carrier and on the instrument.
4. Move the stages so that the objective lens is under the tissue of interest.
5. Set a scan area around the pointer location in the carrier view window. Use the grid to set a square analysis area of a fixed size for each of the tissues in the series.
6. Perform a test scan of the tissue. Observe the scatter images in the Field View window.
7. Perform auto-focus offset corrections if necessary to sharpen the image.
8. Locate a vein, or another scan view in which there are areas of blank space as well as tissue. Either use the cursor to obtain the intensity level of the pixels in the blank area, or use the Profile tool to estimate the current values for areas of the slide with no tissue. Desirable settings for areas of the slide where there is no tissue area around 11,000.

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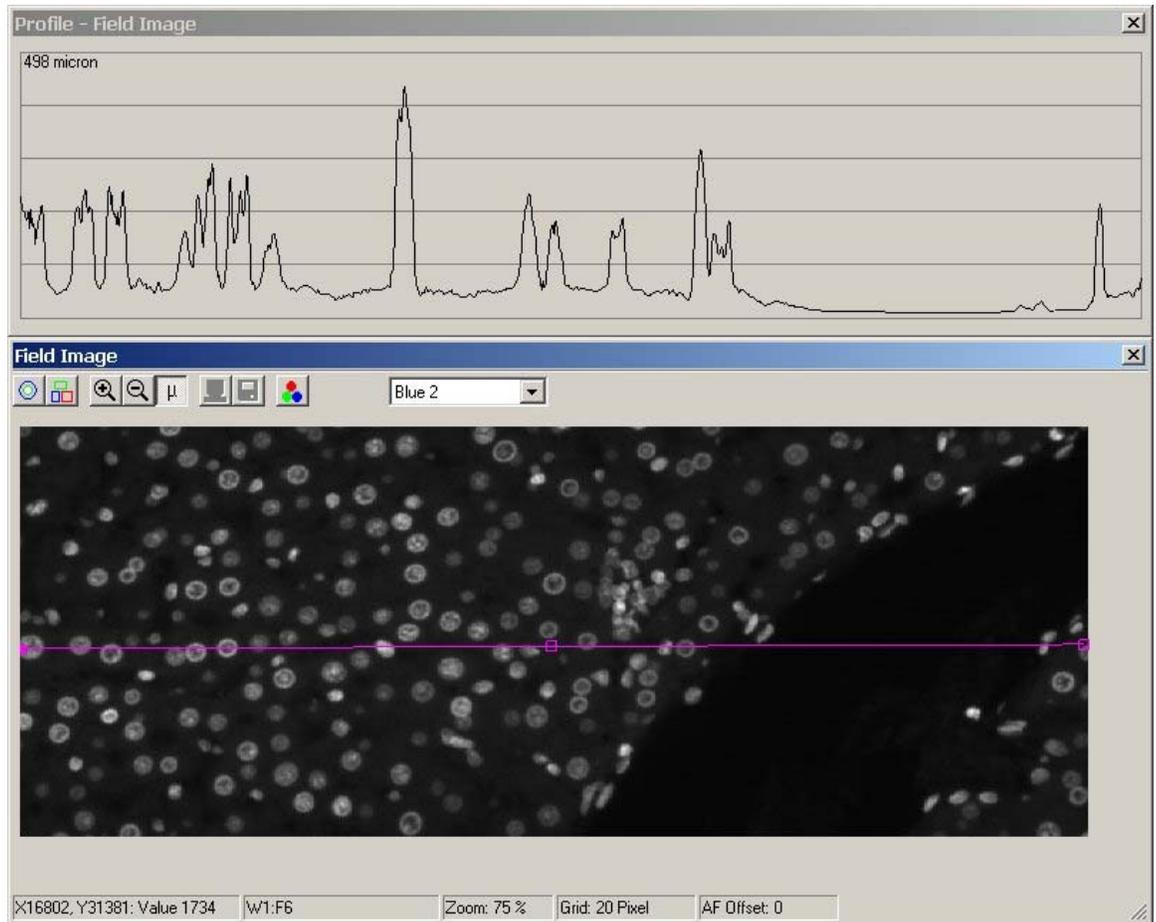
9. Adjust the gain either higher or lower to bring the current values to the desired level and verify by rescanning the same field of view.

Adjusting the blue photomultiplier tube

10. Adjustments to the blue photomultiplier tube can be done concurrently with adjustments to the scatter detector.
11. The scan profile line should be adjusted so that it crosses through a representative sampling of the nuclei, including larger hepatocytes and the smaller dense nuclei.

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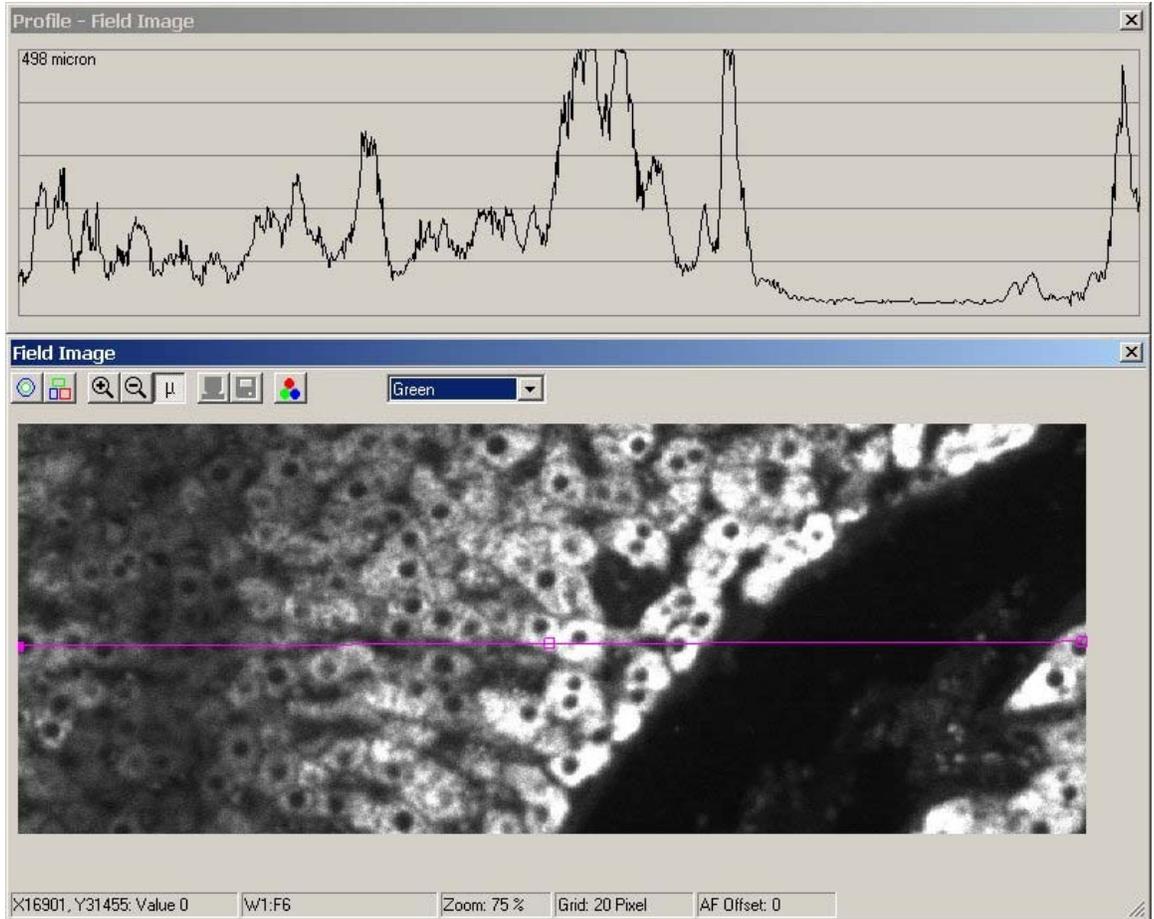
12. The PMT voltage should be adjusted so that the hepatocyte nuclei are at about the 40% level of the profile scale, and the denser nuclei are about 80%.
13. The offset setting should be adjusted so that the values for areas of the slide with no tissue should be close to the baseline with pixel values between 0 and 300.

Adjusting the green photomultiplier tube

14. Adjustments to the green photomultiplier tube also can be done concurrently with adjustments to the scatter detector and blue photo multiplier tube.
15. Green fluorescence will be a combination of the fluorochrome specific fluorescence as well as the tissue auto-fluorescence. Evaluations should be done in areas of high staining, if their locations are known. In this case, it is convenient because the protein of interest is expressed maximally around veins in the tissue sections. The photomultiplier tube should be set so that the brightest values are close to, but not exceeding saturation (although depending on the experiment, some saturated values are tolerable).
16. The offset setting should be adjusted so that the pixel values for areas of the slide with no tissue should be close to the baseline with pixel values between 0 and 300.

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17. Once the photomultiplier tube and scatter detector settings have been established, the samples can be scanned saving the raw data files. Subsequent setting of segmentation and other analysis criteria can be done from the raw data files.

Opening Runs

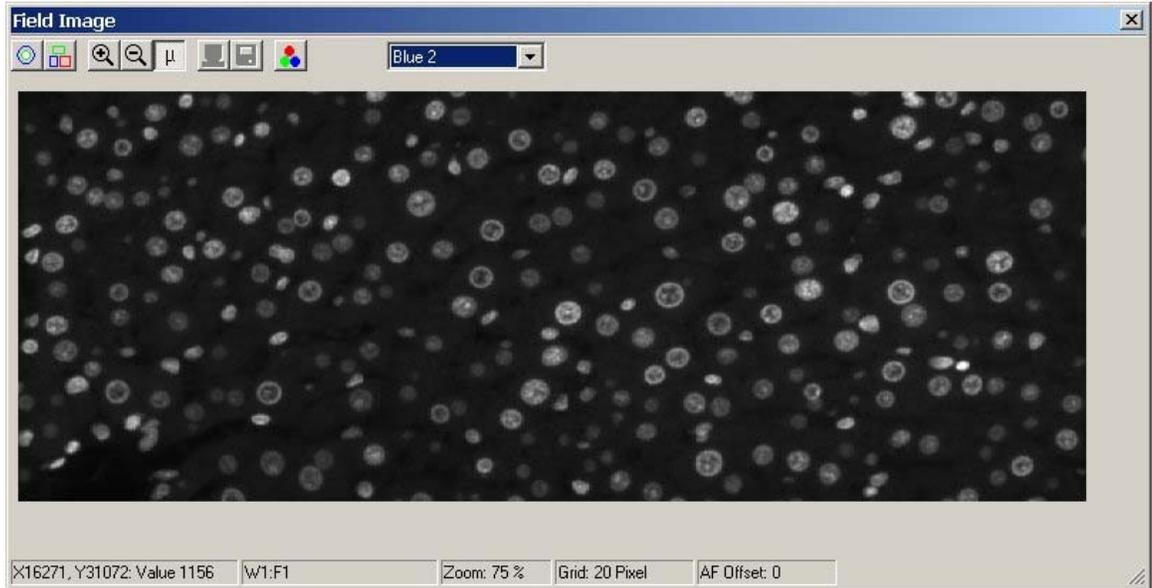
For this part of the tutorial, insert the iCyte/iCys Revision 3.0 Tutorial data disk into the CD-ROM drive of the computer.

1. Select **Open Run** from the side Task bar, the Open Run window will be displayed
2. For this exercise click on the **Browse** button and navigate to the computers CD-ROM drive, select Tutorial Exercise 3 File 1 and **Open**.
3. Select Run.xml and **Open**.
4. Select **Reanalyze** in the Analysis Mode box. This is analogous selecting the Active Data – Raw data in software version 2.6. (Selecting Analyze Events is similar to setting the active data to Event data in version 2.6).
5. Select **OK**, the workspace from the run will open.
6. Select File Open Workspace from the top menu bar.

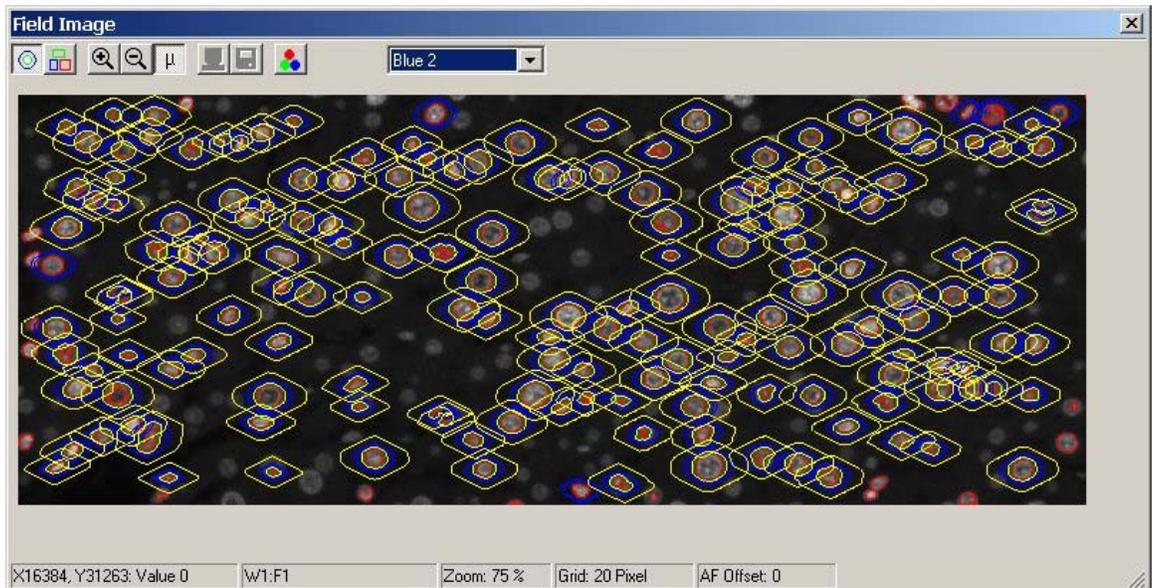
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7. Select *Tutorial Exercise 3* workfile that was saved earlier in this exercise.
8. Select **Single Field** to start the reanalysis of the data file; the Field Image window will display the first scan field
9. View the Blue2 (contouring) channel.



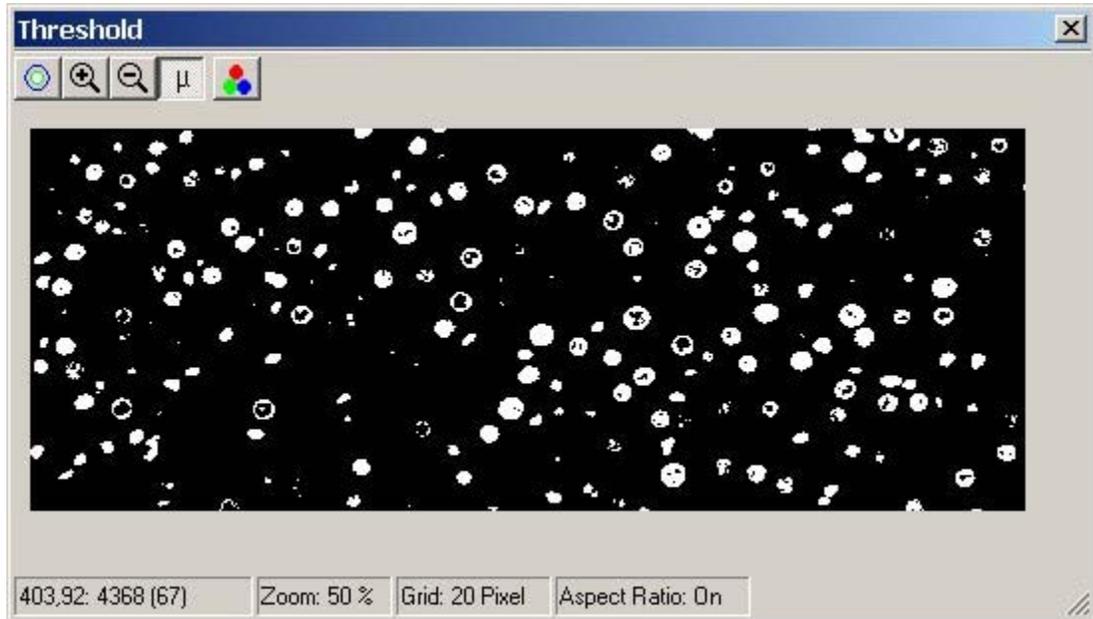
10. Select Draw Contours to view the cellular segmentation. Note that at 5000 not all of the hepatocytes are contoured.



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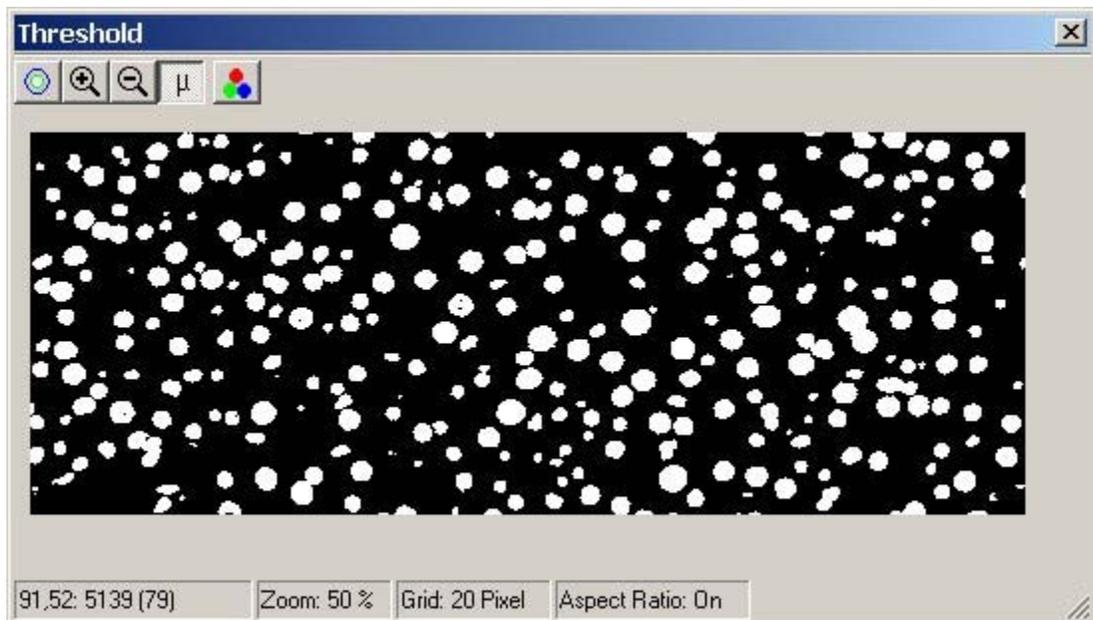
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11. Select the Threshold module in the Protocol window to display its details.
12. Click on the View Image icon () in the Protocol Tool Box window. This will open up the Interactive Threshold window as shown below.



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.

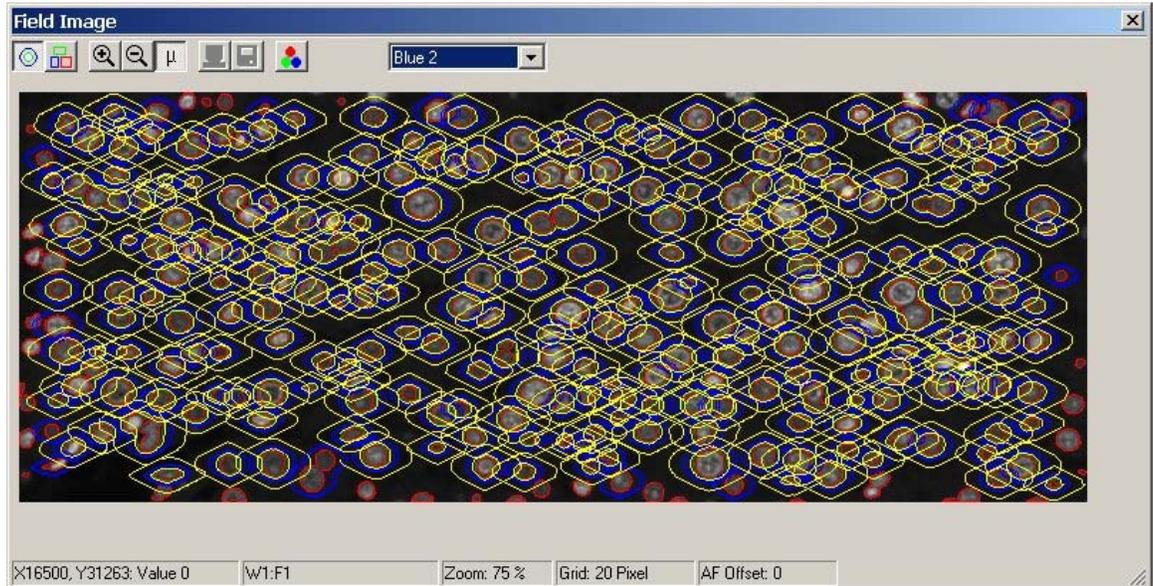
13. Use the slide bar in Threshold module to decrease the threshold value until dimmer nuclei can be seen in the Interactive Threshold window. The example below is at a threshold value of 2500.



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



15. Continue adjusting the threshold value to achieve optical segmentation.
16. Stop the Test scan.

Scan and Save

1. Start a scan by selecting **Scan and Save** from the side Task Menu bar.
2. Enter a Run name in the Run name field.
3. Enter any notations about the specimen or scan in the Annotation field.
4. Select how you want the raw data to be saved, either Jpeg or Flat, these remain the same as in revision 2.6 software.
5. Select **Save Field Scan Mosaic Image** to save a mosaic image of all scan fields together (Well Image)
6. Click OK to initiate the Scan and Save.
7. At the completion of the scan click on OK.

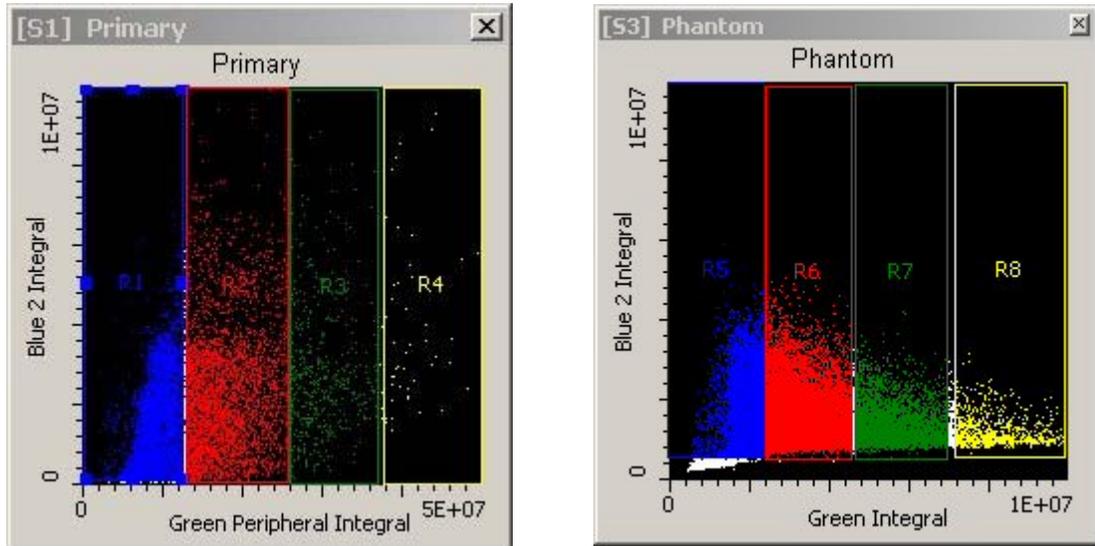
Data Analysis - Positive Tissue Section

1. Open the Data tab on the desk top. It may be necessary to change the scale on the X or Y axis to better view the data.
2. Draw 4 rectangular regions from top to bottom in the scattergram 1 (Green Peripheral Integral vs Blue2 Integral). All four regions should be about the same size.
3. Color Region 1 blue, Region 2 red, Region 3 green and region 4 yellow.
4. Repeat the above two steps in the Phantom Green Integral vs Blue 2 Integral scattergram.

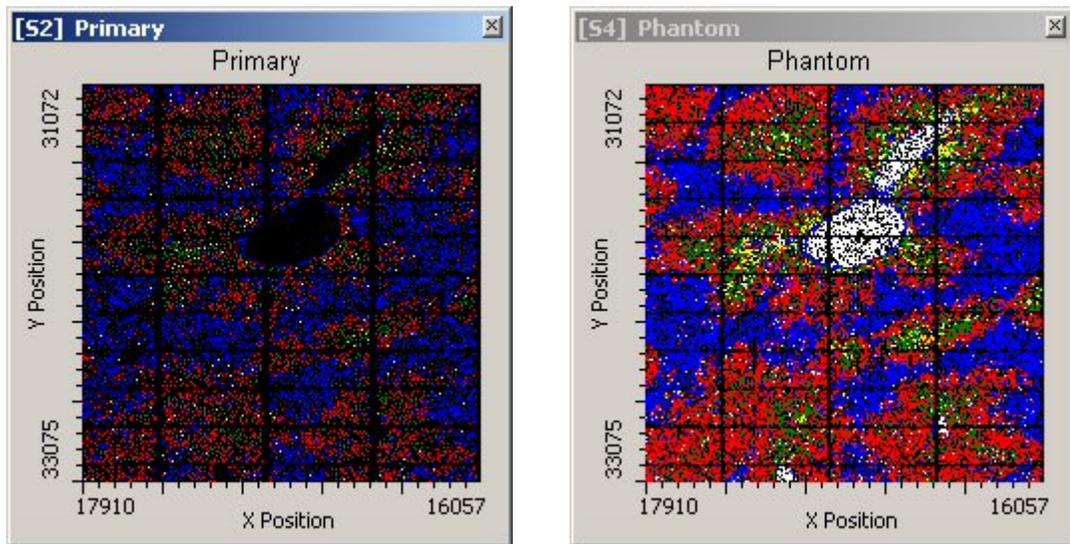
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- Adjust the regions in the Phantom scattergram (S3) so that they are approximately in the same position as in the Primary (S1) scattergram.



- X-Y plots of the colored events shows location of the events with the most intense antibody staining (green and yellow regions)

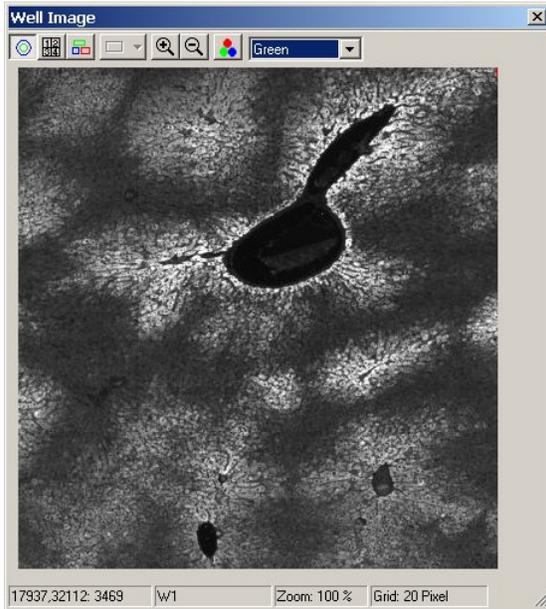


- Well images can be obtained and viewed by detector or in CompuColor

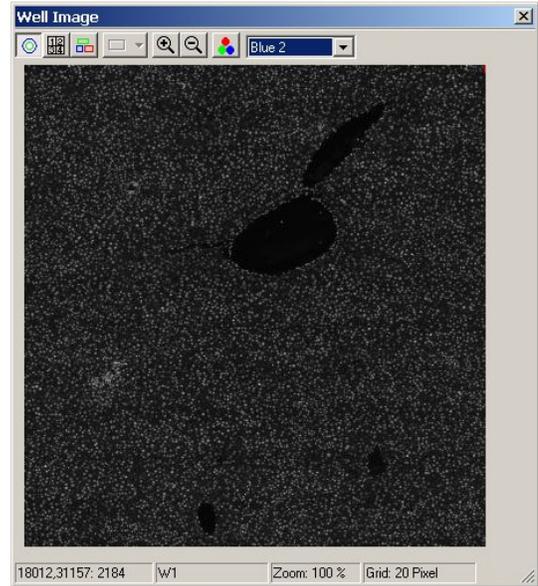
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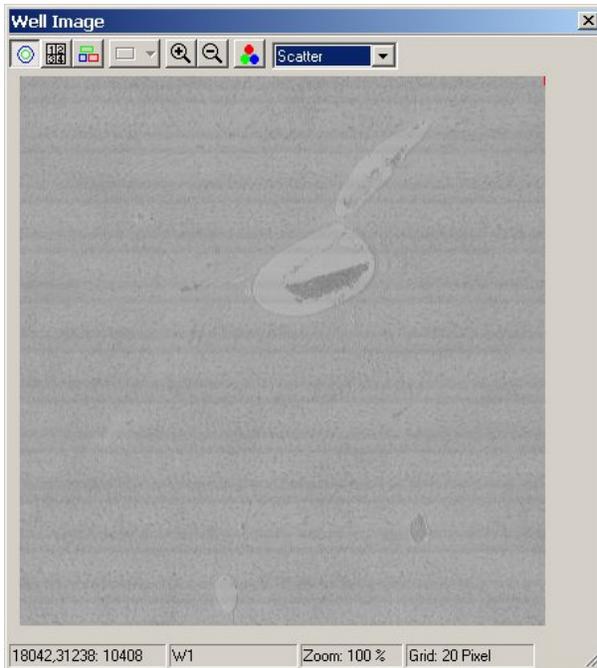
Green



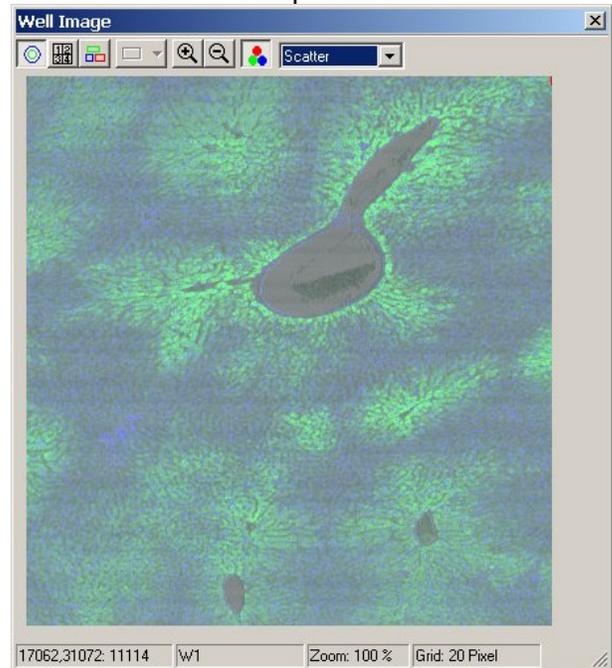
Blue 2



Scatter



CompuColor



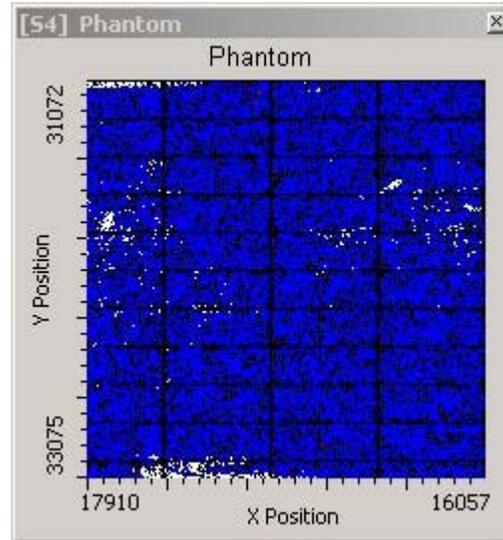
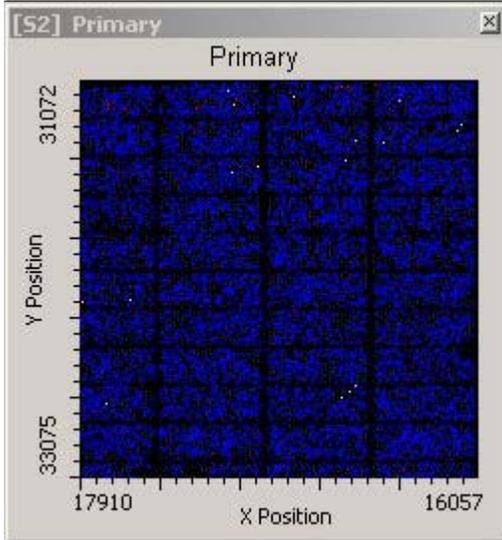
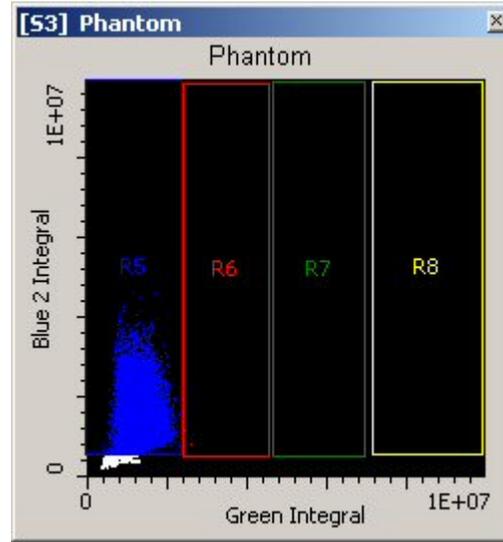
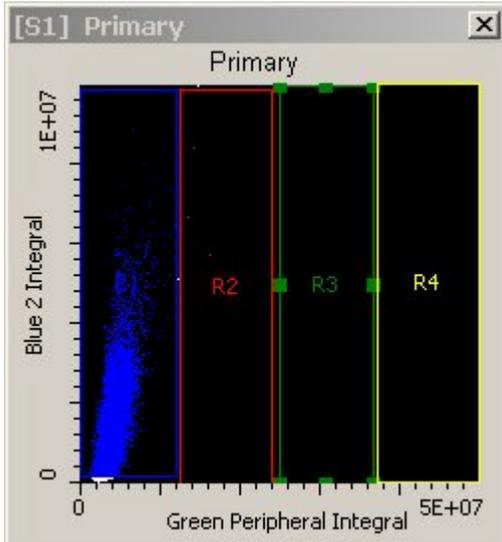
Data Analysis - Negative Tissue Section

1. Select **Open Run** from the side Task Menu
2. Select browse and navigate to the CD-ROM drive.
3. Highlight the file Tutorial Exercise 3 File 2 which is a scan of a negative tissue.
4. Highlight the **Run.xml** file and select **Open**.
5. Select **Reanalyze results** in the Analysis mode window.

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6. Select **Other** in the Workspace window and navigate to the Workfile folder where the Tutorial Exercise 3 workspace was saved.
7. **Select Active well** from the 4 Slide Carrier window and click on the scan area in slide one.
8. Open the data tab and review the Primary and Phantom scattergrams and histograms.



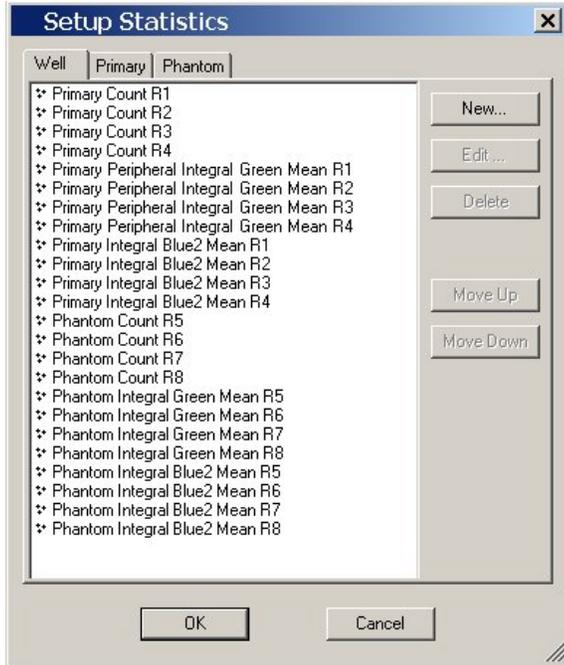
Note that both the Primary and Phantom scattergrams show no events in the red, green and yellow regions.

Run Statistics

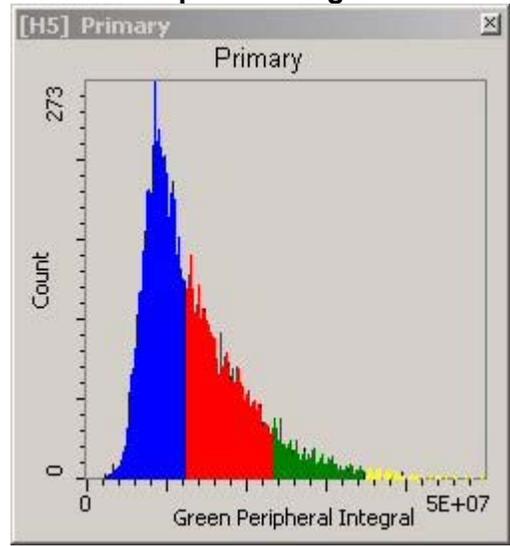
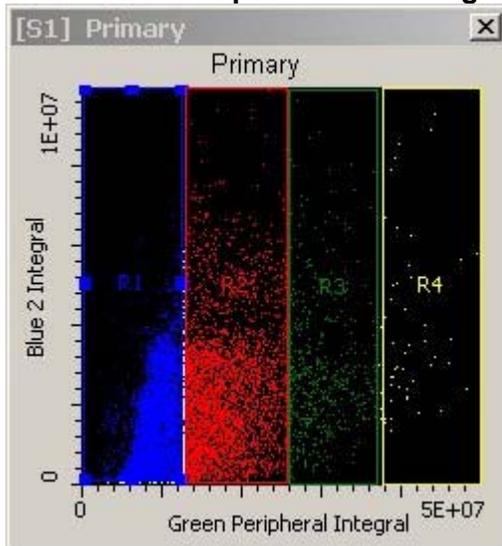
1. Statistics for both the cellular segmented events and phantom segmented events can be obtained for the run by setting up Run Statistics. These statistics can be viewed in iBrowser.

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Positive specimen showing elevated Green Peripheral Integral values



#	Well	Primary Peripheral Integral Green Mean R1	Primary Peripheral Integral Green Mean R2	Primary Peripheral Integral Green Mean R3
1	1	8,871,976	16,715,090	27,466,070

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Negative specimen showing low Green Peripheral Integral

