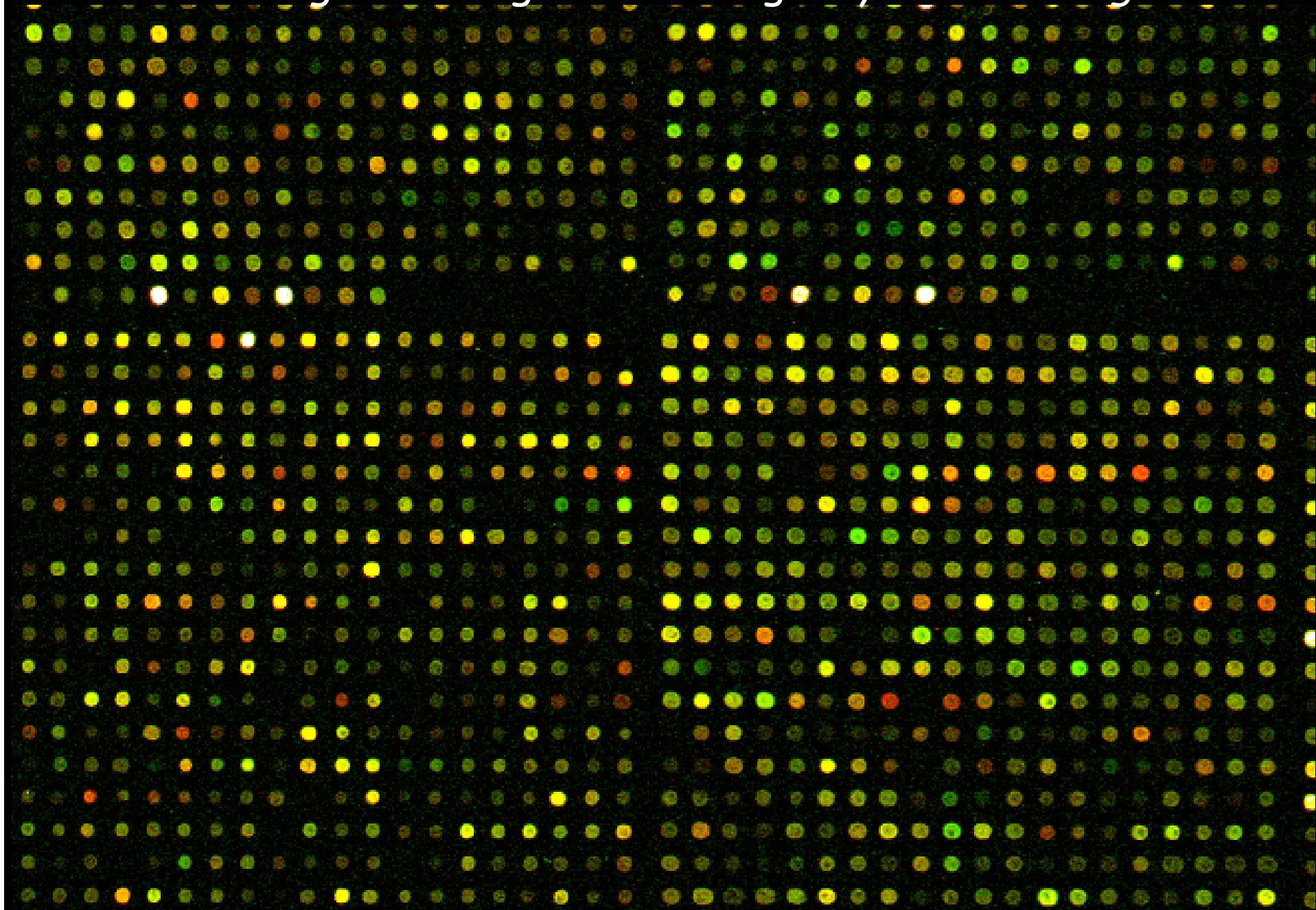
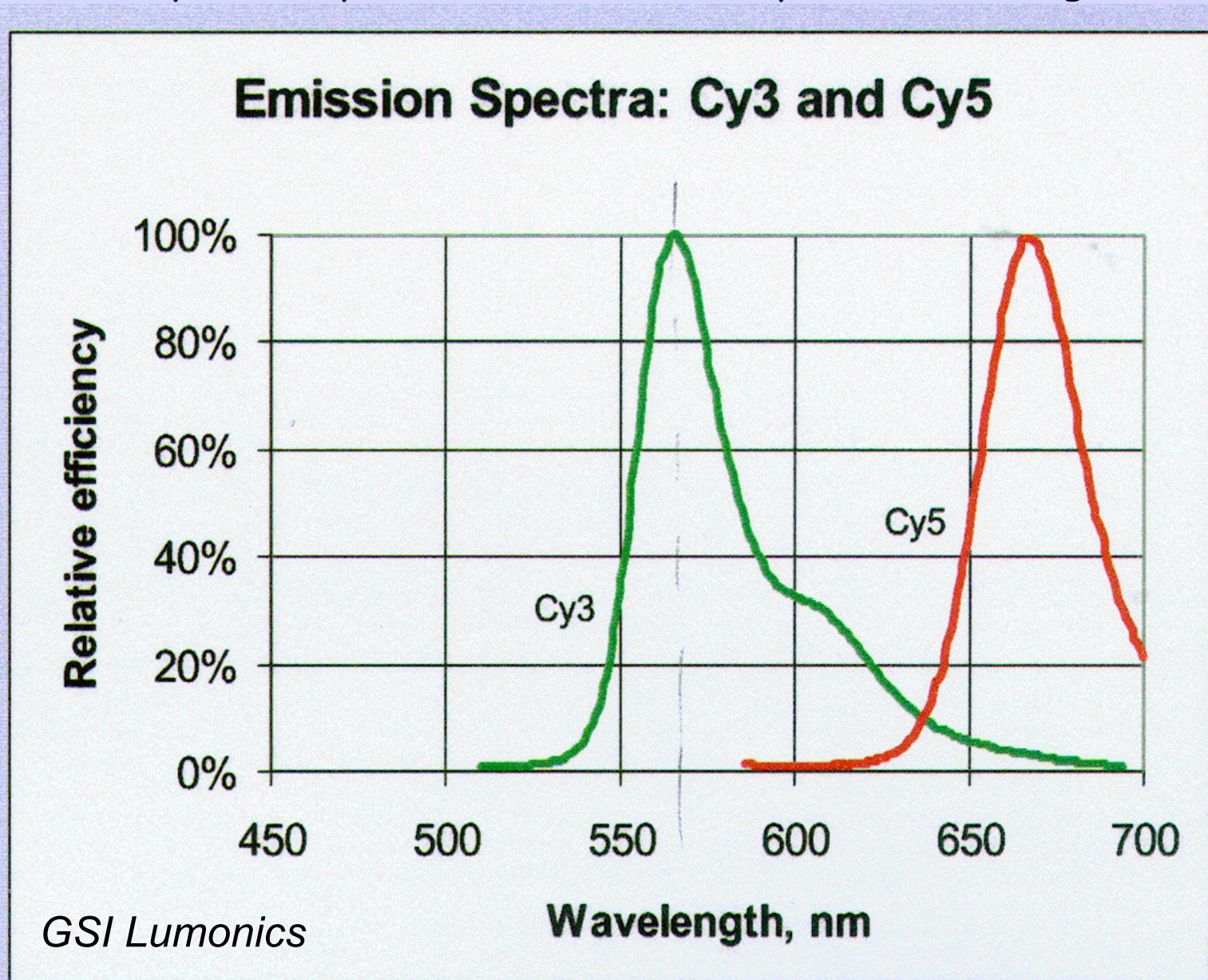


Scanning and Image Processing -by Steve Clough



cDNA microarrays use two dyes with well separated emission spectra such as Cy3 and Cy5 to allow direct comparisons on single slide



Ratio of Expression of Genes from Two Sources

Cells from condition A

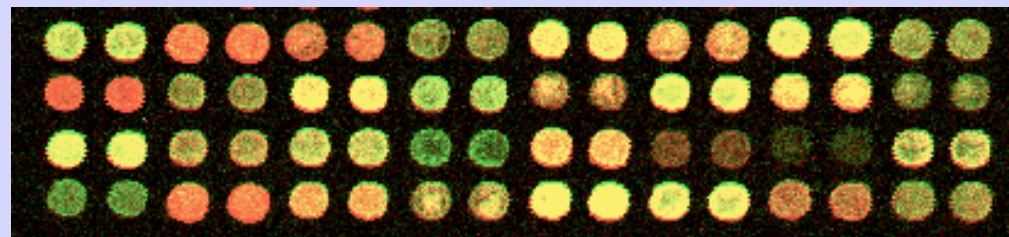
Cells from condition B

mRNA

Label Dye 1

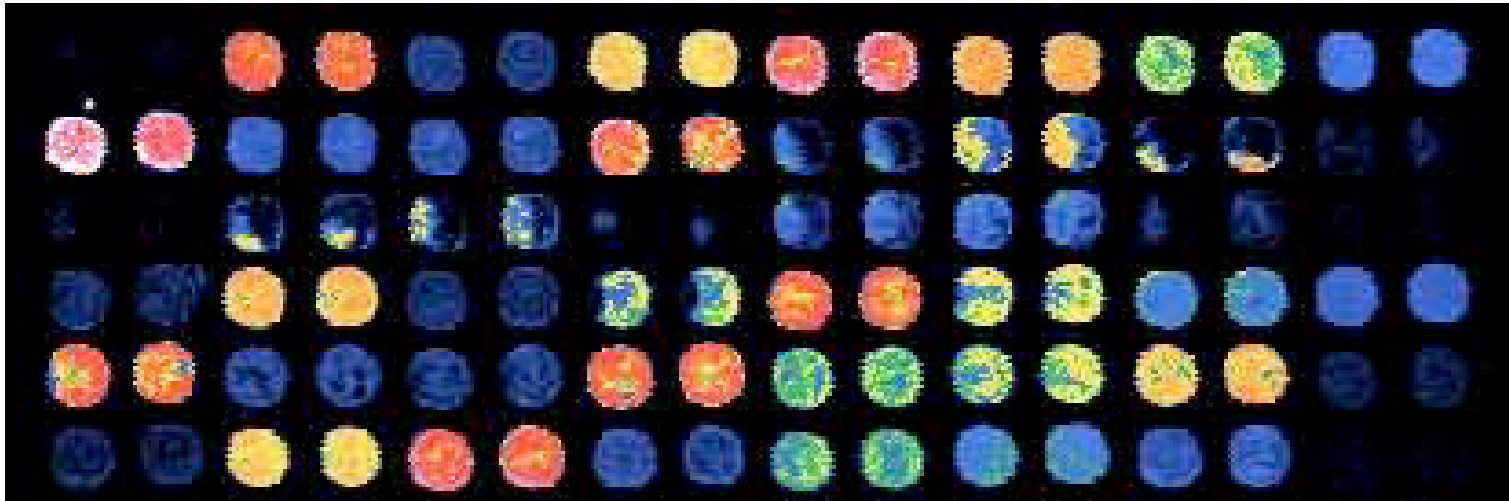
Label Dye 2

cDNA



● equal ● higher in A ● higher in B

False Coloring of Fluorescent Signal

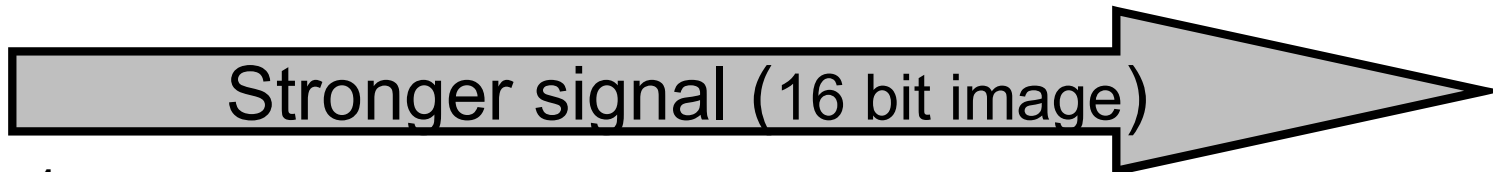


Scale of increasing fluorescent intensities



2

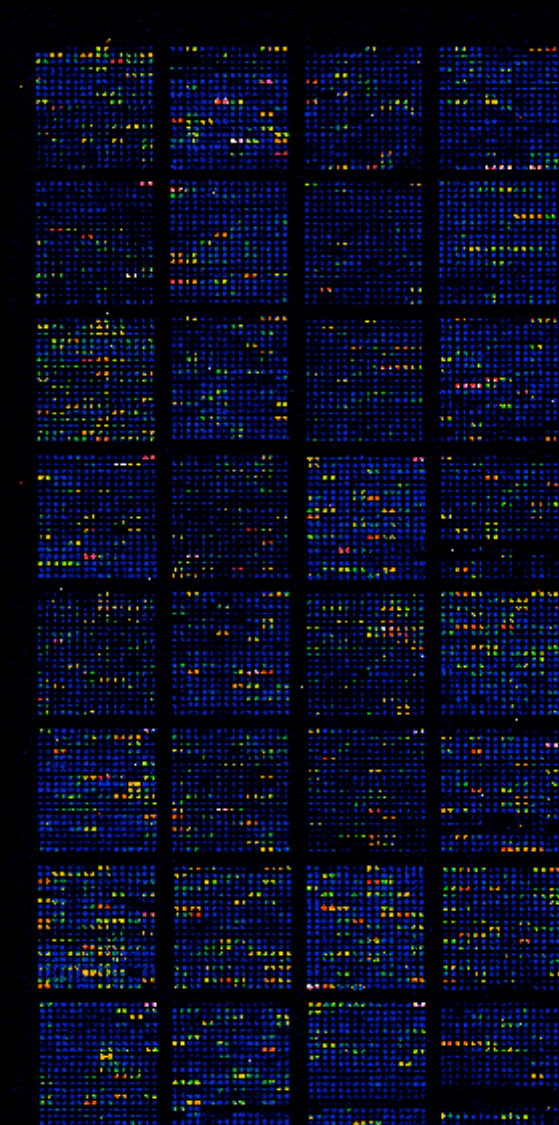
65,536



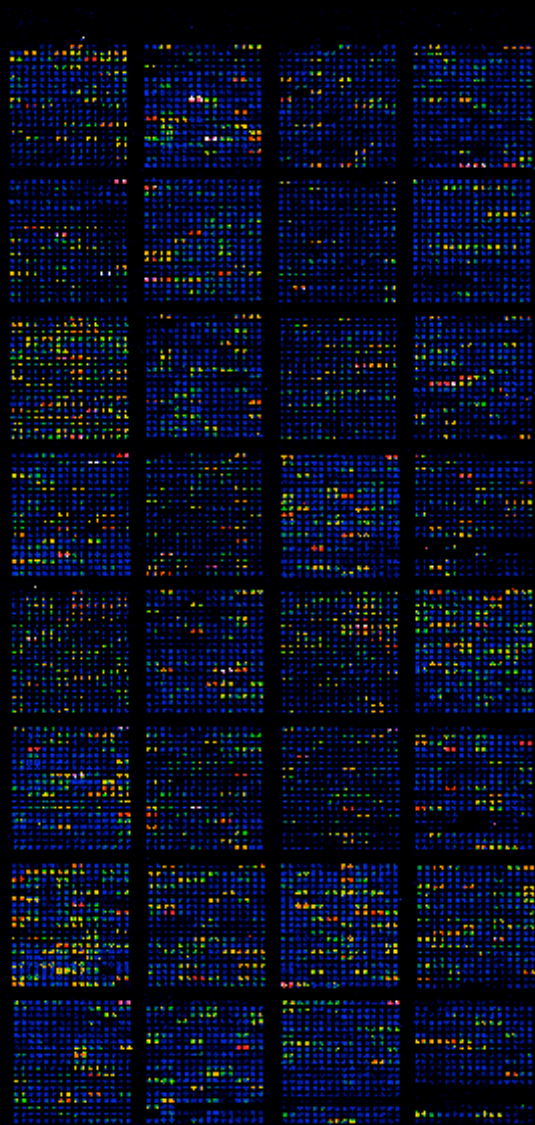
2^1

2^{16}

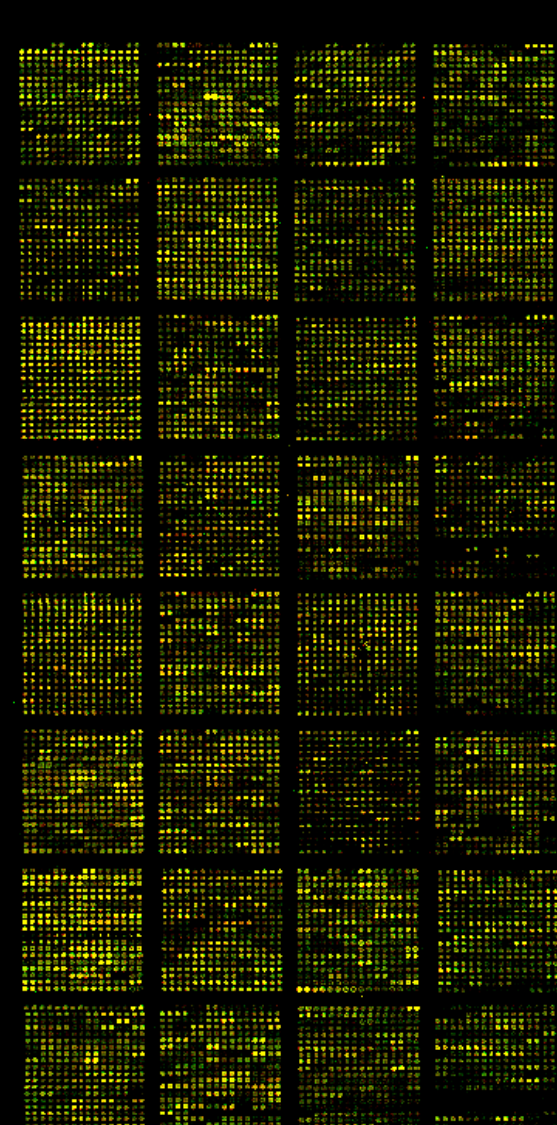
Cy3 Scan



Cy5 Scan



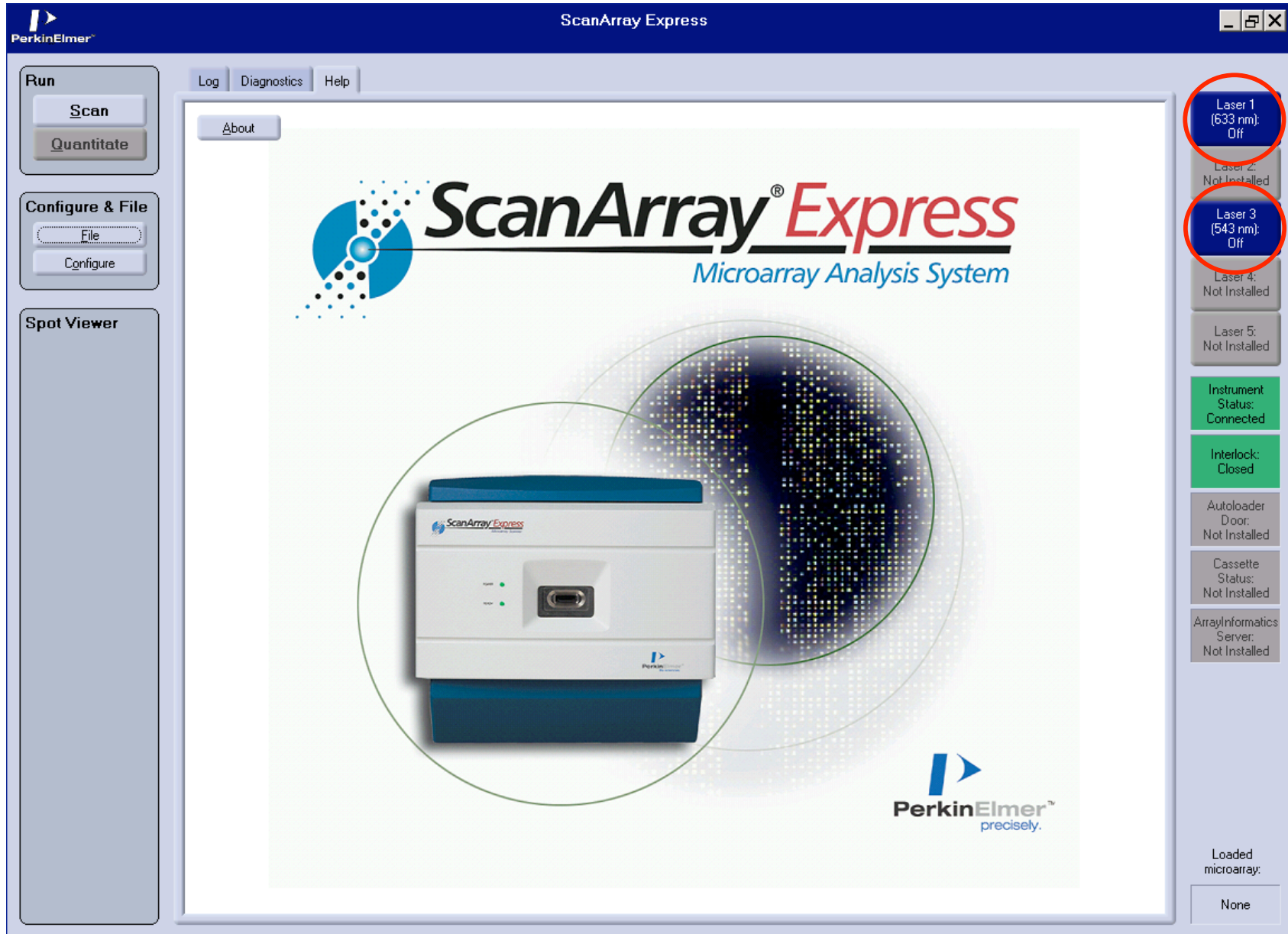
Overlay



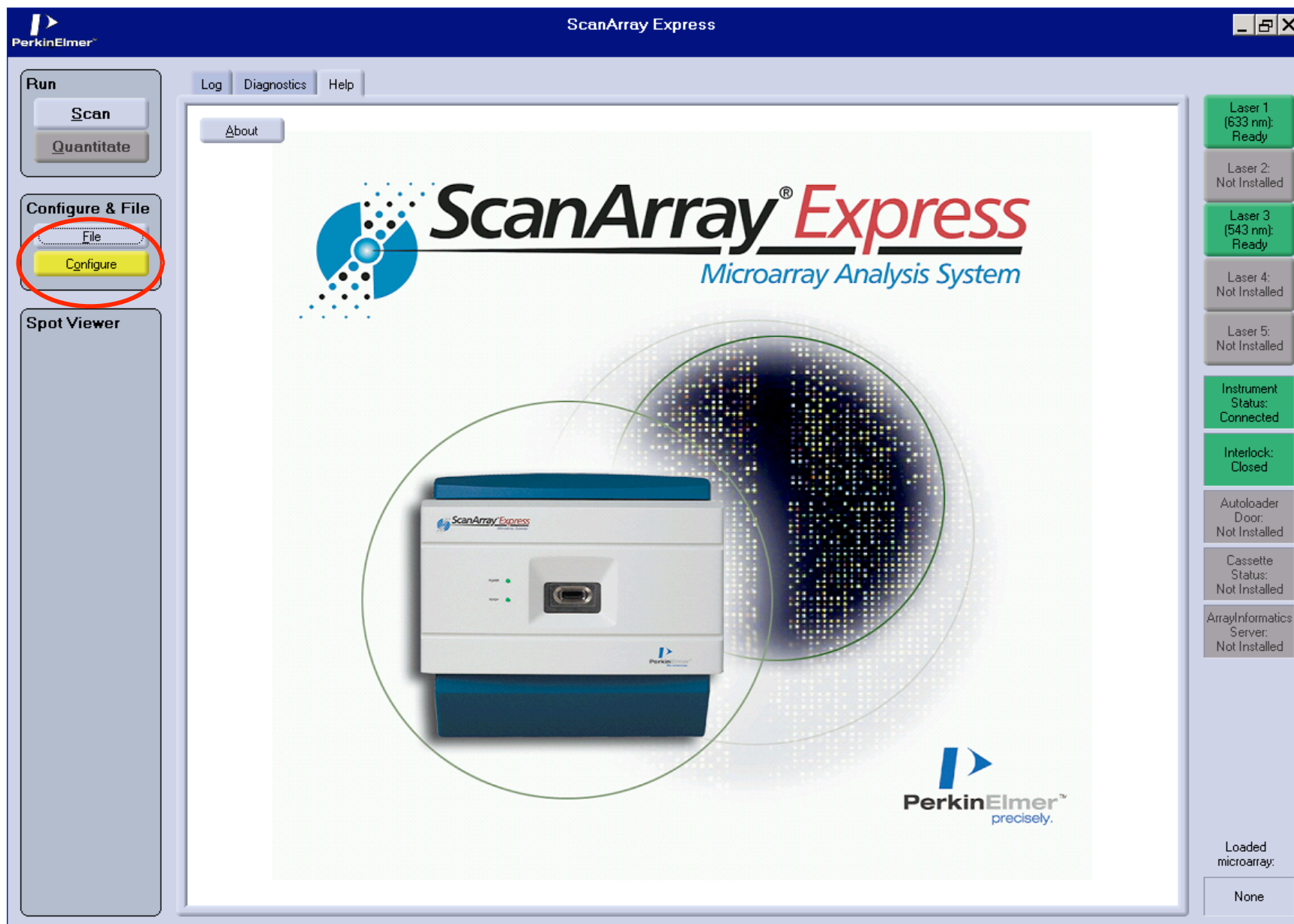
We use a ScanArray Express from Perkin Elmer



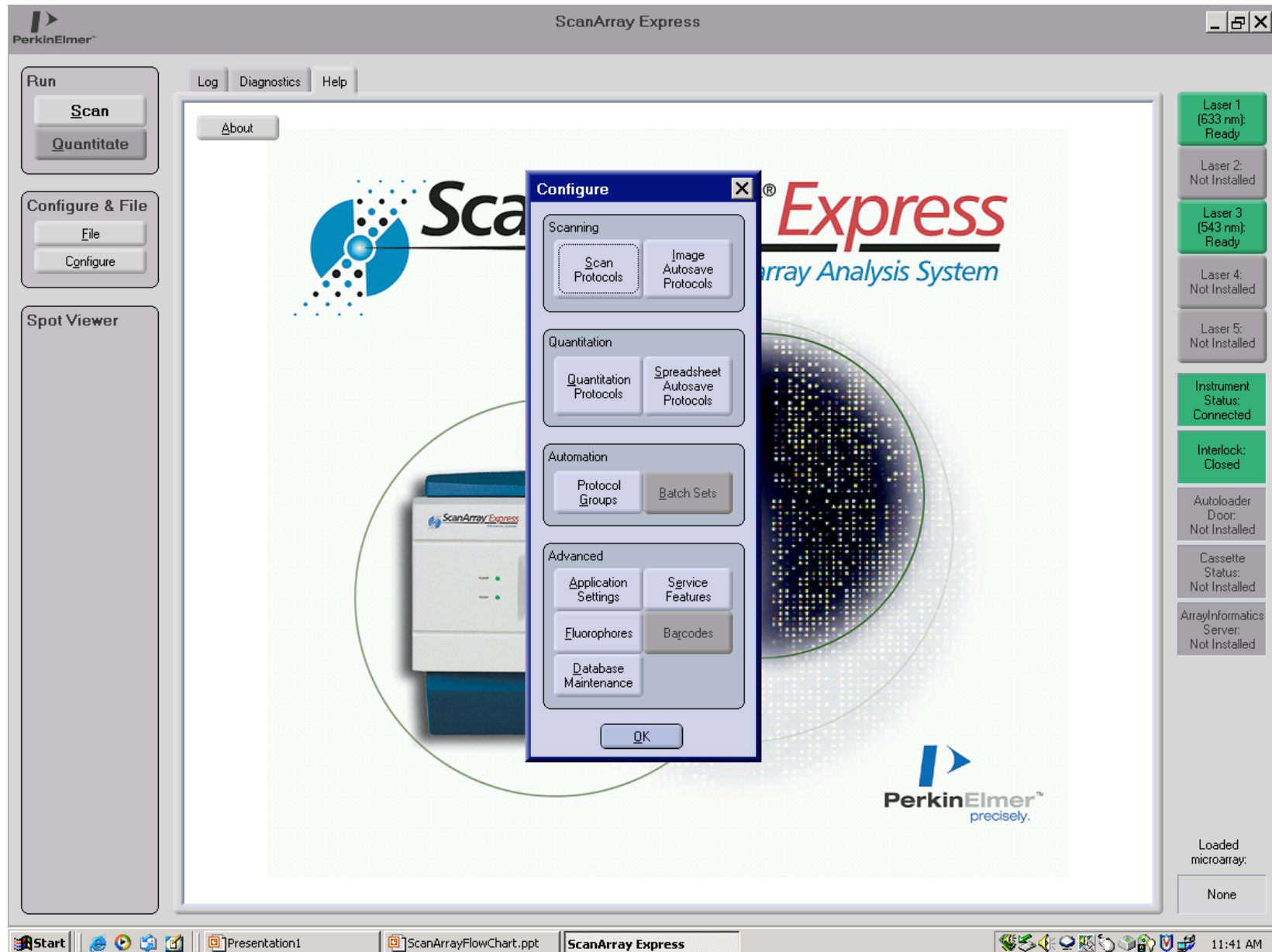
After double clicking the ScanArray icon on the desktop, the following window will appear. Click on the Laser 1 and Laser 2 tabs to turn on the lasers.



After lasers have warmed up, the laser tabs turn green.
Click on the 'Configure' button on the left.



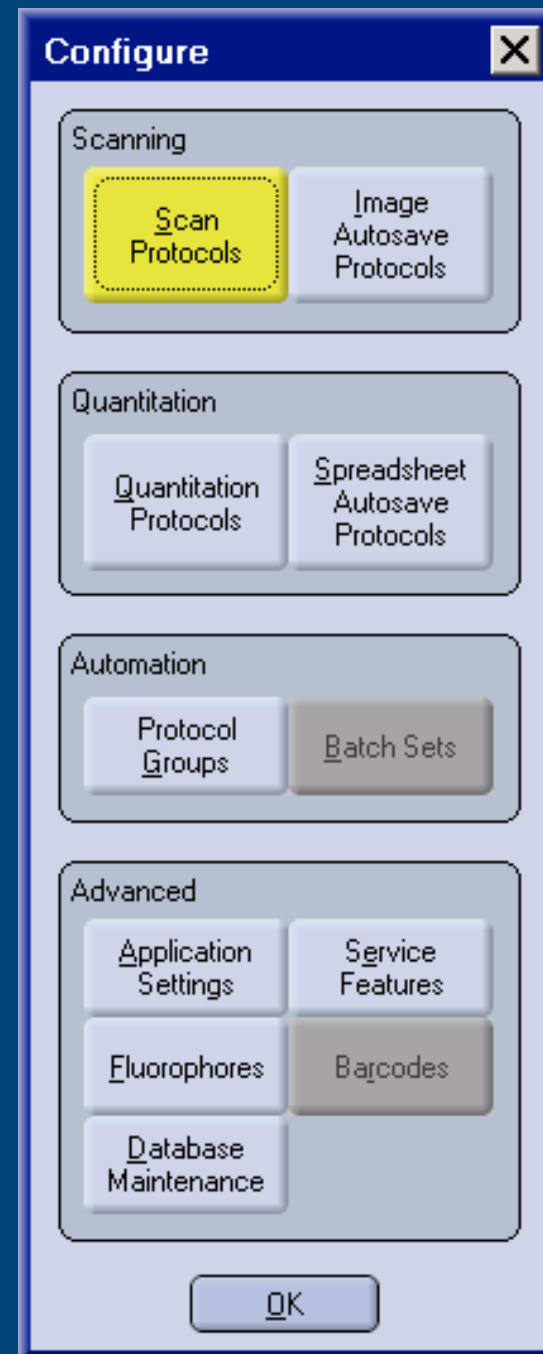
The following small 'Configure' window will appear.



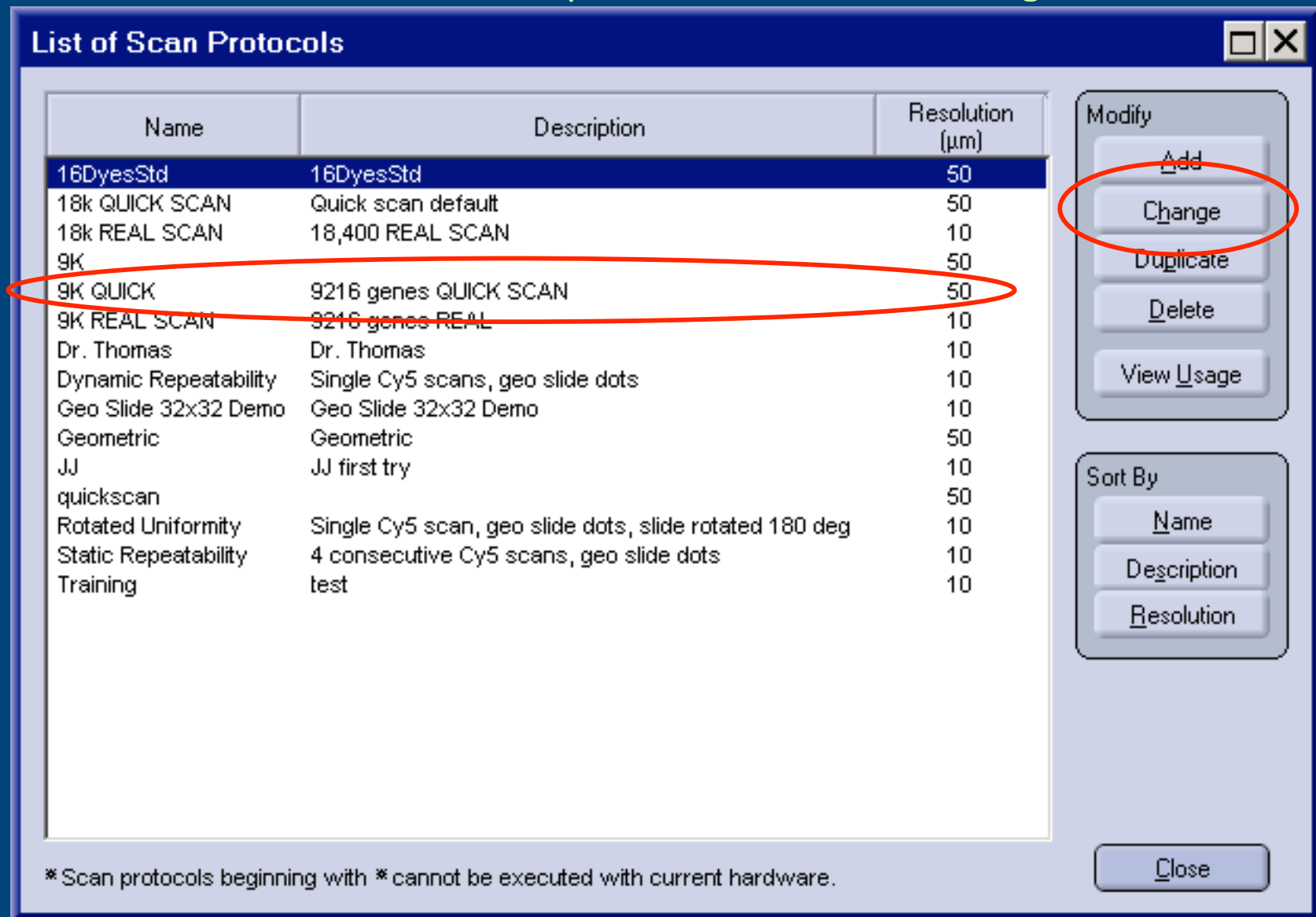
Click on 'Scan Protocols' to select a protocol to use for Quick Scanning.

The purpose of Quick Scanning is to determine the correct laser levels needed to have balanced intensities between Cy3 and Cy5 channels and to find levels that provide a nice spread of intensities across the spectrum without having too many saturated spots.

Note: the scans do not have to be perfectly balanced between the 2 dyes as a mathematical correction called 'normalizing' or 'smoothing' will be performed later. However, having the scan levels approximately balanced will minimize the magnitude



In this example we are using one of the Vodkin soybean 9k arrays such as 1021/83, 1070, or 1088. To begin the Quick Scanning process, highlight the '9K QUICK' protocol and click 'Change'



These are default settings. Verify that nobody has changed them and click 'Next'.

Scan Protocol - Basic Information

1. Basic Information
2. Scan Area
3. Fluorophores
4. Sensitivity Calibration Areas
5. Sensitivity Calibration
6. Tools

Name: 9K QUICK

Description: 9216 genes QUICK SCAN

Focus position (μm): -2

Scan resolution:
☐ 5 μm
☐ 10 μm
☐ 20 μm
☐ 30 μm
☒ 50 μm

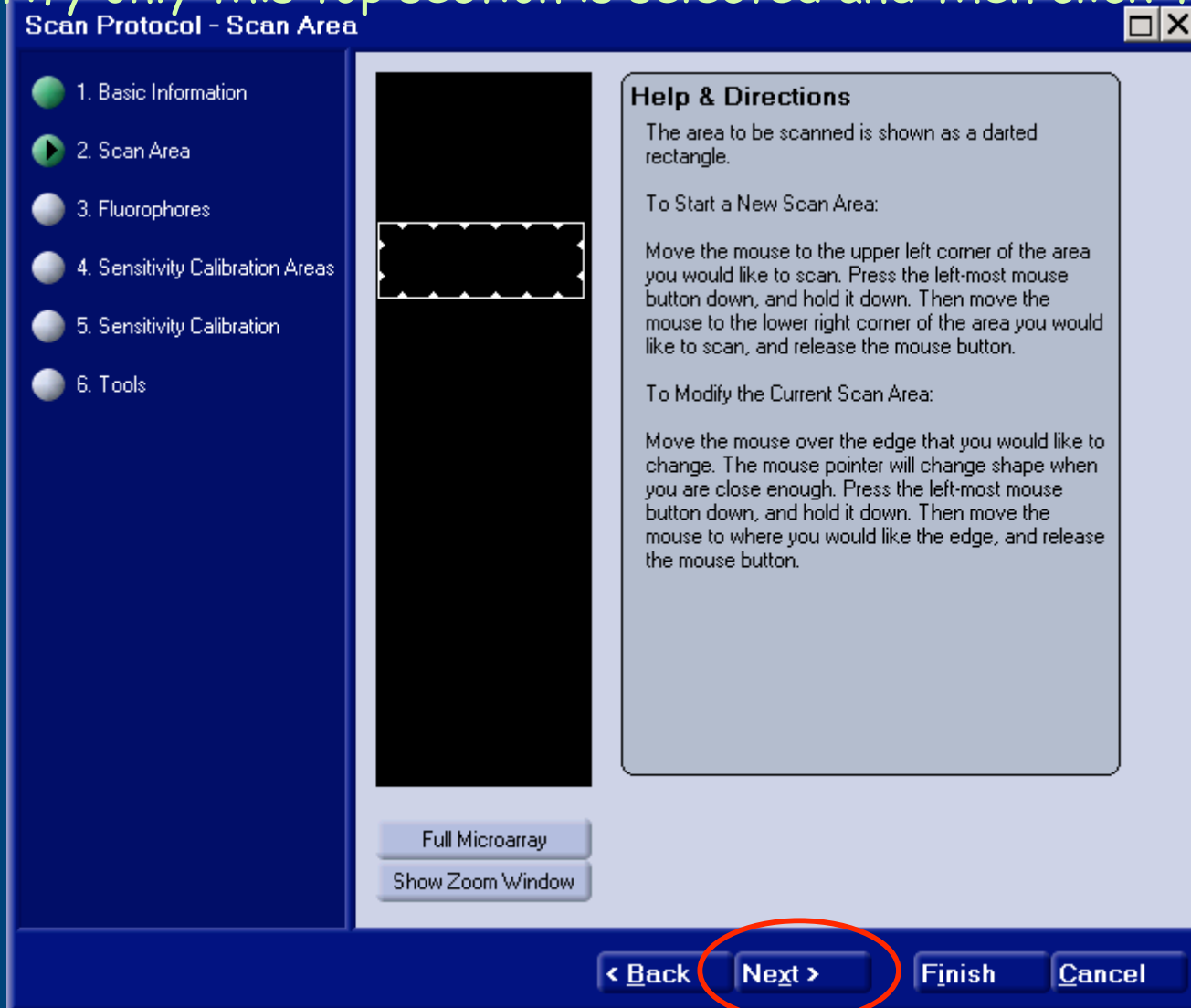
Scan speed:
☐ Half
☒ Full

Help and Directions

Focus position - should generally be 0 μm .
Scan resolution - should be about 1/10th of the spot diameter.
Scan speed - should be 'Full', unless you specify a resolution of 5 μm , or unless you particularly desire low signal-to-noise ratios at high PMT gains.

< Back **Next >** Finish Cancel

The quick scan will only cover a small portion of the slide as this reduces the likelihood of bleaching the dyes and you only need a small sampling to determine if the colors are balanced. Verify only this top section is selected and then click 'Next'.

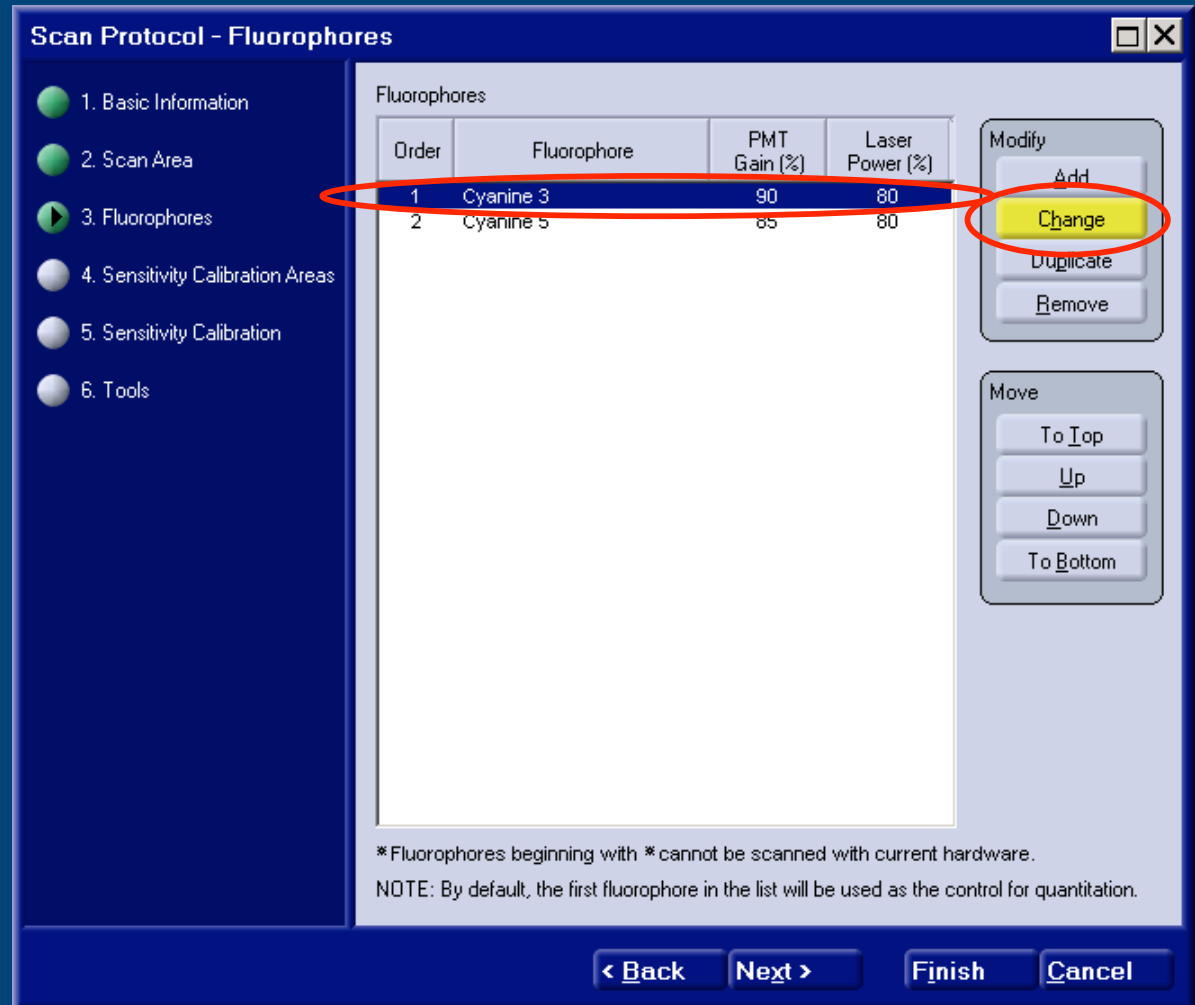


For our scanner, we found
that using a default of:

Cy3: 80 PMT and 70 Laser
Cy5: 70 PMT and 70 Laser

works well as an initial
starting point for scanning.

Change values as needed by
highlighting the dye that
needs changing and clicking
'Change'



Enter new values
And click 'OK'

Fluorophore [X]

Laser power (%): 80

PMT gain (%): 90

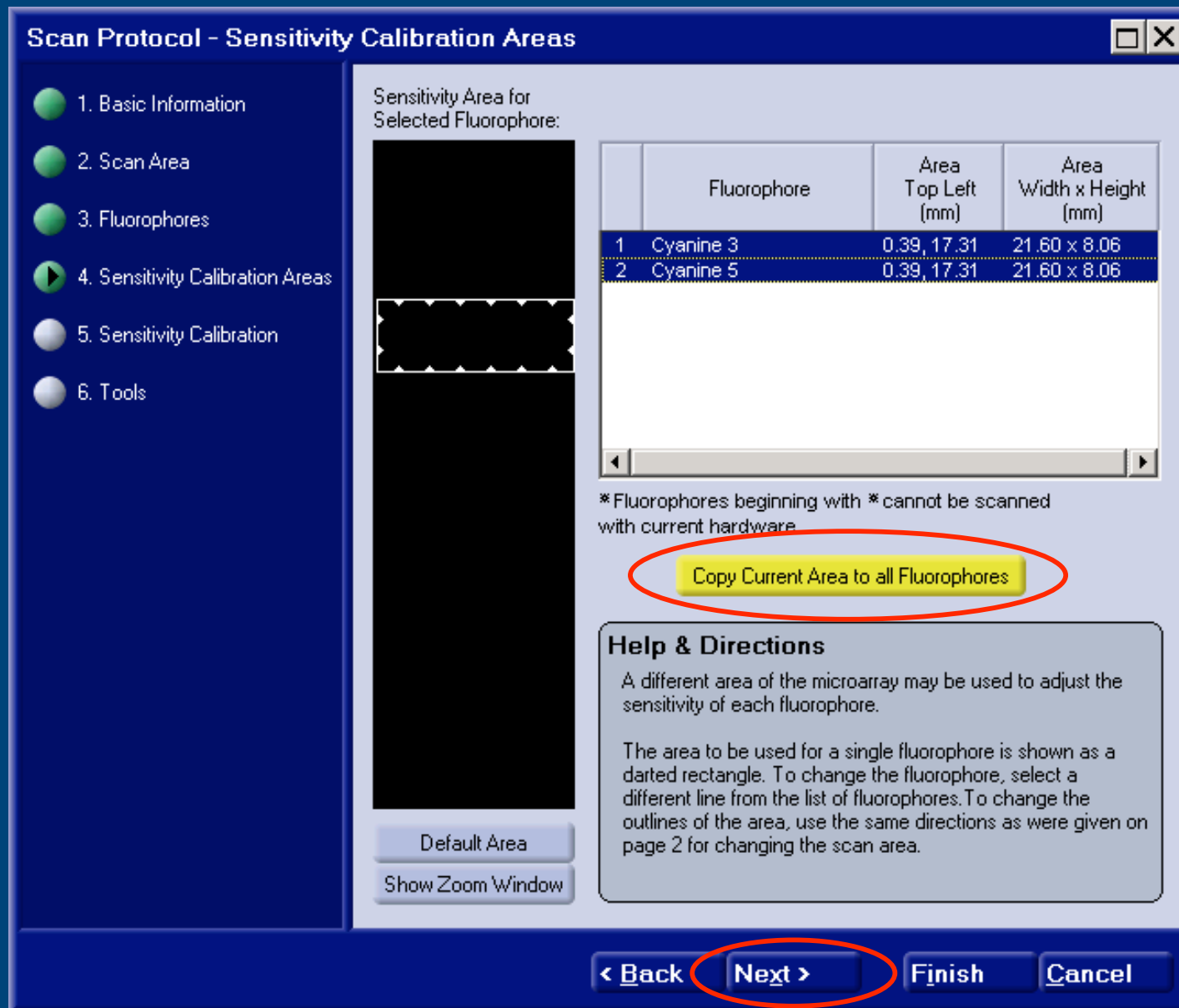
Fluorophore

- *5-FAM
- *Alexa 488
- Alexa 532
- Alexa 546
- Alexa 555
- Alexa 568
- *Alexa 594
- Alexa 647
- *Alexa 660
- Allophycocyanin (APC)
- *BODIPY 530-550
- BODIPY 558-568
- BODIPY 564-570
- BODIPY 630-650
- BODIPY TMR
- *Calcein
- *Calcium Crimson
- *Calcium Green-1
- Calcium Orange
- *Cyanine 2
- Cyanine 3

* Fluorophores beginning with * cannot be scanned with current hardware.

OK Cancel

Highlight the two dyes and then click on the 'Copy Current Area to all Fluorophores' to set the scan areas.



These are default settings. Verify that nobody has changed them and click 'Next'.

The screenshot shows a software window titled "Scan Protocol - Automatic Sensitivity Calibration". On the left is a vertical sidebar with six steps: 1. Basic Information, 2. Scan Area, 3. Fluorophores, 4. Sensitivity Calibration Areas, 5. Sensitivity Calibration (highlighted with a green play button icon), and 6. Tools. The main area contains a "Automatic Sensitivity Calibration" section with the following settings: an unchecked checkbox for "Automatically calibrate sensitivity for each microarray", "Average spot size (µm):" set to 100, "Target signal intensity (%):" set to 90, and "Sensitivity adjustment method:" with two radio buttons. The first radio button, "Keep laser power fixed, vary PMT gain", is selected. The second radio button is "Keep PMT gain fixed, vary laser power". At the bottom of the window are four buttons: "< Back", "Next >" (which is circled in red), "Finish", and "Cancel".

Scan Protocol - Automatic Sensitivity Calibration

1. Basic Information
2. Scan Area
3. Fluorophores
4. Sensitivity Calibration Areas
5. Sensitivity Calibration
6. Tools

Automatic Sensitivity Calibration

☐ Automatically calibrate sensitivity for each microarray

Average spot size (µm): 100

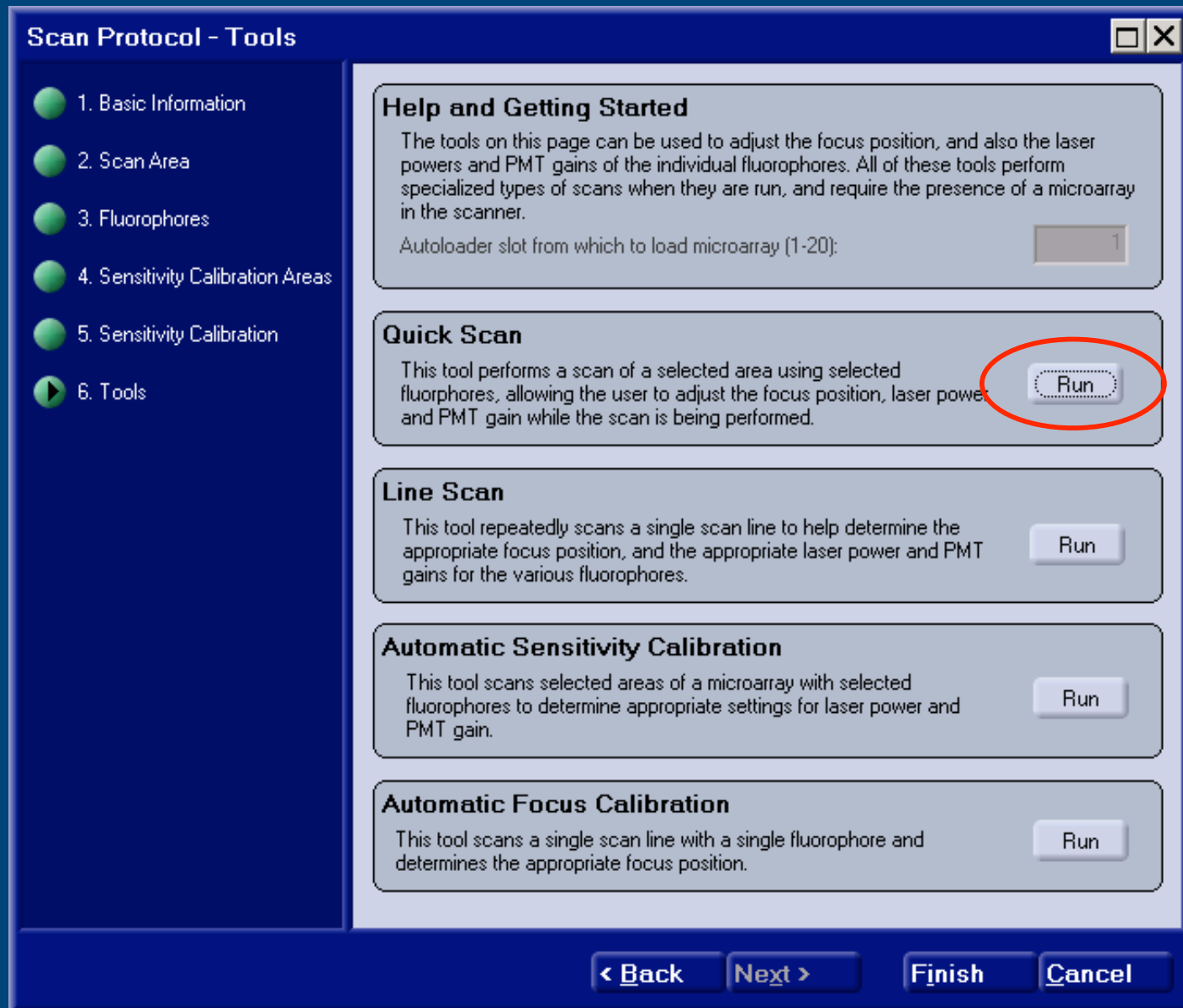
Target signal intensity (%): 90

Sensitivity adjustment method:

☒ Keep laser power fixed, vary PMT gain
☐ Keep PMT gain fixed, vary laser power

< Back Next > Finish Cancel

Click 'Run' in the Quick Scan window.

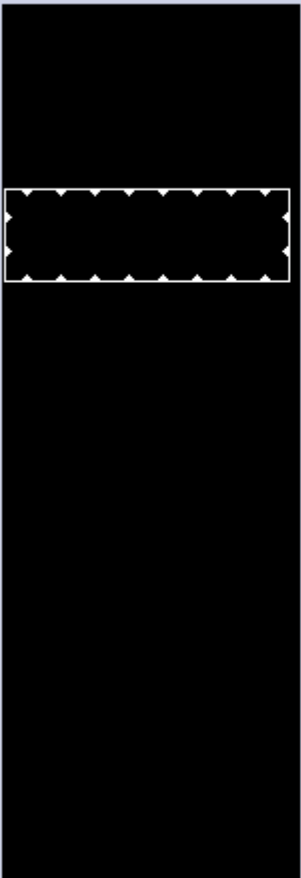


The settings should be as they appear below. Be sure resolution is set at 50 μm or else you're more likely to bleach dyes.

Click 'Start'

Quick Scan [X]

Area to Scan:



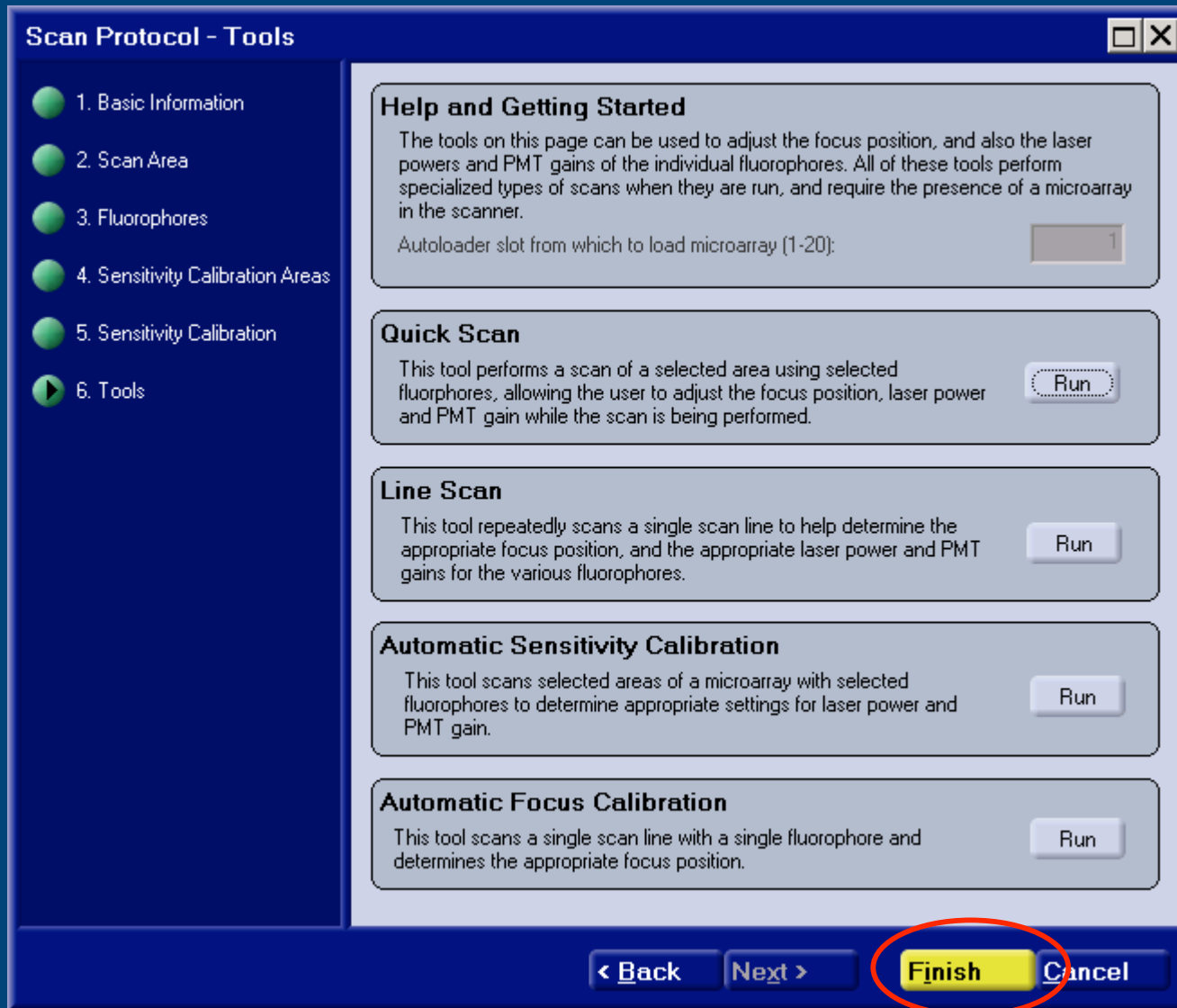
Scan resolution (μm):
☐ 5 ☐ 10 ☐ 20 ☐ 30 ☒ 50

Focus position (μm):

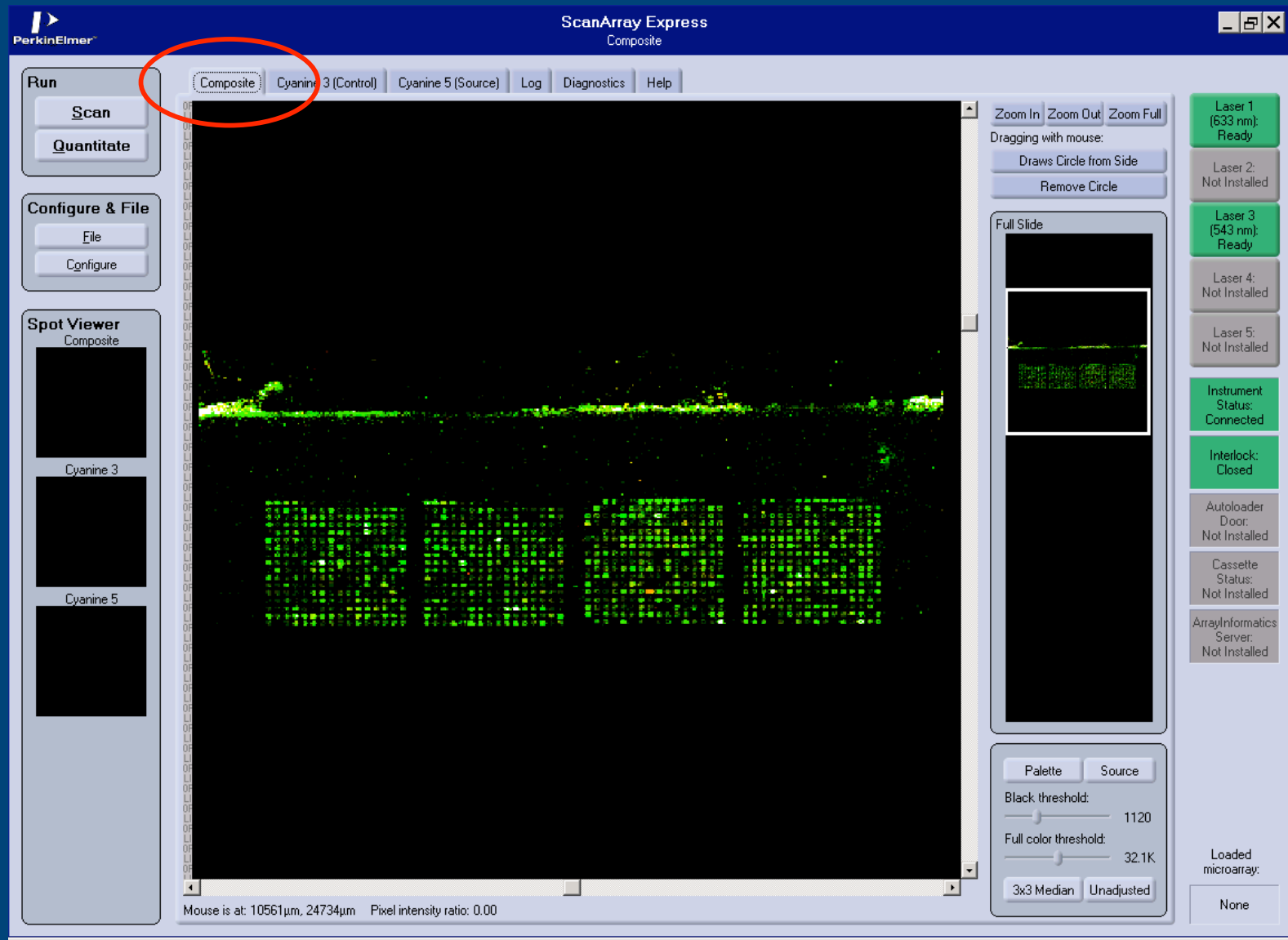
| | Fluorophores | PMT Gain (%) | Laser Power (%) |
|---|----------------------|--------------|-----------------|
| <input checked="" type="checkbox"/> Use | Cyanine 3 | 80 | 70 |
| <input checked="" type="checkbox"/> Use | Cyanine 5 | 70 | 70 |
| <input type="checkbox"/> Use | Select a Fluorophore | 70 | 90 |
| <input type="checkbox"/> Use | Select a Fluorophore | 70 | 90 |
| <input type="checkbox"/> Use | Select a Fluorophore | 70 | 90 |

Help & Directions
The area to scan is shown as a dashed rectangle.
For instructions on how to select an area, click 'Show Zoom Window.'

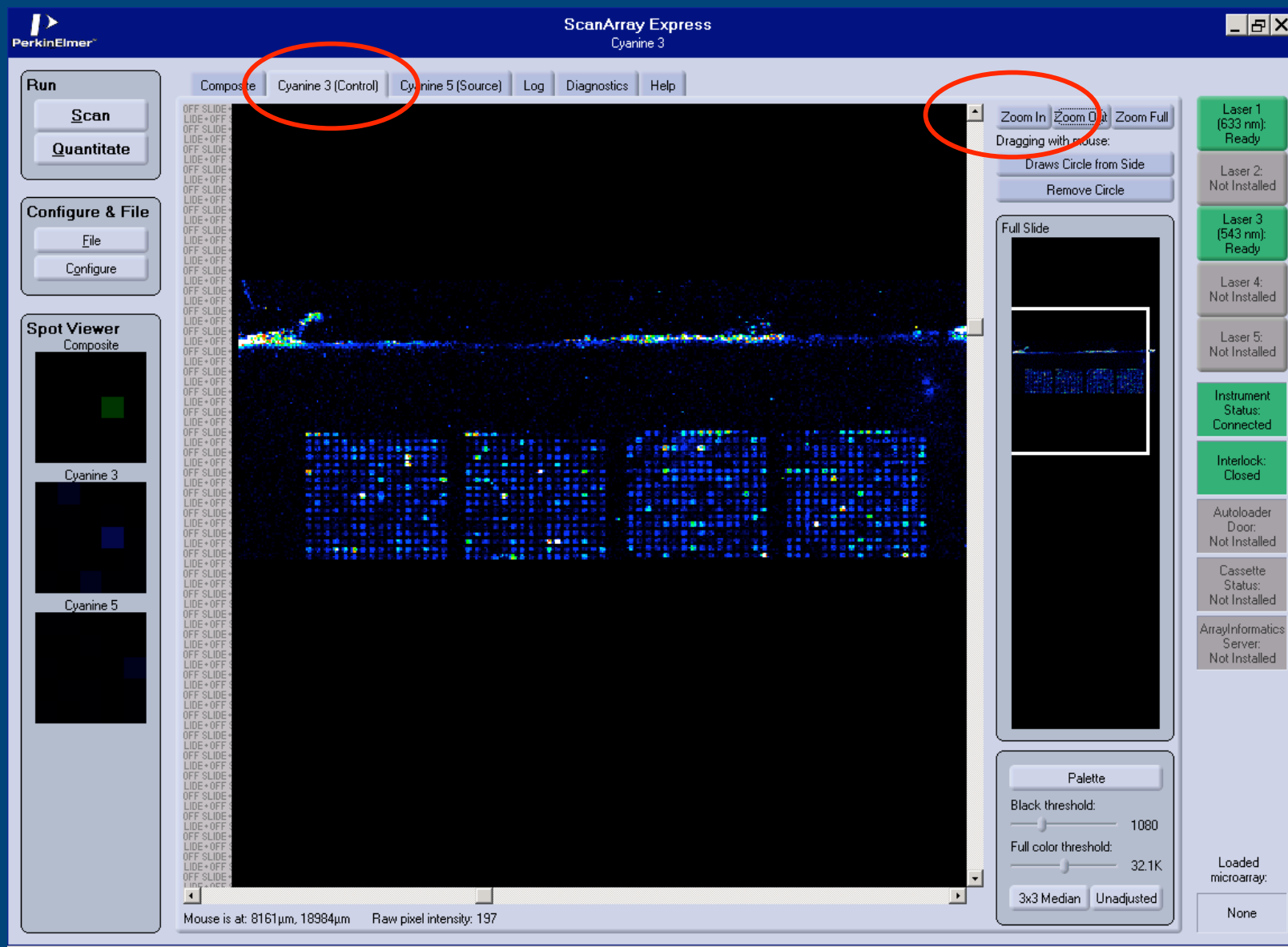
When run ends, click 'Finish' to view the results and determine what adjustments will need to be made to balance the signals between the two dye channels.



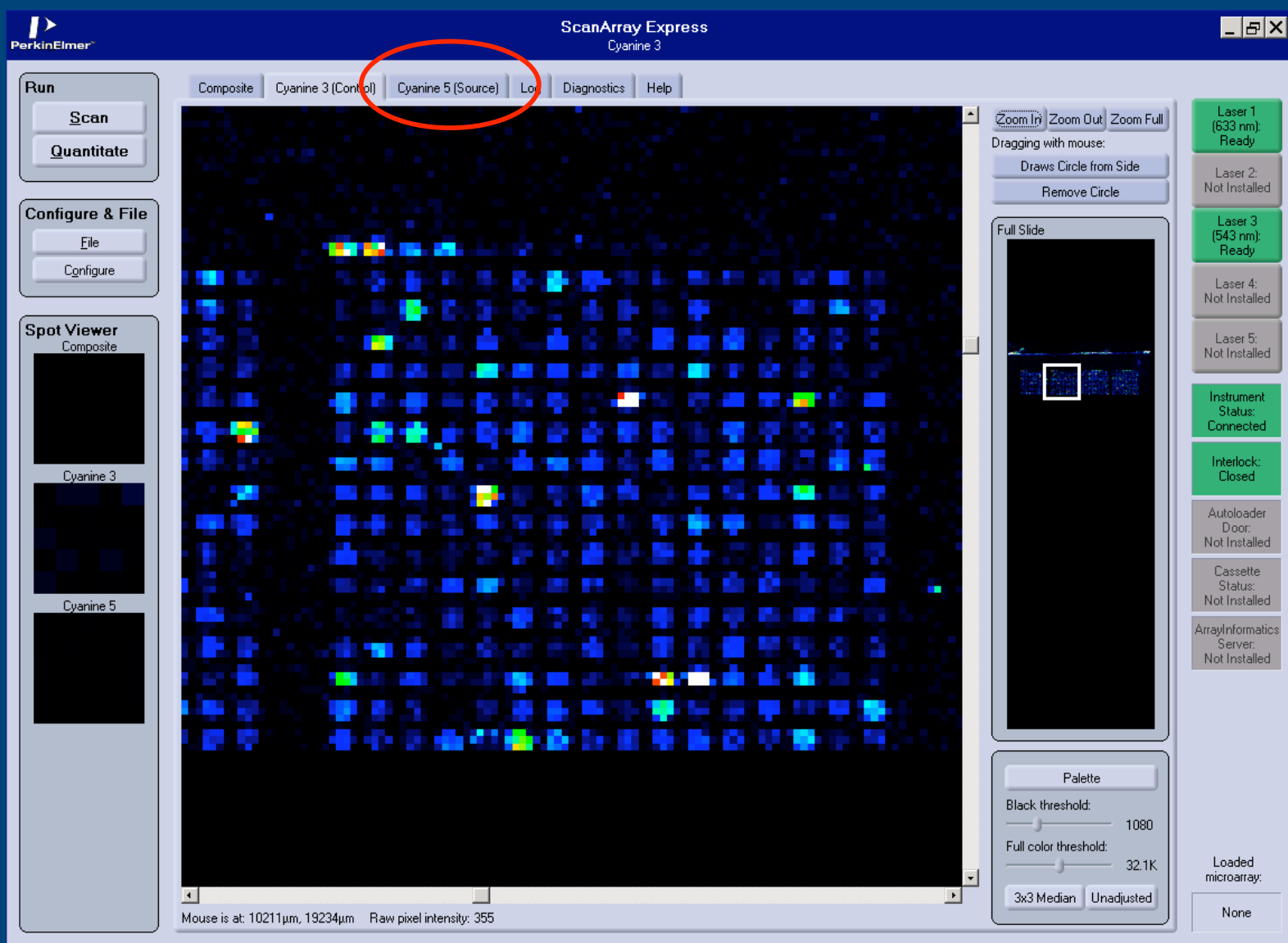
In this example, the composite Cy3_Cy5 image is too green indicating that the overall fluorescence level of Cy3 labelling was much stronger than Cy5. Therefore, we need to either adjust the Cy3 down or the Cy5 up to balance them.



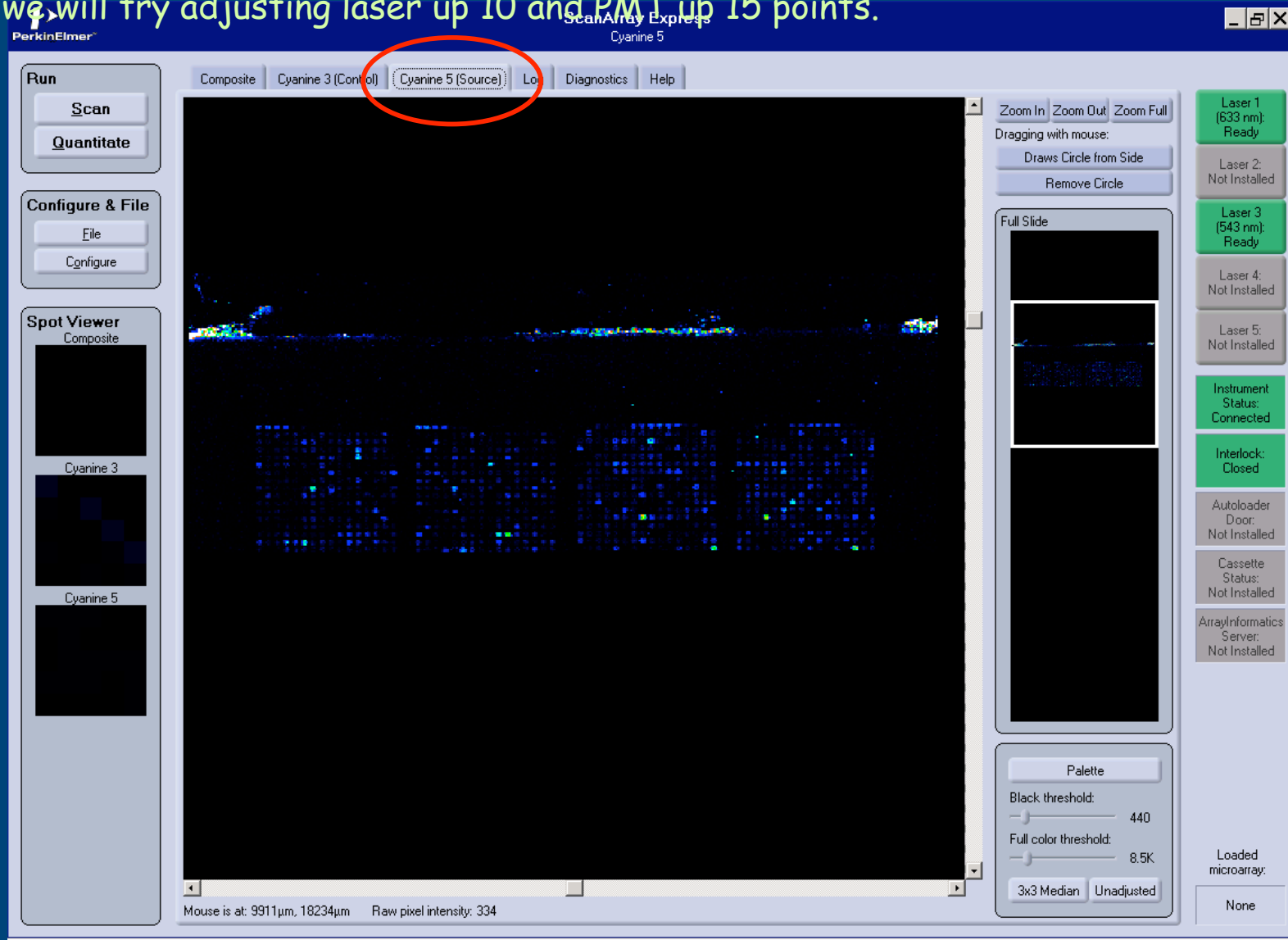
Clicking on the 'Cyanine 3 (Control)' tab shows the intensity related to the Cy3 label. Check to determine the level of saturation. We believe that about 1-3 saturated spots per subgrid is a good level. To see the spots more clearly, click the 'Zoom In' tab.



Close up of a sub grid shows that we are in this range of having about 1-3 saturated spots per subgrid. Therefore, we'll keep this Cy3 setting and adjust the Cy5. Click the Cyanine 5 (Source) tab to see the Cy5 levels.



Clicking on the 'Cyanine 5 (Source)' tab shows the intensity related to the Cy5 label. In this example, the levels are low with none approaching saturation. We will need to increase the Cy5 laser and/or PMT levels to be closer to that of the Cy3 levels. It takes some experience to determine how much to adjust (and this may vary with machine). Here we will try adjusting laser up 10 and PMT up 15 points.

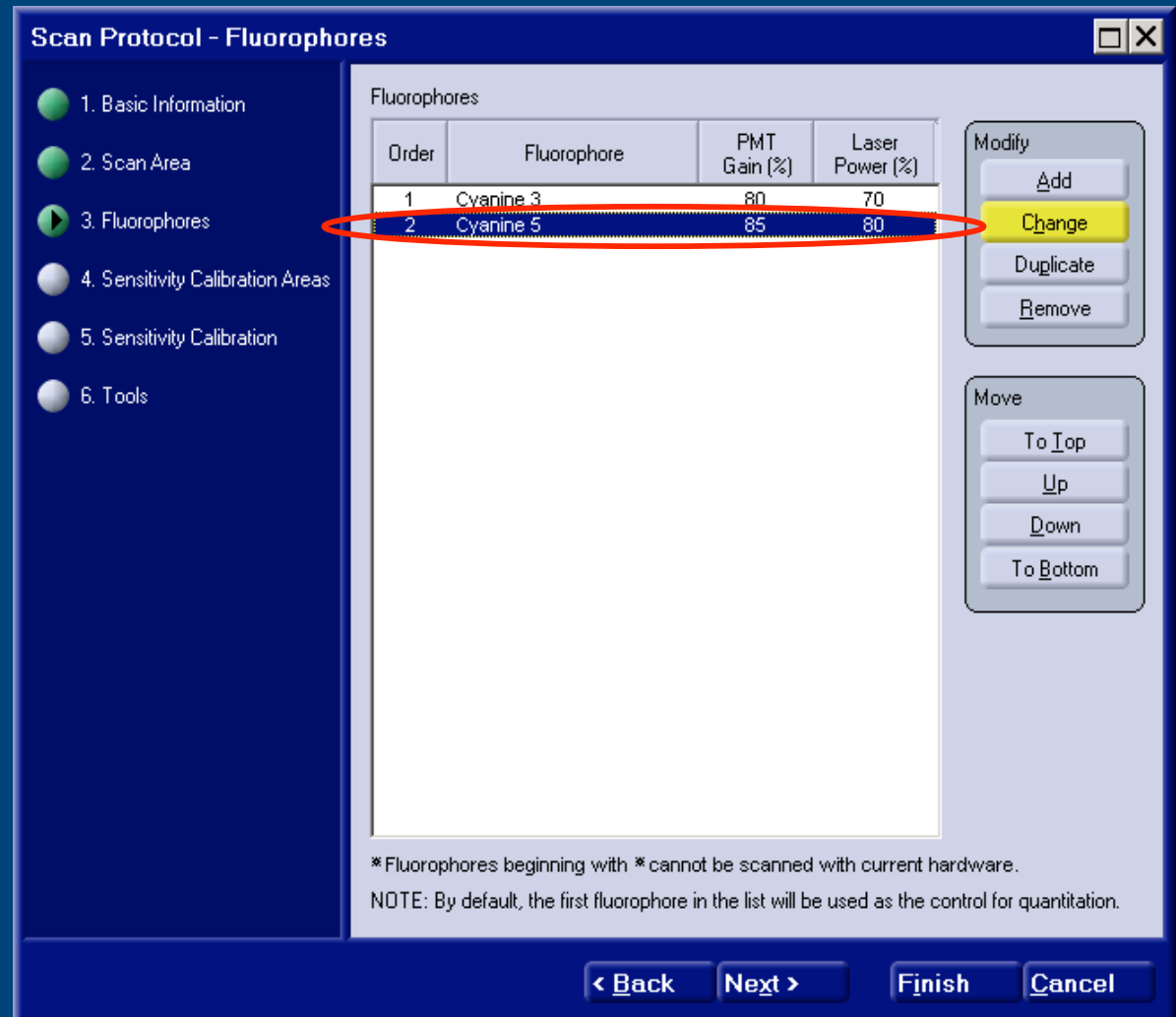


To change Cy5 value:

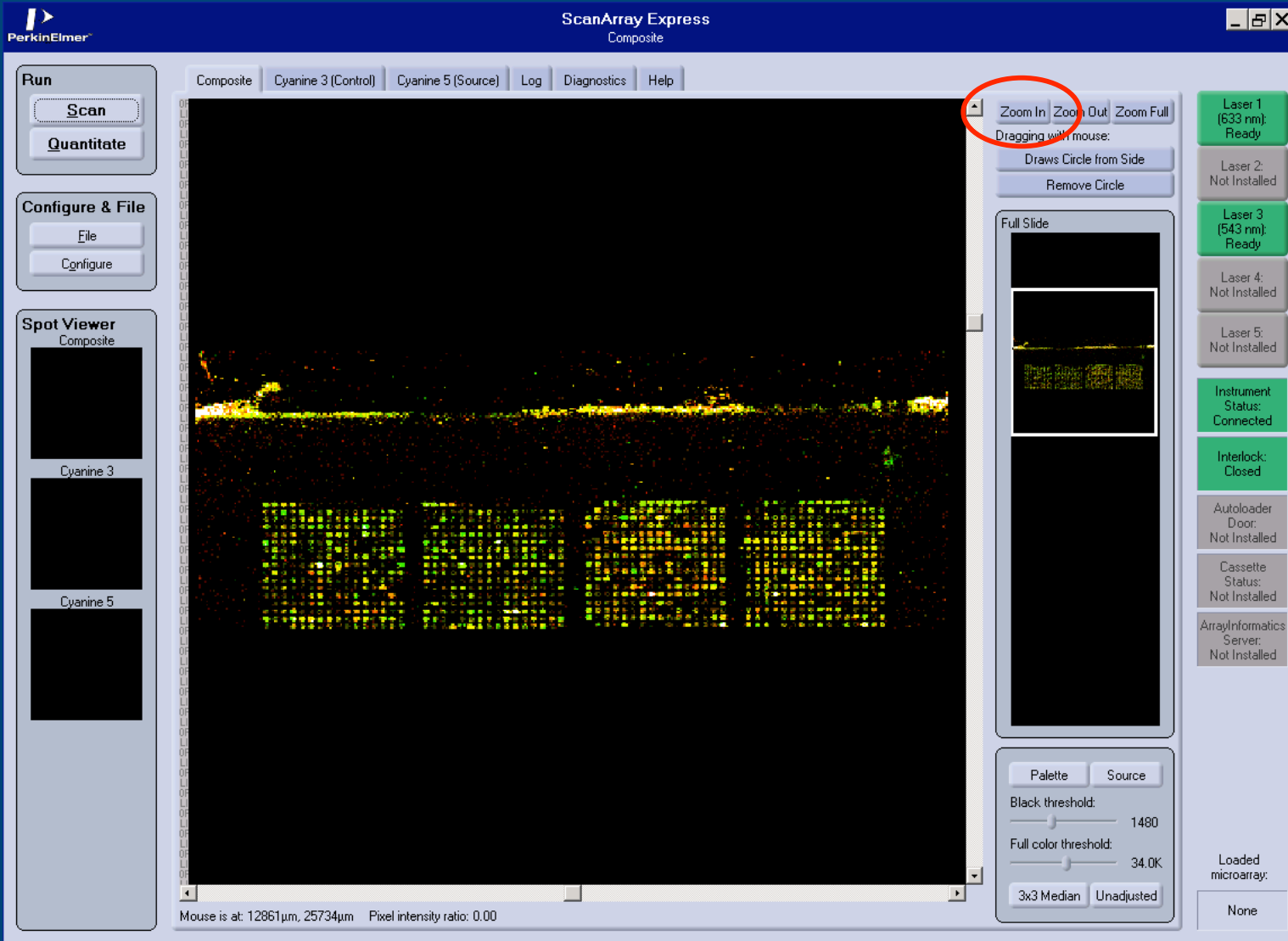
Go back to Step 3, 4, 5, 6.

Change Cy5 values as needed by highlighting Cyanine 5 and clicking 'Change'.

Based on the first quick scan we decided to change the Cy5 to 85 PMT and 80 laser and to leave Cy3 at 80 PMT and 70 Laser

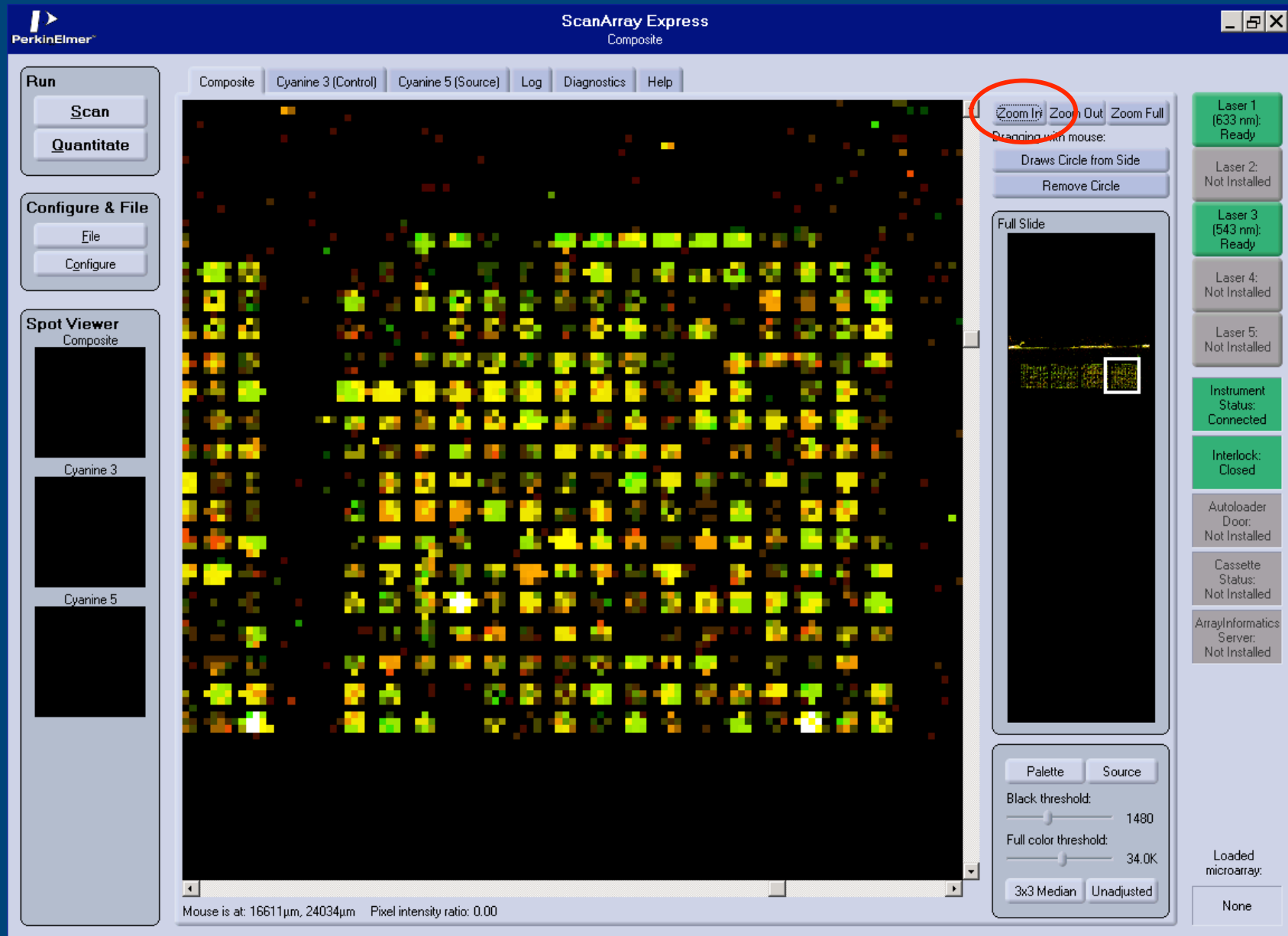


Repeating the quick scan with these new values for Cy5 produced an image with good balance between the two dyes as most spots look yellow with a fairly similar number of reddish and greenish spots. Can click on the 'Zoom In' tab to have a better view.

- 

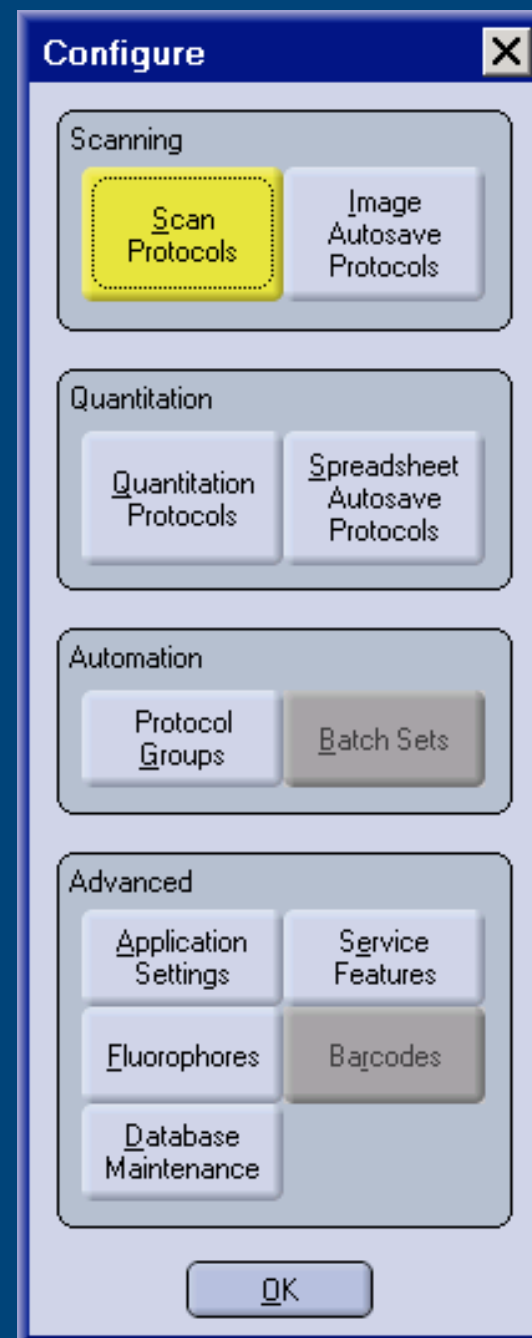
The screenshot displays the ScanArray Express software interface. The main window shows a composite image of a microarray scan with yellow and green spots. The 'Zoom In' button is circled in red. The interface includes a 'Run' section with 'Scan' and 'Quantitate' buttons, a 'Configure & File' section with 'File' and 'Configure' buttons, and a 'Spot Viewer' section with 'Composite', 'Cyanine 3', and 'Cyanine 5' tabs. The right side features a status panel with laser status (Laser 1: Ready, Laser 2: Not Installed, Laser 3: Ready, Laser 4: Not Installed, Laser 5: Not Installed), instrument status (Connected), interlock status (Closed), autoloader door status (Not Installed), cassette status (Not Installed), and array/informatics server status (Not Installed). The bottom status bar indicates the mouse position at 12861µm, 25734µm and a pixel intensity ratio of 0.00.

Use the 'Zoom In' tab to get better picture of a given subgrid.

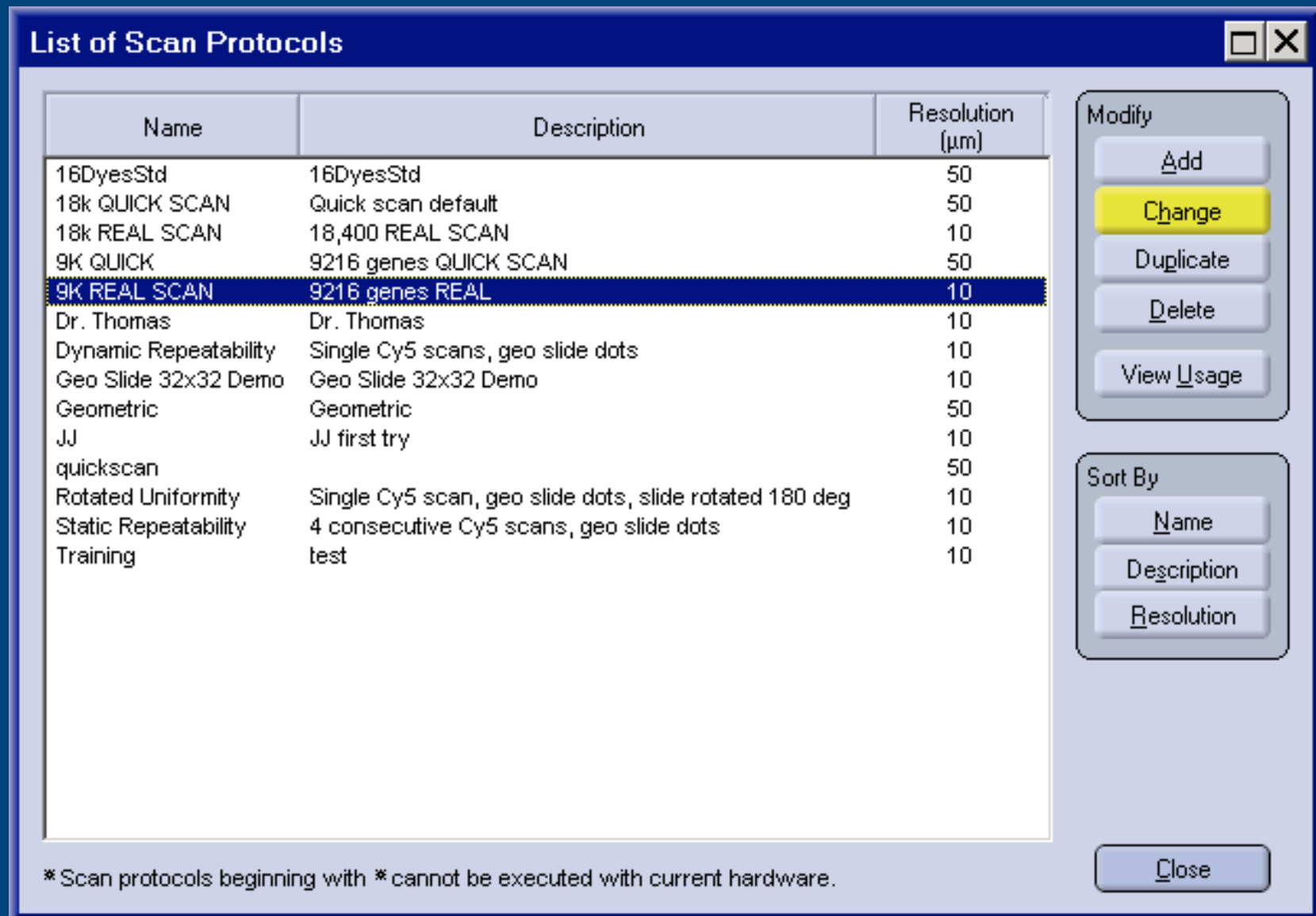


Now that the Quick Scans has given us an acceptable balance between Cy3 and Cy5 images, we need to do a 'real' scan at higher resolution at these same laser and PMT levels.

Go back to the 'Configure' tab and click on the 'Scan Protocols'



Change on the appropriate real scan protocol. This example uses one of the 9K slides (ex: Gm-1021/83, Gm-r1070 or Gm-r1088) so highlight the '9K Real Scan' and hit the 'Change' tab.



Verify that these default settings are in place and click 'Next'.

Scan Protocol - Basic Information [] [X]

1. Basic Information

2. Scan Area

3. Fluorophores

4. Sensitivity Calibration Areas

5. Sensitivity Calibration

6. Tools

Name: 9K REAL SCAN

Description: 9216 genes REAL

Focus position (μm): -2

Scan resolution:

- ☐ 5 μm
- ☒ 10 μm
- ☐ 20 μm
- ☐ 30 μm
- ☐ 50 μm

Scan speed:

- ☐ Half
- ☒ Full

Help and Directions

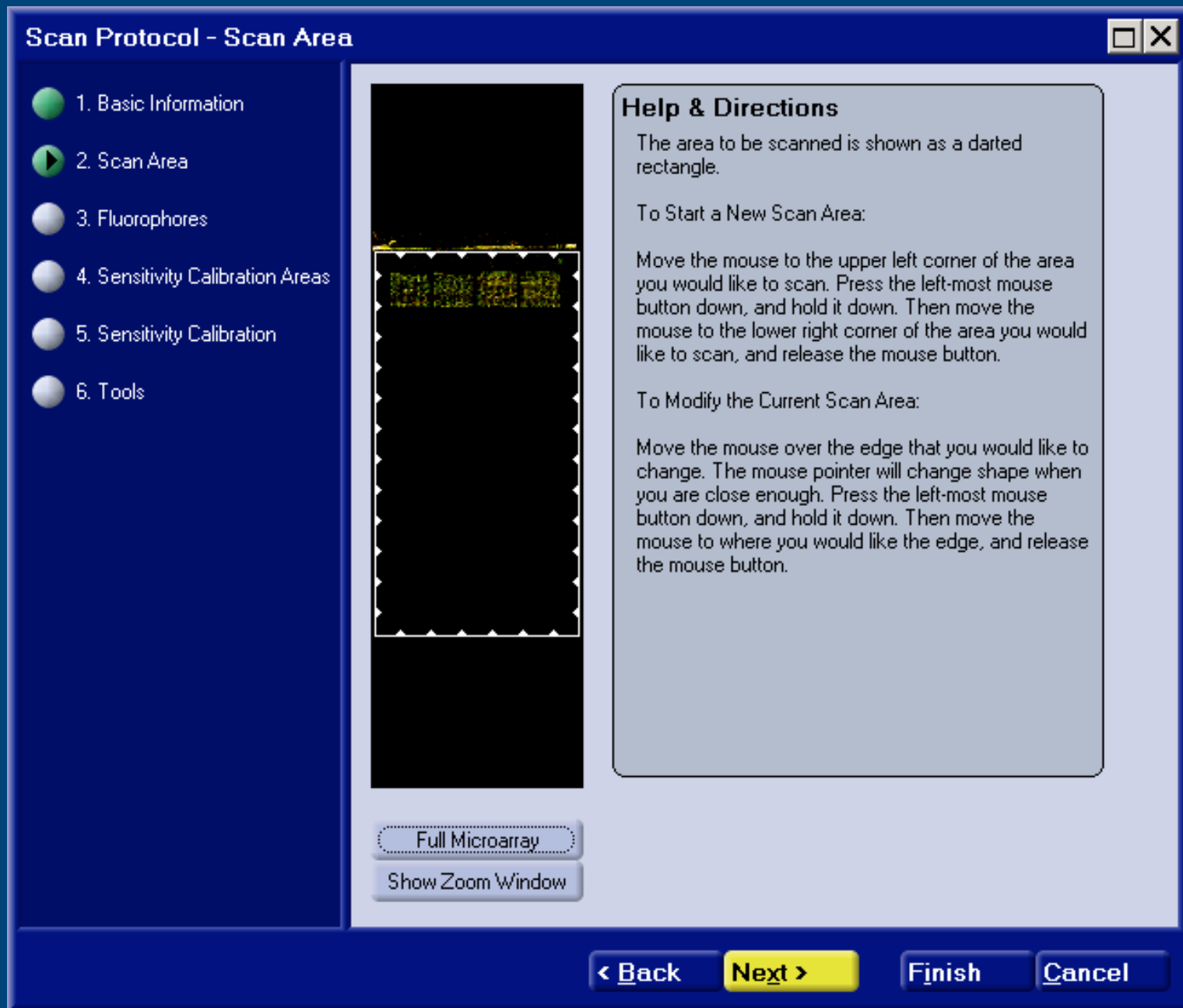
Focus position - should generally be 0 μm .

Scan resolution - should be about 1/10th of the spot diameter.

Scan speed - should be 'Full', unless you specify a resolution of 5 μm , or unless you particularly desire low signal-to-noise ratios at high PMT gains.

< Back Next > Finish Cancel

Verify that the default area is set correctly and click 'Next'.



Change the PMT and Laser settings to use levels determined from Quick Scans. In this example the Quick Scans suggested Cy3: 80 PMT and 70 Laser, Cy5: 85 PMT and 80 Laser.

Scan Protocol - Fluorophores

1. Basic Information
2. Scan Area
3. Fluorophores
4. Sensitivity Calibration Areas
5. Sensitivity Calibration
6. Tools

Fluorophores

| Order | Fluorophore | PMT Gain (%) | Laser Power (%) |
|-------|-------------|--------------|-----------------|
| 1 | Cyanine 3 | 80 | 70 |
| 2 | Cyanine 5 | 85 | 80 |

Modify

Add
Change
Duplicate
Remove

Move

To Top
Up
Down
To Bottom

* Fluorophores beginning with * cannot be scanned with current hardware.
NOTE: By default, the first fluorophore in the list will be used as the control for quantitation.

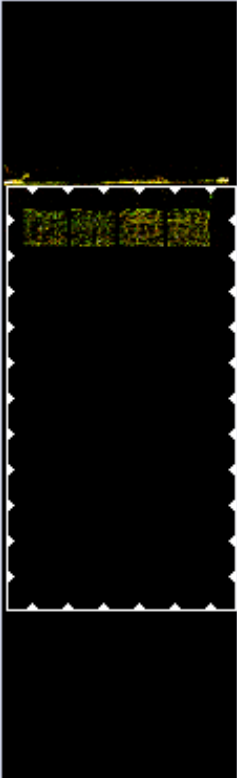
< Back Next > Finish Cancel

Highlight both dyes and click on 'Copy Current Area to all Fluorophores'. Then click 'Next'.

Scan Protocol - Sensitivity Calibration Areas

1. Basic Information
2. Scan Area
3. Fluorophores
4. Sensitivity Calibration Areas
5. Sensitivity Calibration
6. Tools

Sensitivity Area for Selected Fluorophore:



Default Area
Show Zoom Window

| | Fluorophore | Area Top Left (mm) | Area Width x Height (mm) |
|---|-------------|--------------------|--------------------------|
| 1 | Cyanine 3 | 0.39, 17.31 | 21.60 x 39.94 |
| 2 | Cyanine 5 | 0.39, 17.31 | 21.60 x 39.94 |

*Fluorophores beginning with * cannot be scanned with current hardware.

Copy Current Area to all Fluorophores

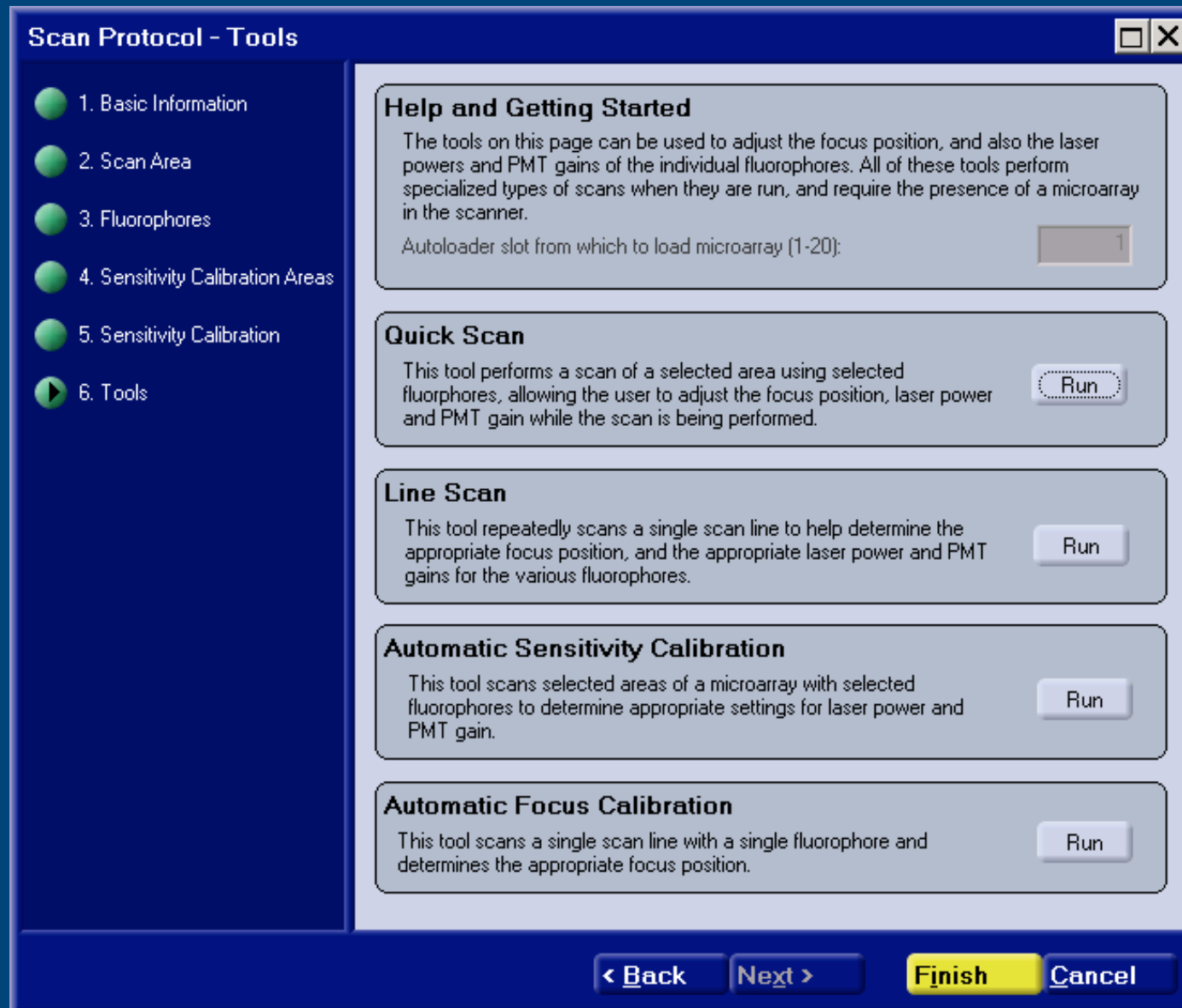
Help & Directions

A different area of the microarray may be used to adjust the sensitivity of each fluorophore.

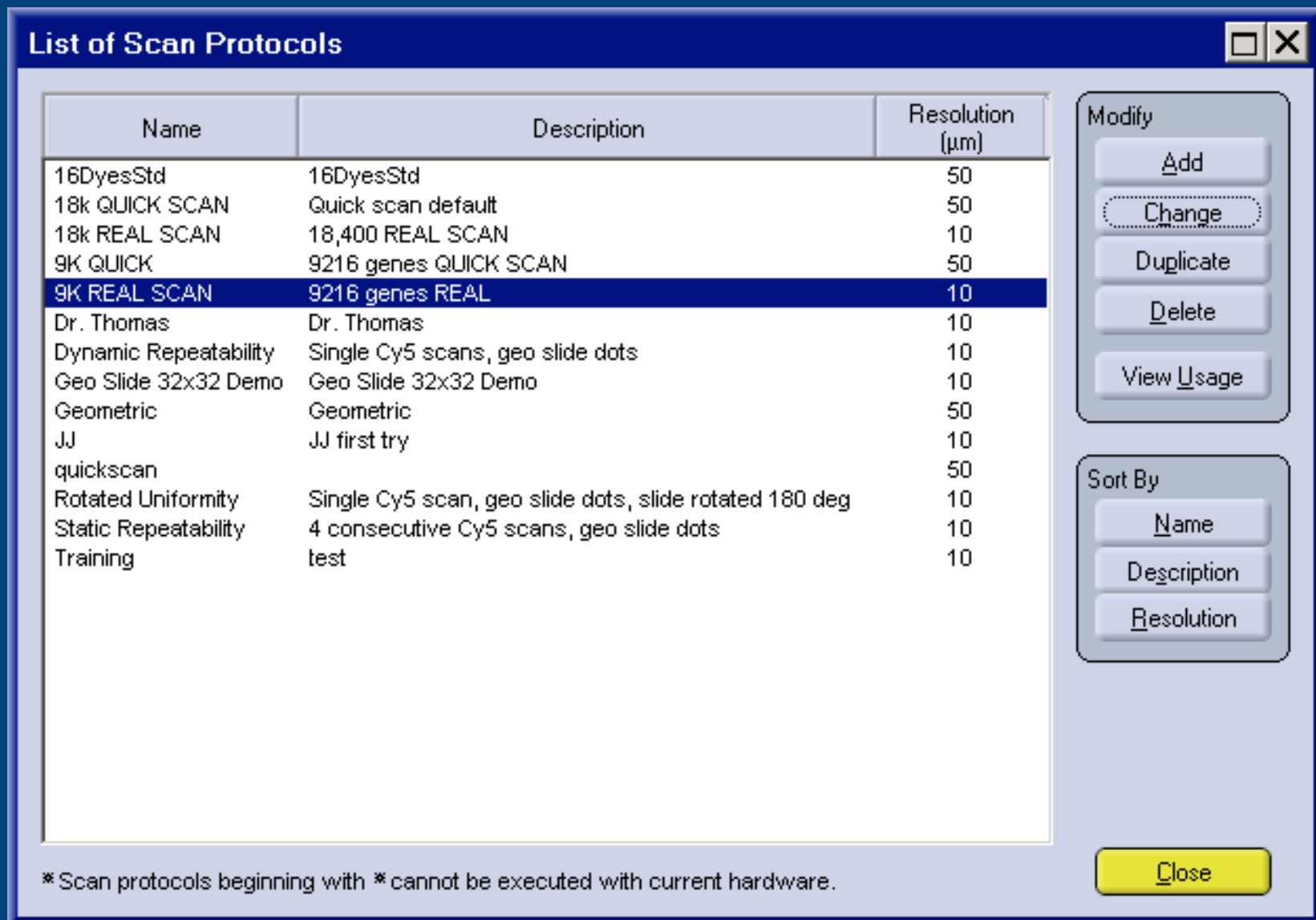
The area to be used for a single fluorophore is shown as a dashed rectangle. To change the fluorophore, select a different line from the list of fluorophores. To change the outlines of the area, use the same directions as were given on page 2 for changing the scan area.

< Back Next > Finish Cancel

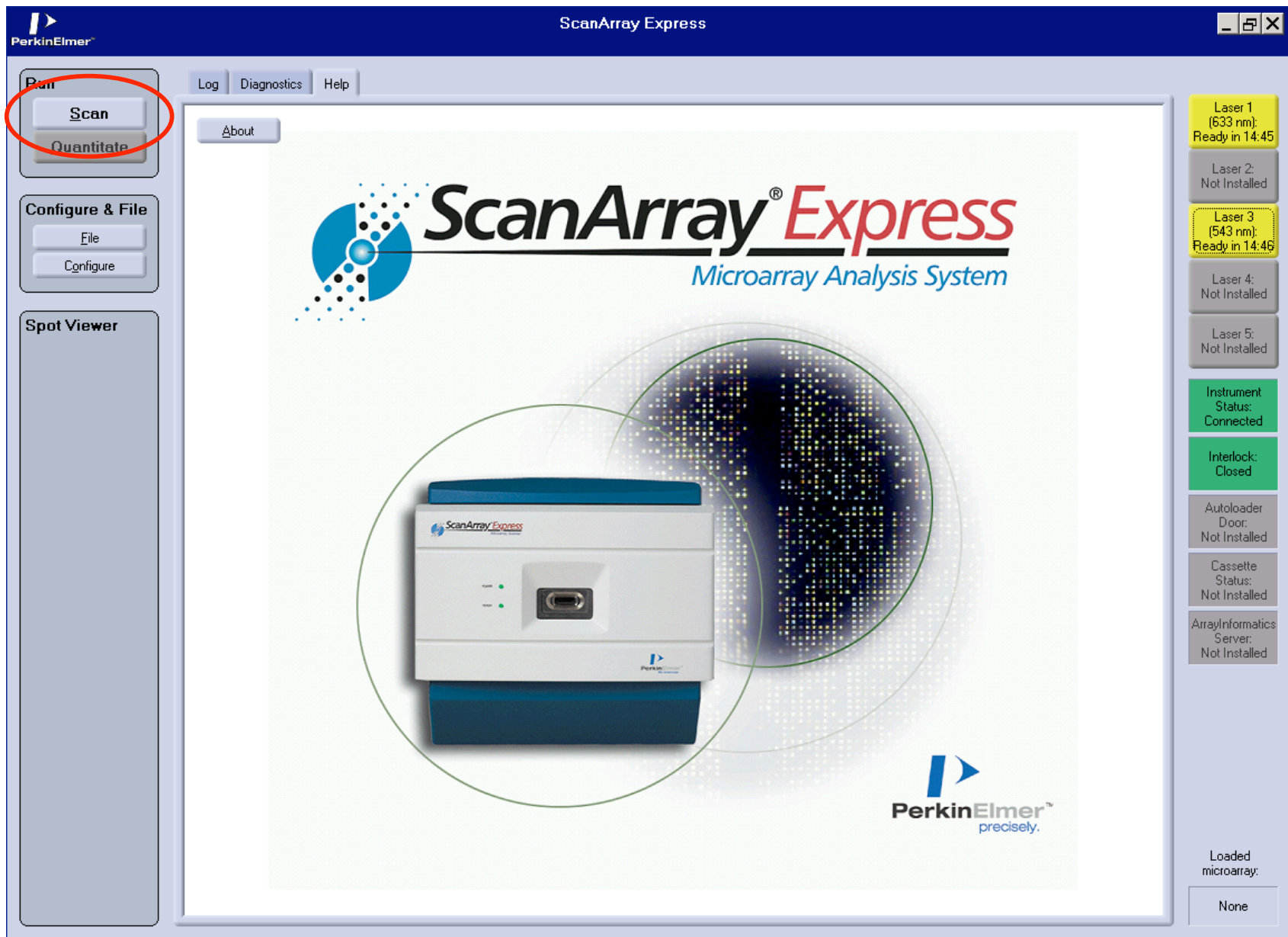
Click 'Finish' tab.



Click 'Close' tab as you have finished setting the scan parameters and are now ready to scan.




Click 'Scan' tab



Select 9K REAL SCAN.
Verify that the default scan
area has not been changed
and click 'Start'

Scan [X]

Scan Area:



Scan type:

- ☐ Run Easy Scan
- ☒ Run a scan protocol
- ☐ Run a protocol group

☐ Obtain scan protocol from barcode

Scan protocol:
9K REAL SCAN

☐ Obtain image autosave protocol from barcode

Image autosave protocol:
Default

☐ Perform automatic quantitation

Quantitation protocol:
Select a Quantitation Protocol

Spreadsheet autosave protocol:
Select a Spreadsheet Autosave Protocol

☐ Automatically save in ArrayInformatics

To change the scan area,
select a different scan protocol.

Start Cancel

The scanner will scan the slide with both the Cy3 and Cy5 lasers and show the composite image, which should be mostly yellowish if the dyes are fairly balanced. The image looks better if click the '3x3 median' tab which averages pixels based on neighboring pixel values.

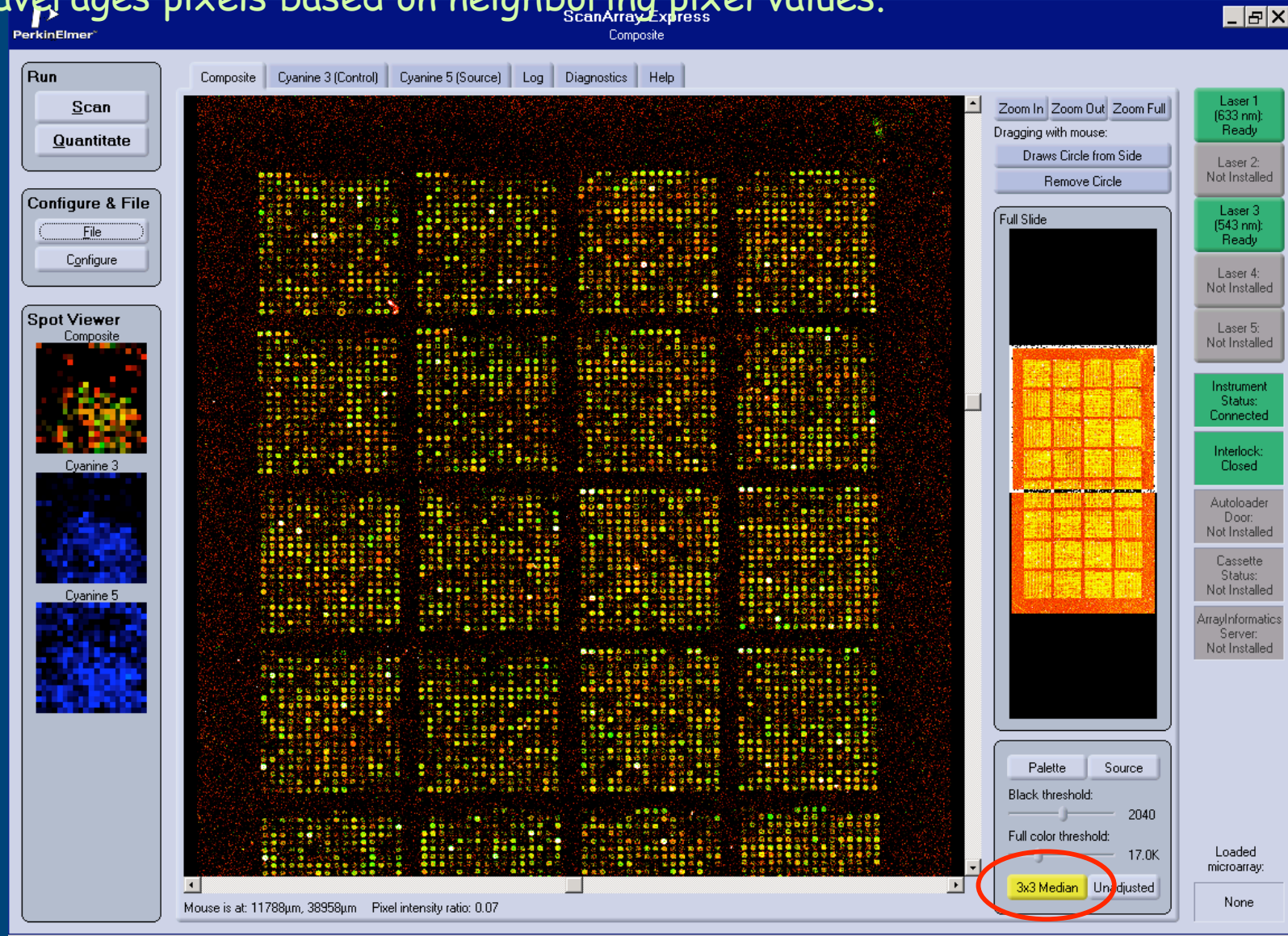
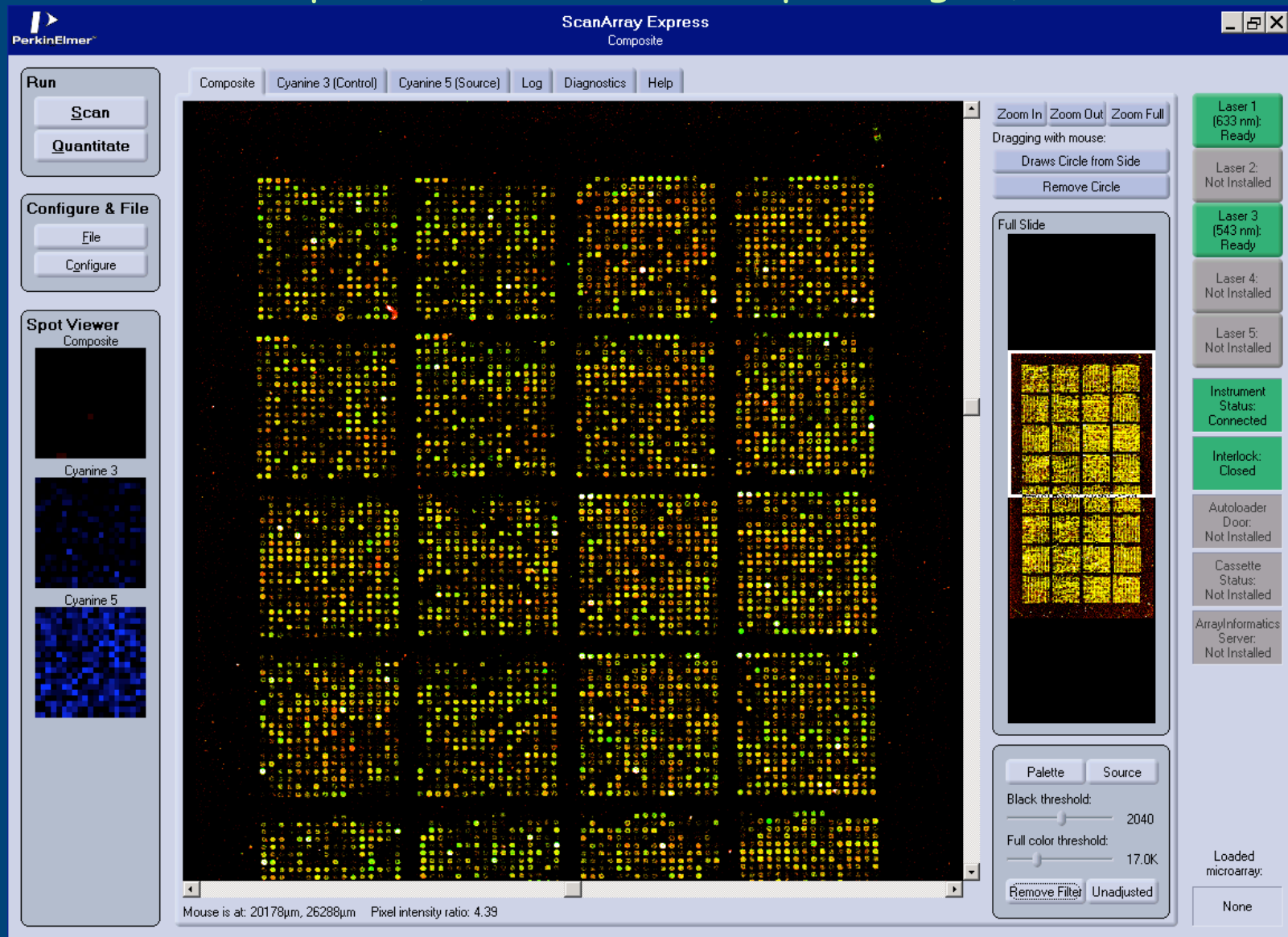
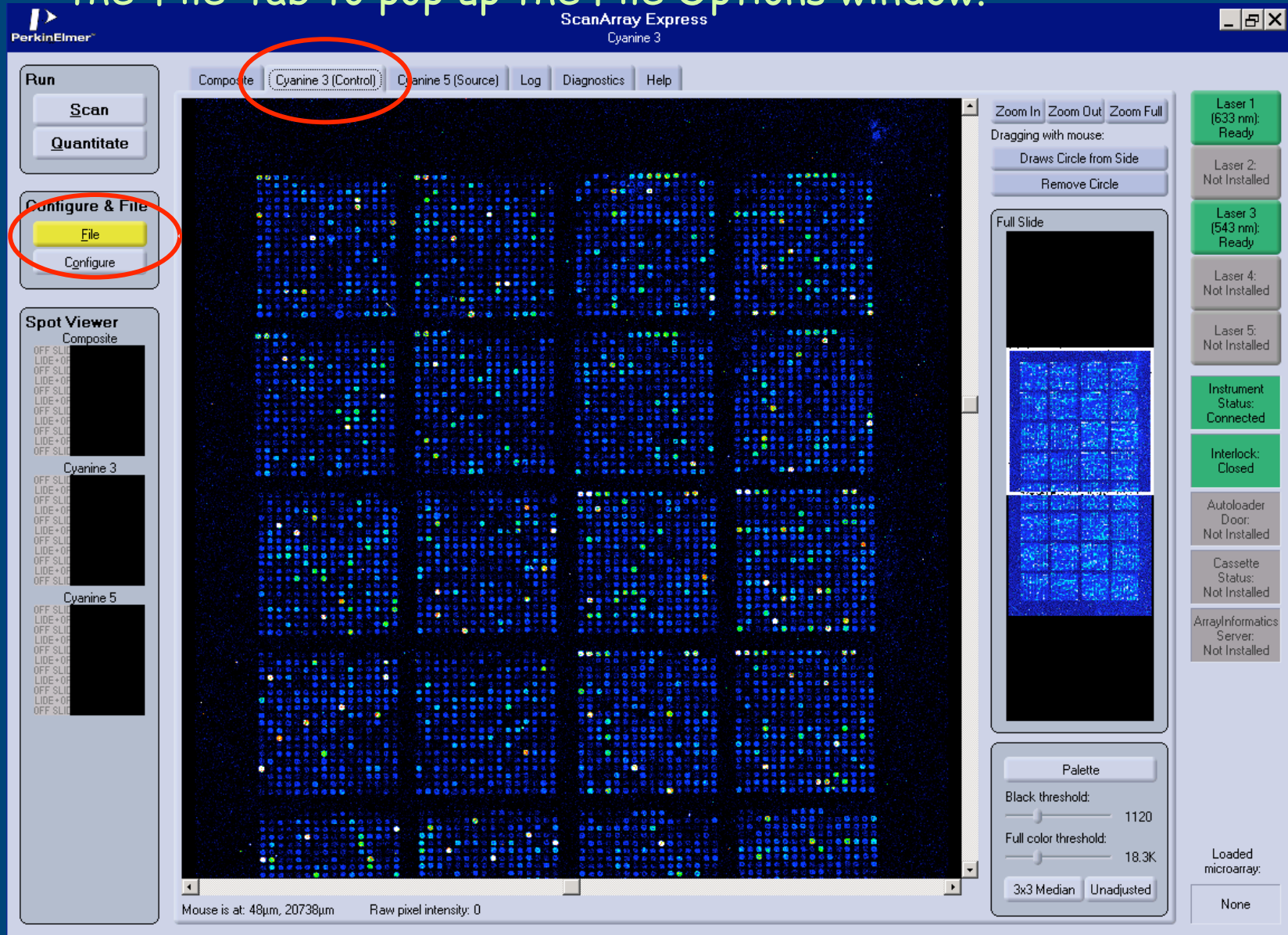


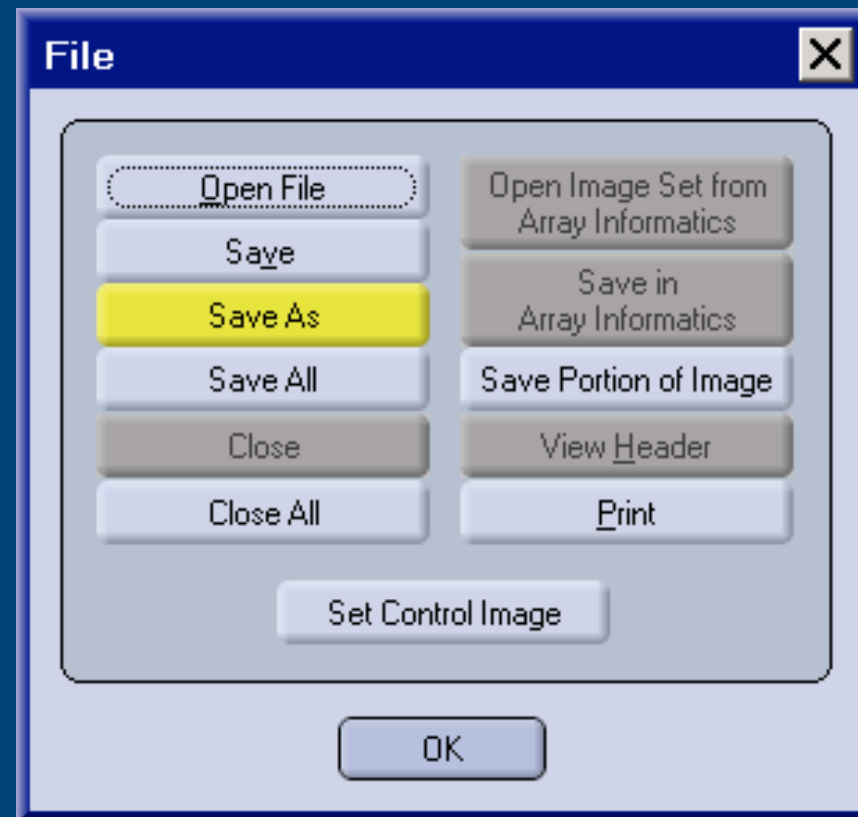
Image after applying the '3x3 Filter'. Note that most spots are fairly yellow and that there are not too many saturated spots (no more than 2-3 per subgrid).



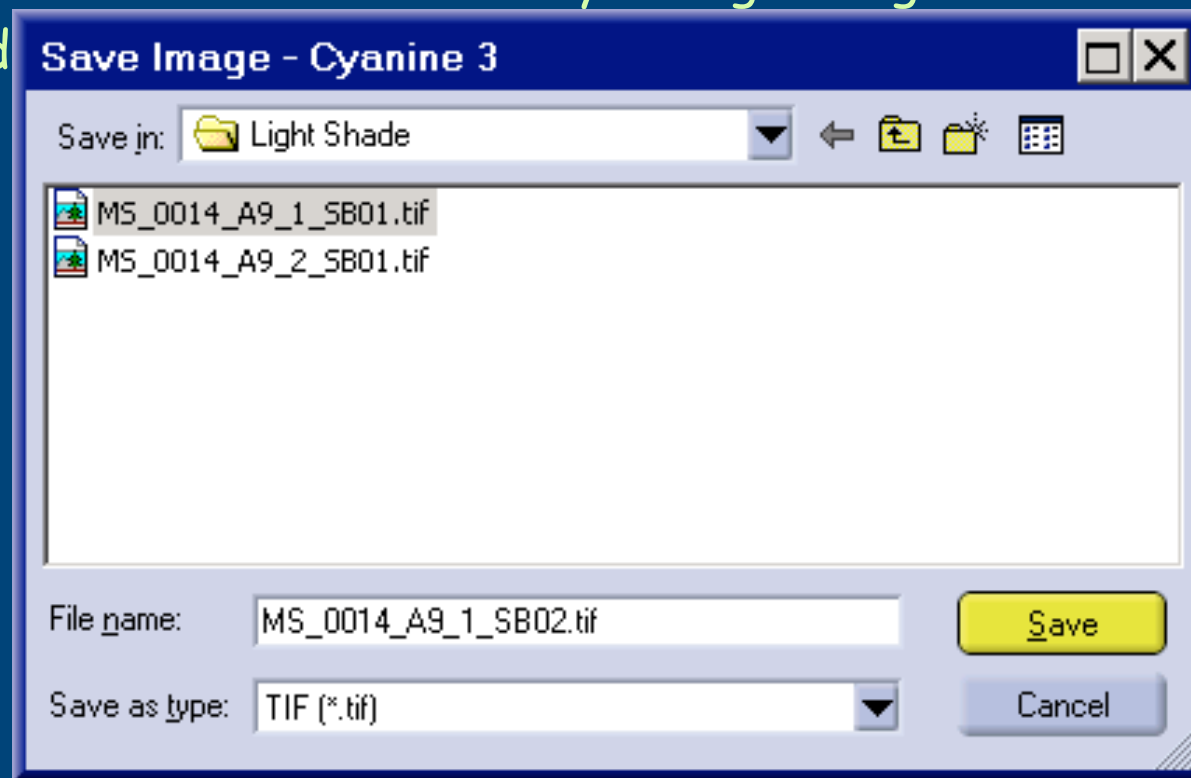
To save the images, you need to save the individual Cy3 and Cy5 scans. Click the 'Cyanine 3 (Control)' tab and then click the 'File' tab to pop up the File Options window.



Click 'Save As' and the then click 'ok'



Identify your folder and type in the file name. To make it easier, you can click on the name of a previous slide and that name will pop into the 'File name' box and you can modify it. The naming convention we use is to follow slide name with channel (1 for Cy3, 2 for Cy5), and then SB followed by the scan for this slide (SB01 for first scan and SB02 for second, etc if one decides they do not like the scan and wish to repeat it). Save every scan as there is always a chance that the next scan will be worse as dyes might begin to fade after repeated



Save Cy5 image

