

Cellometer[®]

PI Cell Cycle Kit

Product Number: CSK-0112

Description: Cellometer™ PI Cell Cycle Kit

Cell Type Markers

Apoptosis

Cell Cycle

Proliferation

Metabolism

Viability



Table of Contents

1. Introduction

1.1 Assay Description	Page 2
1.2 Materials Included	Page 2
1.3 Additional Equipment and Materials Required	Page 2
1.3 Instrument and Software	Page 2

2. Assay Protocol

2.1 Cell Preparation	Page 3
2.2 Staining Procedure	Page 3
2.3 Data Acquisition	Page 3
2.3.6 Focusing	Page 4
2.3.11 Optimizing Exposure Time	Page 5
2.3.13 Confirming Maximum Cell Diameter Setting	Page 6
2.4 Saving and Exporting Data Files	Page 7

3. Data Analysis

3.1 Opening Data Files	Page 7
3.2 Pre-Gate Optimization	Page 8
3.3 Optimization of Gating	Page 9
3.4 Saving Optimized Data Files	Page 14
3.5 Viewing Images	Page 15
3.6 Technical Support	Page 15

4. Software Settings

4.1 Cell Cycle Counting Options Screen	Page 15
4.2 Assay Set-up FOM VB-595-502	Page 16
4.3 Assay Set-up FOM VB-660-502	Page 19

5. Using the Sample Adjustment Calculator

6. Importing FCS Express Layout

1.0 Introduction

1.1 Description of Assay

Cell Cycle:

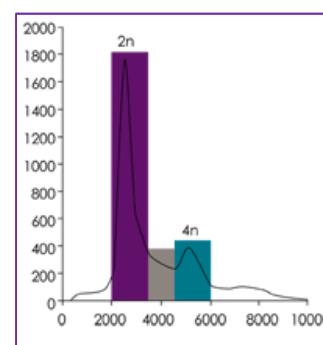
The process of DNA replication and cell division is known as the **Cell Cycle**. The cell cycle is typically divided into five phases: **G₀**, the resting phase, **G₁**, the normal growth phase, **S**, the DNA replication phase, **G₂**, involving growth and preparation for mitosis, and **M**, mitosis.



There are at least two types of cell cycle control mechanisms. The first involves kinase activation to relay a cell from one stage to the next. The second is a type of checkpoint control by which flaws in critical events trigger a delay in cell cycle progression. Because cell cycle events regulate cell proliferation and cancer involves inappropriate cell proliferation, cell cycle and alterations to the cell cycle control mechanisms are at the heart of oncology research¹.

Cell Cycle Analysis using PI

Cell cycle analysis is used to determine the proportion of cells in each stage of the cell cycle for a given cell population based on variations in DNA content. Each cell is stained with a fluorescent dye that intercalates with DNA. Propidium iodide (PI) is a nuclear staining dye that is frequently used to measure cell cycle. Because the dye cannot enter live cells, the cells are fixed with ethanol or methanol prior to staining. All of the cells are then stained. Cells preparing for division will contain increasing amounts of DNA and display proportionally increased fluorescence. Differences in fluorescence intensity are used to determine the percentage of cells in each phase of the cell cycle.



¹Collins, K., *et al.* (1997). Cell Cycle and Cancer. *Proc. Natl. Acad. Sci.* V.94, pp2776-2778.

1.2 Materials Included

- PI/RNase Staining Solution
- Cellometer PI Cell Cycle Kit instructions

1.3 Additional Equipment and Materials Required

- Centrifuge to spin 200 to 400 x g (1000-2000 rpm)
- 37°C incubator
- 1X PBS (pH 7.4)
- Ethanol - 200 proof (absolute), for molecular biology or 100% methanol

1.4 Instrument and Software Requirements

- Cellometer Vision or Vision CBA instrument
- Cellometer Vision CBA software
- De Novo FCS Express 4 software (release 4.01.0018 or newer)
- Fluorescence Optics Module VB-595-502 or VB-660-502
- PI Cell Cycle FCS Express layout (most recent)
 - See section 6.0 for layout import instructions

2.0 Assay Protocol

2.1 Cell Preparation

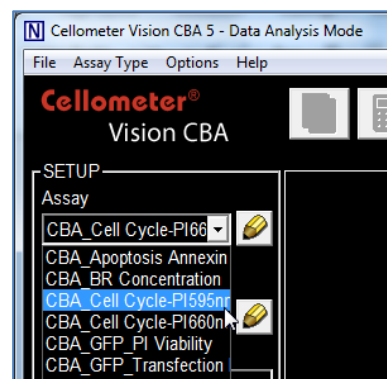
1. Determine the concentration, % viability, and mean diameter of the starting cell preparation using the Vision CBA Analysis System. Target cell viability for the control sample is >90%.
 2. Use the Cellometer Sample Adjustment Calculator to determine the sample volume required to obtain a starting number of 2 million cells. See section 5.0: Using the Sample Adjustment Calculator.
 3. Spin down cell sample at 200 - 400 x g for 5 minutes (using volume calculated in Step 2), then re-suspend cell pellet in 200 μ l of PBS* (for a final concentration of 5×10^6 to 1×10^7 cells/ml.)
 4. Manually pipette cells in and out of a pipette tip until there are no visible cell clumps in the sample (more than ten times).
 5. Gradually add 500 μ l of 200 proof (100%) ice cold ethanol to fix the cells. Gently mix by pipetting in and out at least ten times, and then keep on ice for 15 minutes. (Fixed cells may be stored at -20°C for analysis at a later time. Long term storage is not recommended.)
- *If using methanol to fix, do not re-suspend cell pellet in 200 μ l of PBS. Re-suspend cell pellet in 500 μ l of 100% ice cold methanol, and keep on ice for 15 minutes.

2.2 Staining Procedure

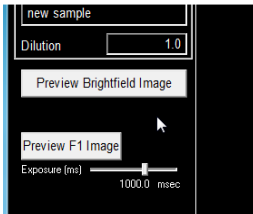
1. Spin down cells sample at 200 - 400 x g (~1,000 to 2,000 rpm) for 8 minutes, remove PBS/Ethanol or methanol solution, re-suspend pellet in 150 μ l of PI staining solution. Gently mix by pipetting in and out until there are no visible cell clumps in the sample (more than ten times).
2. Incubate stained sample inside a 37°C incubator for 40 min.
3. Spin down cells 200 - 400 x g (~1,000 – 2,000 rpm) for 8 minutes. Carefully, without disturbing the pellet, remove the staining solution. Then re-suspend the cells in 200 μ l of PBS.
4. Analyze stained cells within 30 minutes. Store in the dark prior to analysis.

2.3 Data Acquisition

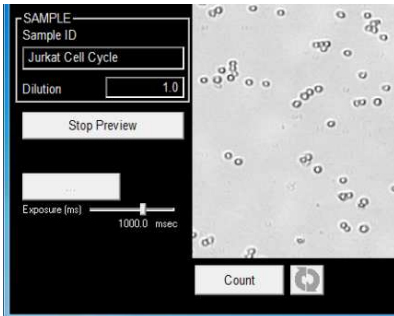
1. Select the assay CBA_Cell Cycle-PI595nm or CBA_Cell Cycle-PI660nm (depending on the fluorescence optics module installed) from the Assay drop-down menu in the upper left corner of the main Vision CBA software screen. The Cell Cycle assay should be run with the default software settings. If you suspect that the settings may have been changed, review the default software settings in section 4.0.
2. Gently mix the cell sample by pipetting in and out until no cell clumps are visible in the sample (more than ten times), slowly vortex if necessary. Load 20 μ L into the Cellometer imaging / counting chamber and insert slide.
3. Wait 60 seconds for the cells to settle in the chamber
4. Type a name for your sample into the Sample ID text box



5. Click Preview Brightfield Image at the bottom left of the main Vision CBA screen.

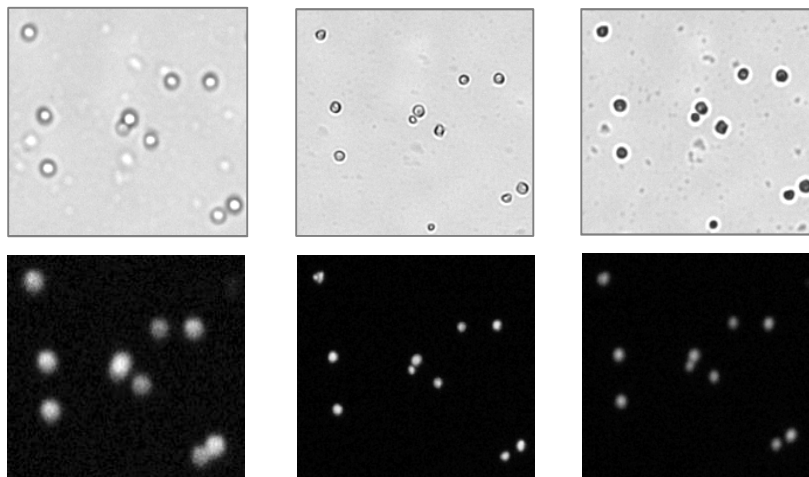


6. Turn the focus knob and adjust focus for the bright field image. Cells in focus for the cell cycle assay will have a bright center and dark outline. There should be a crisp contrast between background and the cell membrane. See Focus Guide in Step 8.

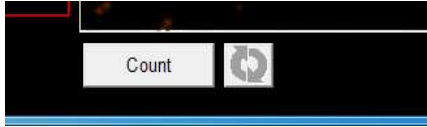


7. Click Stop Preview
8. Click Preview F1 Image button (bottom left of screen) and verify that the fluorescence image is in focus. The fluorescent spots should have sharp edges. If the edges appear fuzzy, the focus should be adjusted. See Focus Guide below. Bright field images are displayed in the top row. Fluorescent images are displayed in the bottom row.

Optimal Focus

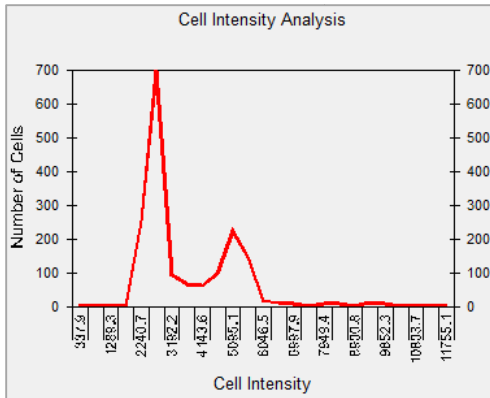


- Click the Count button at the bottom of the screen

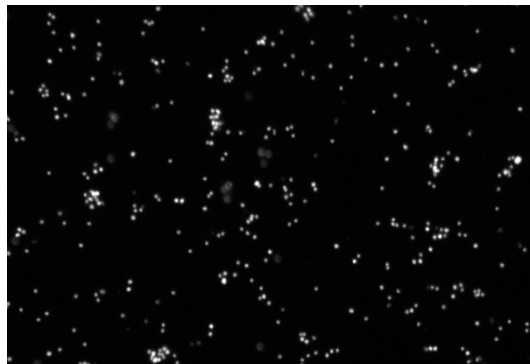


- When counting is complete, an initial Results Table will appear on the screen. For optimal results, the Total Count should be >4,000. If the Total Count is <4,000 repeat the analysis with a more concentrated sample. To concentrate your sample, spin down the cells at 1,000 – 2,000 rpm for 8 minutes, remove the PBS and re-suspend pellet in 50 to 100 µl of PBS. (Re-suspending the pellet in 50 µl will make your sample 4x more concentrated). For cells >15 microns in diameter, a minimum of 2,000 cells is acceptable.

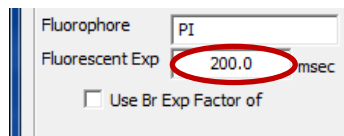
- Verify Exposure Time by clicking on the Intensity Distribution button in the Counting Results window. (see above) Verify that the peak intensity is within the optimal range in the table below for the cell size analyzed (see Mean Diameter in the Counting Results window above). An example intensity plot and image (correct focus and exposure time) are shown below.



Mean Cell Diameter	Target Intensity Distribution
8 - 10 µm	3,000 - 4,000
11 - 13 µm	5,000 - 7,000
14 - 16 µm	8,000 - 10,000
17 - 20 µm	10,000 - 12,000



If the peak intensity is too low, increase the exposure time in the Dialog Screen (see section 4.2.1 or 4.3.1) by 200 msec (for 595 nm emission) or by 500 msec (for 660 nm emission). If the peak intensity is too high, decrease the exposure time by 100 msec (for 595 nm emission) or by 200 msec (for 660 nm emission), then re-analyze the sample.



- Click the **Close** button at the bottom right corner of the Counting Results table. Select the F1 Counted image at the right-hand side of the screen.



- Image 1.** When the maximum cell diameter is set correctly, large cell clumps will be outlined in yellow (not counted) and single cells will be outlined in green.

Image 1.

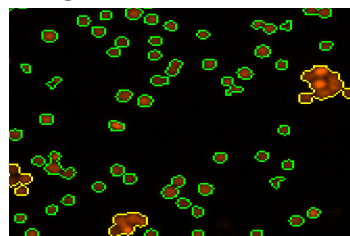


Image 2. If single cells are yellow, increase the maximum diameter in the Fluorescent Cell Type page by 5 microns*, then click re-count.



*See section 4.2.2 or 4.3.2. Adjust the maximum cell diameter in the fluorescent tab only. Repeat if necessary.

Image 2.

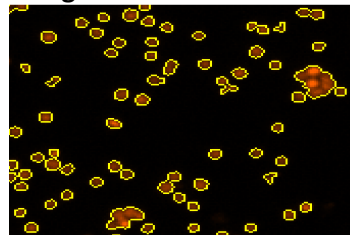
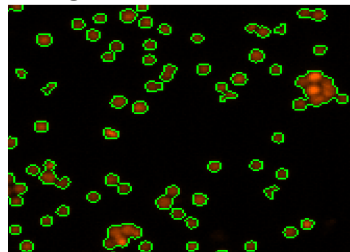


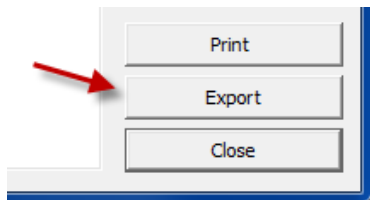
Image 3. If the maximum cell diameter is set too high, cell clumps will be outlined in green and counted as single cells. In this case, the maximum cell diameter can be reduced and the image re-counted. (See section 4.2.2 or 4.3.2. Adjust the maximum cell diameter in the fluorescent tab only.) Fluorescent signal from counted cell clumps will show up at the far right of the cell cycle histogram and will not be included in the G0/G1, S Phase, and G2/M Phase data following optimization of gating.

Image 3.

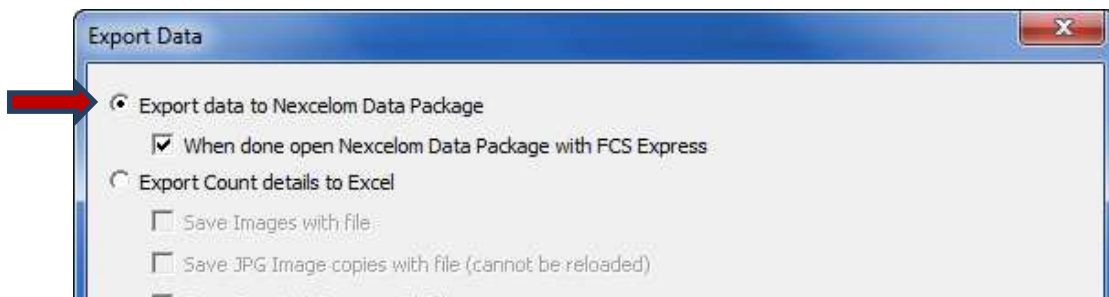


2.4 Saving and Exporting Data Files

1. Once cells are correctly counted, click the Export button at the bottom right of the Counting Results screen.



2. Confirm that “Export data to Nexcelom Data Package” is selected, and then click Continue.



3. Select a location and enter a name for the data package. Three items will be saved in the designated location with the file name entered:



Raw Nexcelom data file (.nxdat)



Folder with cell images



FCS Express 4 launch file

*The .nxdat and launch files must be kept in the same location to access data

3.0 Data Analysis

3.1 Opening Data Files

If the Nexcelom Data Package is not selected to automatically open, click on the launch icon in the designated data package location to open the data file in the FCS Express 4 software.



3.2 Pre-Gate Optimization

1. We utilize a pre-gate to define the counted cells to be analyzed for cell cycle distribution. The pre-gate should be manually adjusted to encompass the main cell population, but eliminate cell clusters exhibiting higher levels of fluorescence (large red circle in Figure 3). Inclusion of cell clusters will result in a long tail to the right of the G2/M peak and gate in the resulting histogram. These un-gated cells will be included in the total cell population and will skew the population percentage calculations.
2. As indicated in Figure 3, the area below the main cell population (small red circle) includes very late stage apoptotic/necrotic cells and cellular debris. This population may be included or excluded from the pre-gated population based on user preference, as it is gated with a separate Sub-G1 percentage in the final histogram.
3. A default pre-gate is shown in Figure 1. To move the pre-gate to better define the cell population, move the cursor over the circle area and left click to select it. Drag and move it toward the cell population. (Figure 2.)

Figure 1.

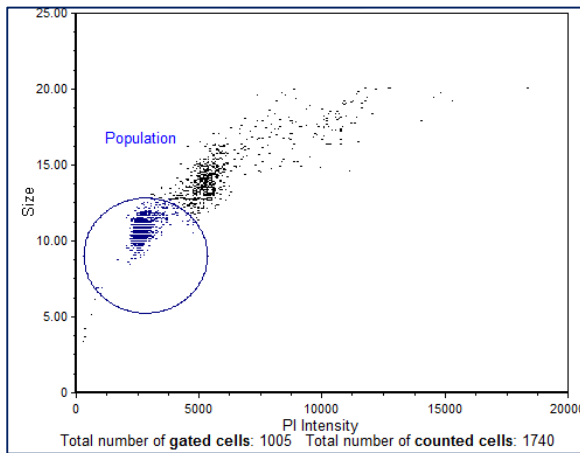
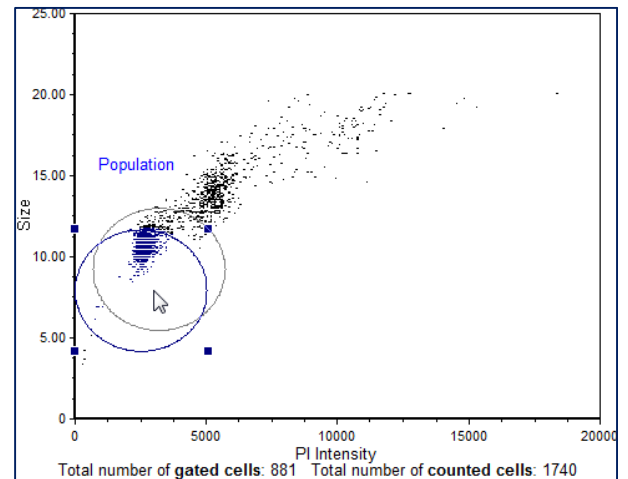
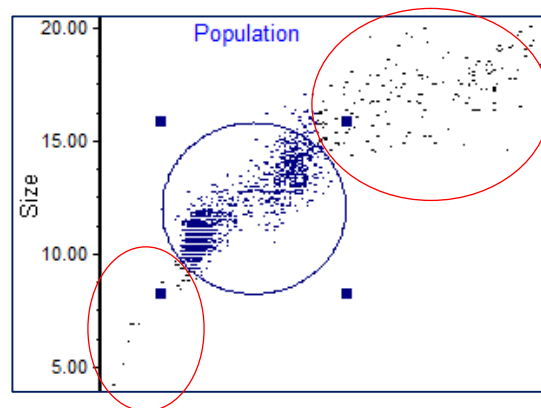


Figure 2.



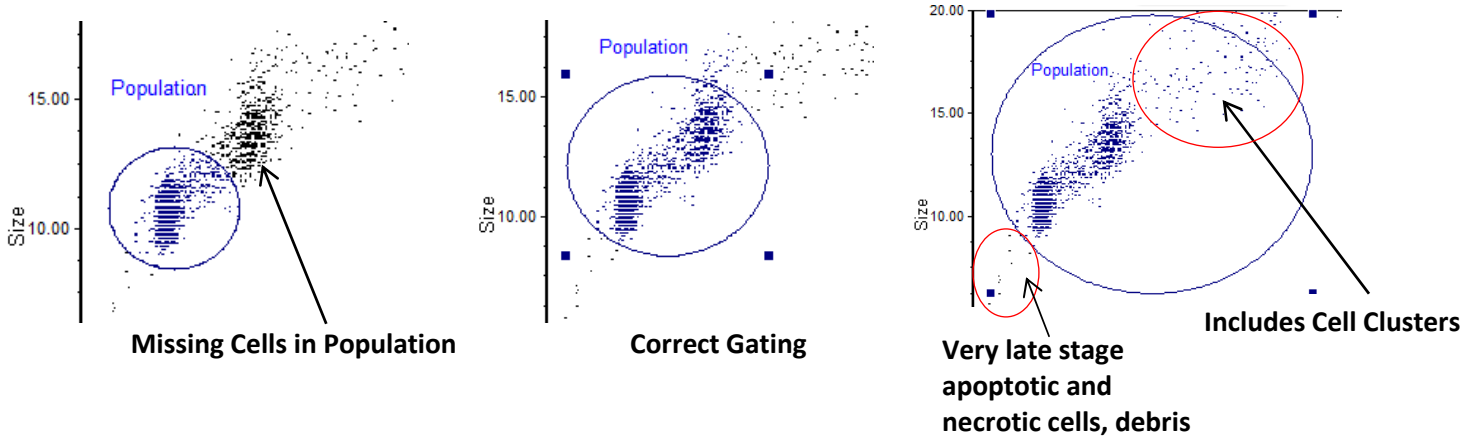
4. To adjust the size of the gate, left click on the circle area (the circle is selected when 4 squares appear at the corners), left click on and hold one of the squares, then drag it to make the circle bigger. Release the left click button when the circle encompasses the desired cell population. See Figure 3.

Figure 3.



- Examples of correct and incorrect gating are shown in Figure 4 below. The population containing very late stage apoptotic/necrotic cells and debris may or may not be included in the pre-gate, depending upon user preference.

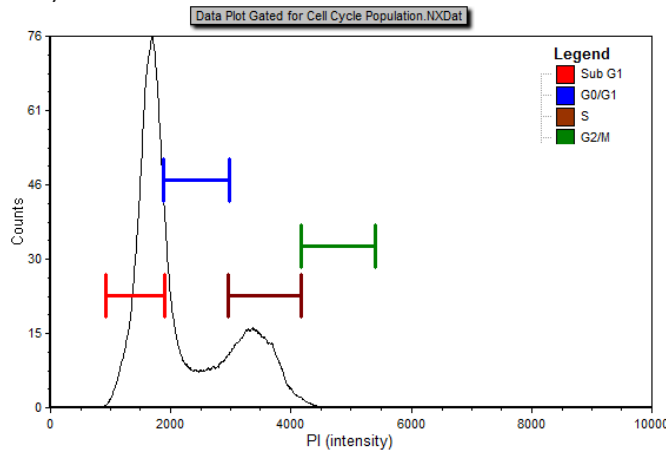
Figure 4.



3.3 Optimization of Gating and Verification of Exposure Time

- A preliminary cell cycle plot using default gating and cell size parameters is automatically displayed. (Figure 5.)

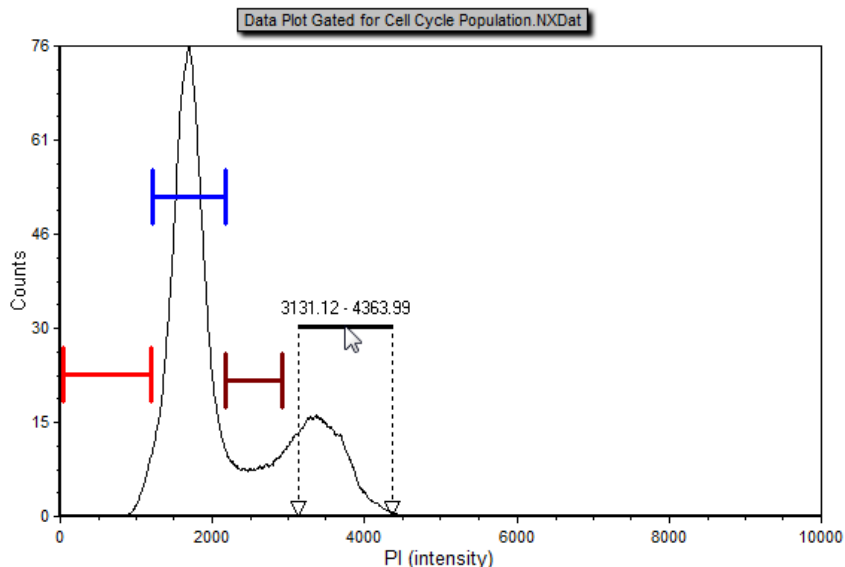
Figure 5.



Cell Population	% of Gated Cells	CV	Concentration (10 ⁶ cells/mL)
Total	100		10.0
Sub G1	37.6	7.3	3.7
G0/G1	46.4	10.4	4.6
S	22.3	10.5	2.2
G2/M	9.8	7.4	1.0

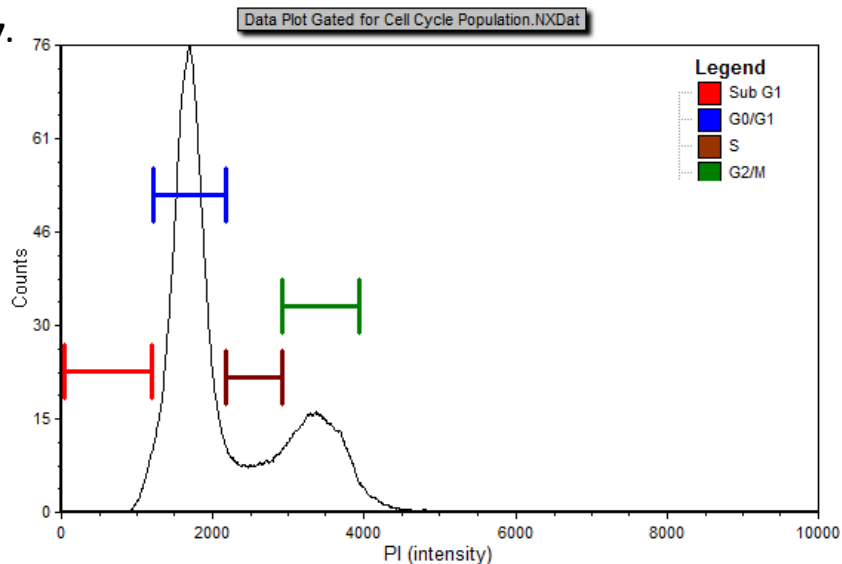
- To adjust a gate, click on the graph. Click on the gate to be moved. Hold the left mouse button and drag the gate to the desired location. (See Figure 6.)

Figure 6.



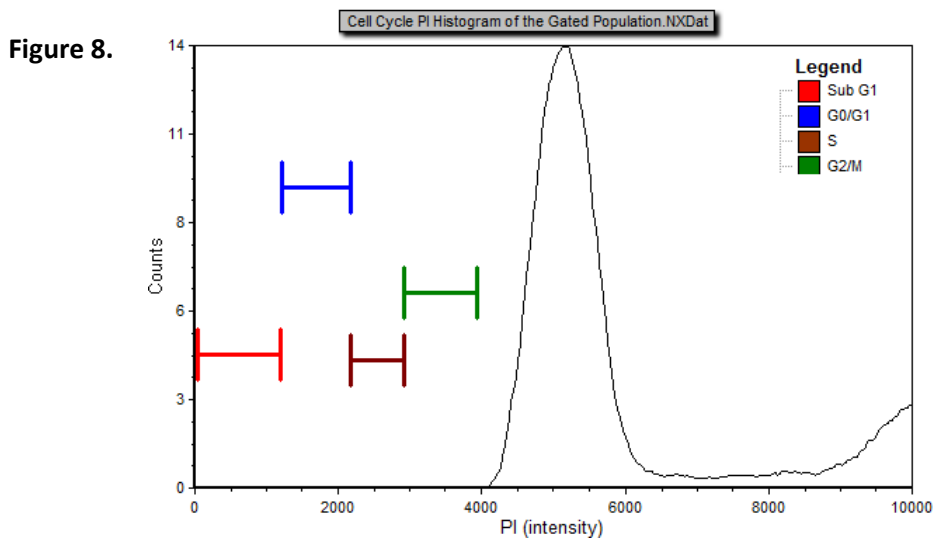
- Manually drag the gates in the data plot and the phase text boxes to more accurately define the peaks and the phases in between peaks. The left-hand peak represents the G0/G1 phase. The right-hand peak represents the G2/M phase. The area between the peaks is the S Phase. The area to the left of the G0/G1 phase includes very late apoptotic/necrotic cells and cellular debris. The area to the right of the G2/M phase includes cell clusters that were not removed in the pre-gate step. This population can be reduced by optimizing the pre-gate. Data in the table displaying cell percentages and concentration will automatically update as gates are adjusted. The data below (Figure 7.) shows the updated cell cycle gating and revised percentages following manual optimization.

Figure 7.

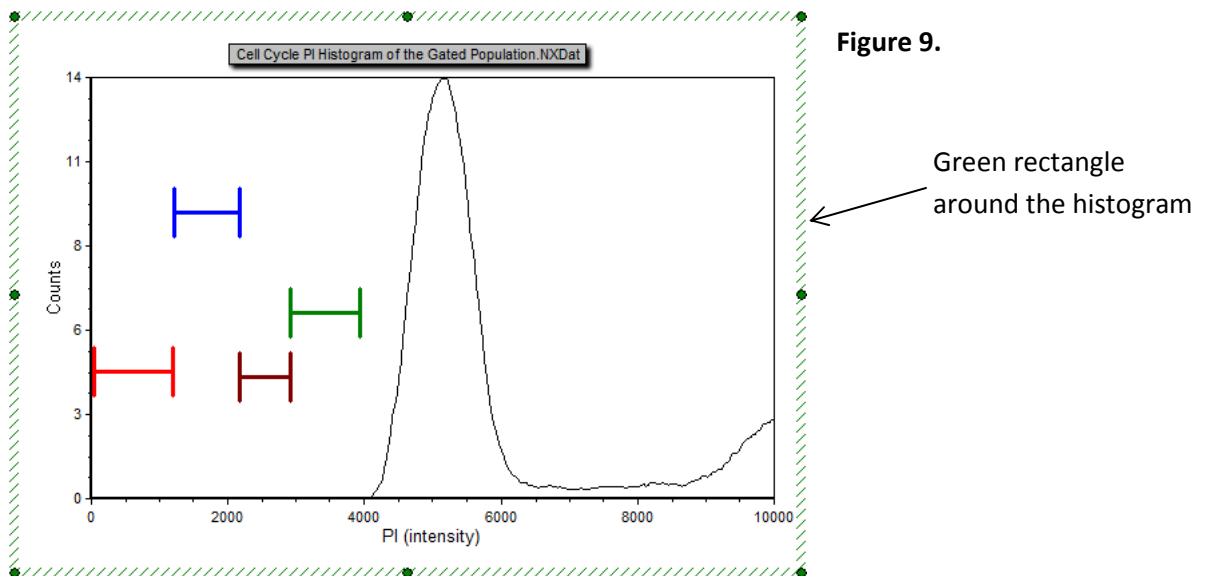


Cell Population	% of Gated Cells	CV	Concentration (10 ⁶ cells/mL)
Total	100		9.2
Sub G1	1.2	5.1	0.1
G0/G1	63.4	10.5	5.8
S	13.9	11.3	1.3
G2/M	21.0	8.2	1.9

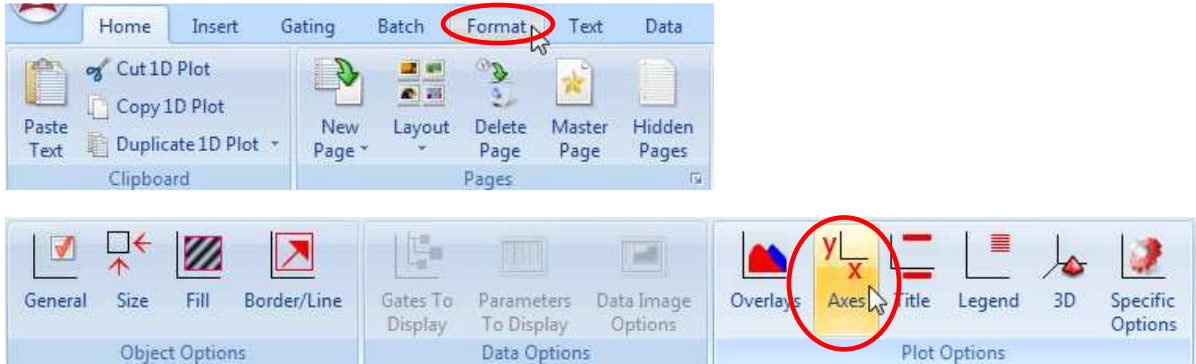
- An insufficient number of cells, poor focus, incorrect exposure time, a large number of counted cell clusters, an error in sample preparation / staining, or an unhealthy cell population prior to fixing can affect the quality of the cell cycle histogram.
- If larger cells (such as PC3) were used for the cell cycle experiment and have a larger intensity distribution (see section 2.3 #11) the x-axis may need to be adjusted in order to include all of the population data. Figure 8 shows a default histogram where the G2/M peak is cut-off at 10,000.



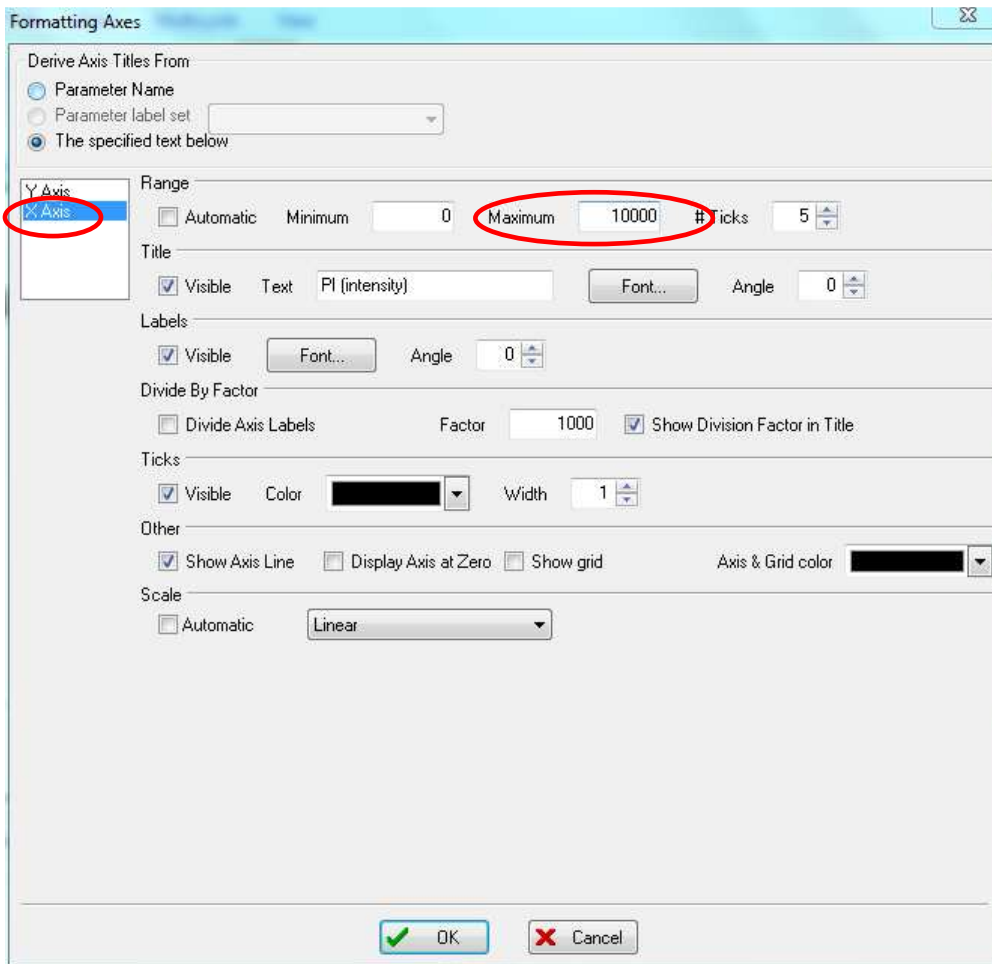
- To adjust the x-axis, first select the histogram by clicking on it. You will know that it is selected when a green rectangle appears around the histogram (See figure 9.)



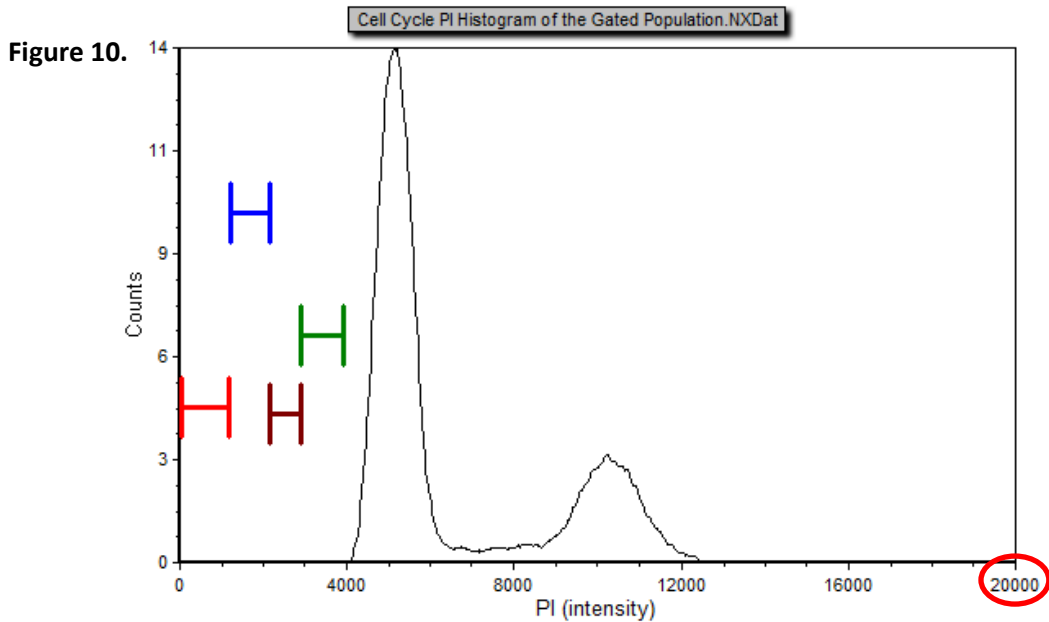
7. Navigate to and click on the “Format” tab at the top of the page. Then locate and click on the “Axes” icon in the Plot Options group (shown below).



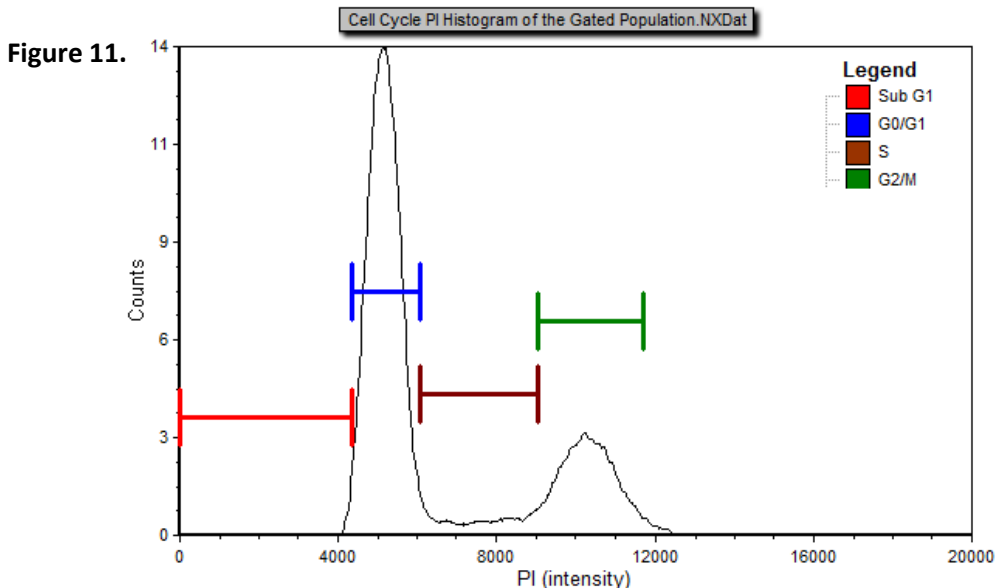
8. The Formating Axes pop-up window has now appeared. Make sure the X Axis is selected, then change the Maximum value from 10000 to 20000. Press OK at the bottom of the window.



9. The X-axis has now been changed to 20000 and both peaks are clearly visible.



10. Adjust the gates to fit the data as described in step 2 of this section.



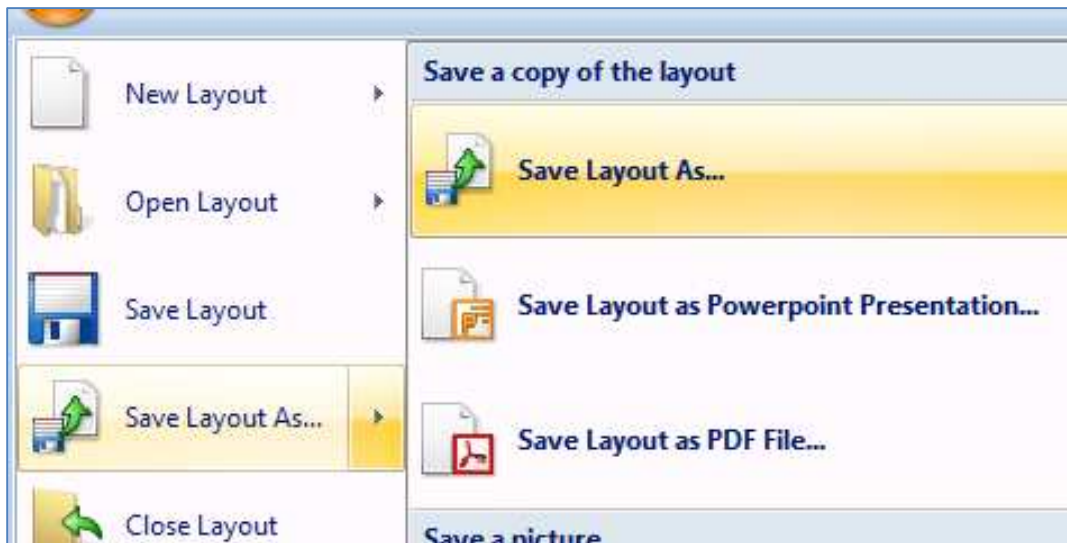
11. The appearance of the histogram can be optimized for presentation or publication. Settings for Smoothing and/or Resolution can be adjusted to “smooth out” the curve. These modifications do not affect the underlying data. Please contact Nexcelom Technical Support or refer to the FCS Express 4 Flow Instructions for more information.

3.4 Saving Optimized Data Files

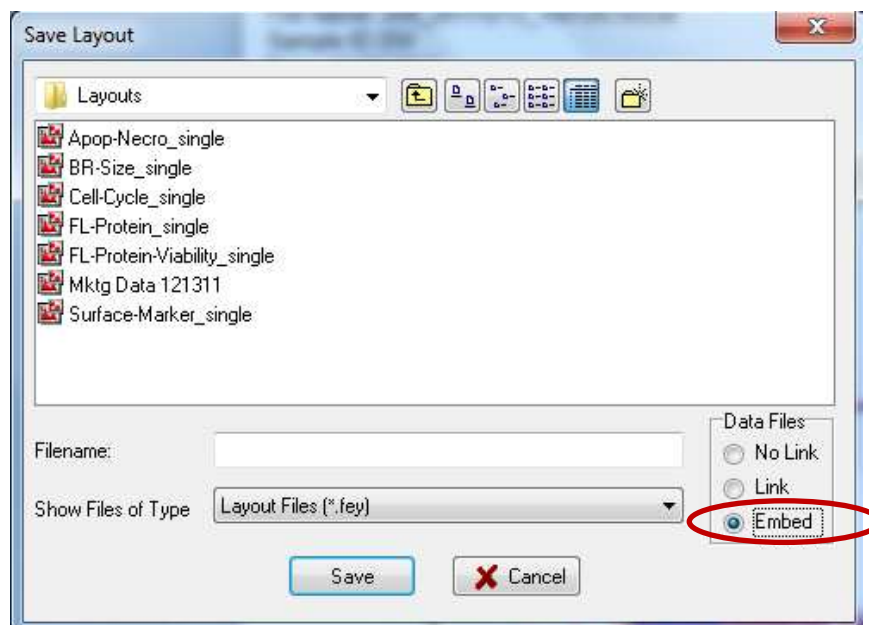
1. Following gating and cell size optimization, click on the FCS Express 4 icon in the top left corner of the screen.



2. Click Save Layout As...



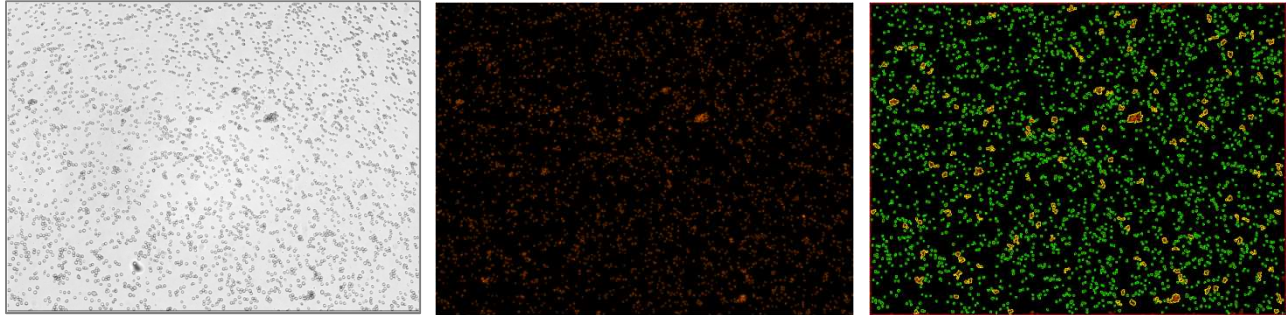
3. Click "Embed" in the bottom right of the Save Layout window under Data Files. Enter the desired file name and location, then click Save.



3. The new .fey file will contain the final analyzed data set.

3.5 Viewing Images

To view the bright field and fluorescent cell image report, open the image folder in the original data package location. Bright field, fluorescent, and fluorescent counted images are captured for cell cycle assays.



3.6 Technical Support

Nexcelom Technical Support is available from 9am to 5pm EST.

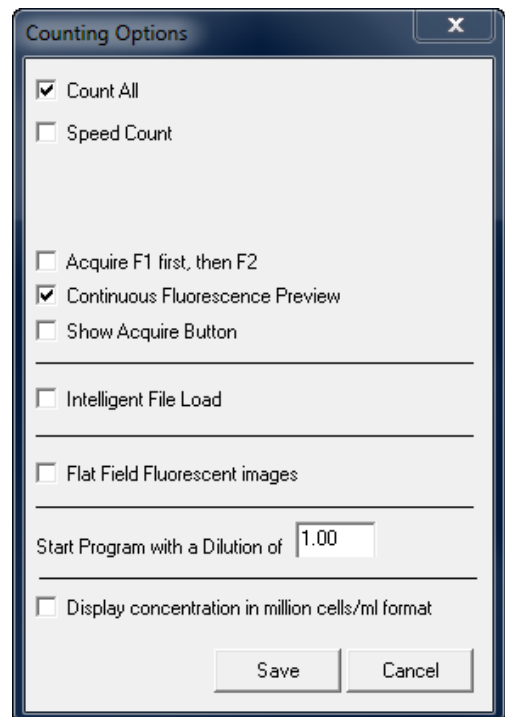
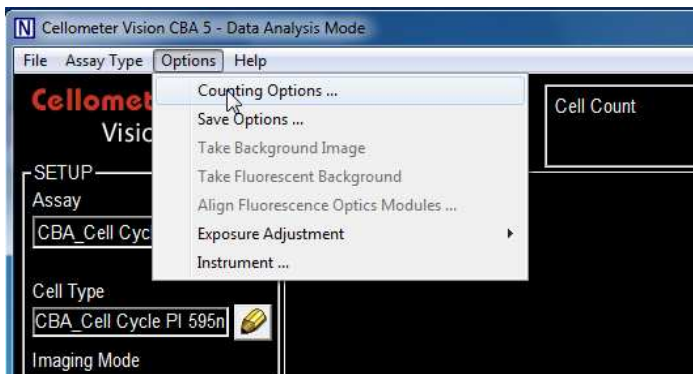
E-mail: support@nexcelom.com

Phone: 978-327-5340

4.0 Software Settings

4.1 Review Cell Cycle Counting Options Screen

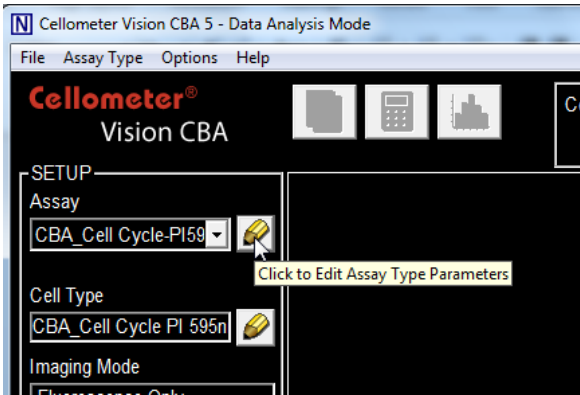
Click on the Options Page and select Counting Options shown at right). Verify that all selections on the instrument screen match the default settings below.



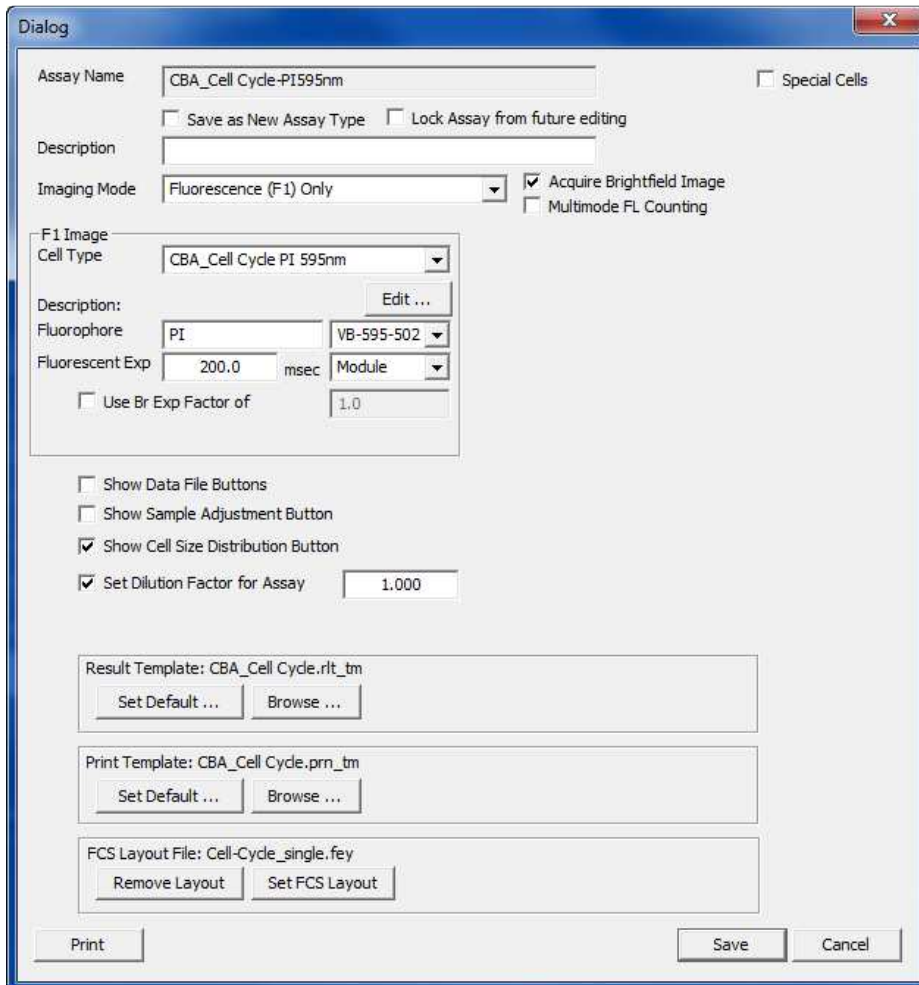
4.2 Default Software Settings for FOM (fluorescence optics module) VB-595-502

4.2.1 Check Dialog Screen Settings

1. Click on the pencil icon under Assay on the main Vision CBA screen.

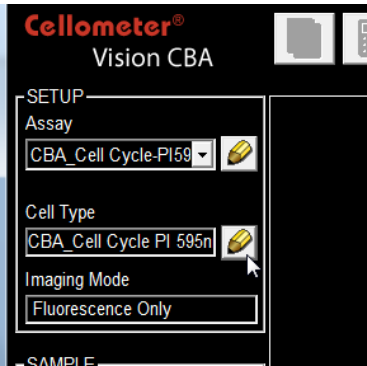


2. Verify that all selections on the instrument screen match the default settings below. If fluorescent exposure time needs to be increased (section 2.3.11). Locate Fluorescent Exp. (200 msec. default) and change it per recommendation in section 2.3.11.

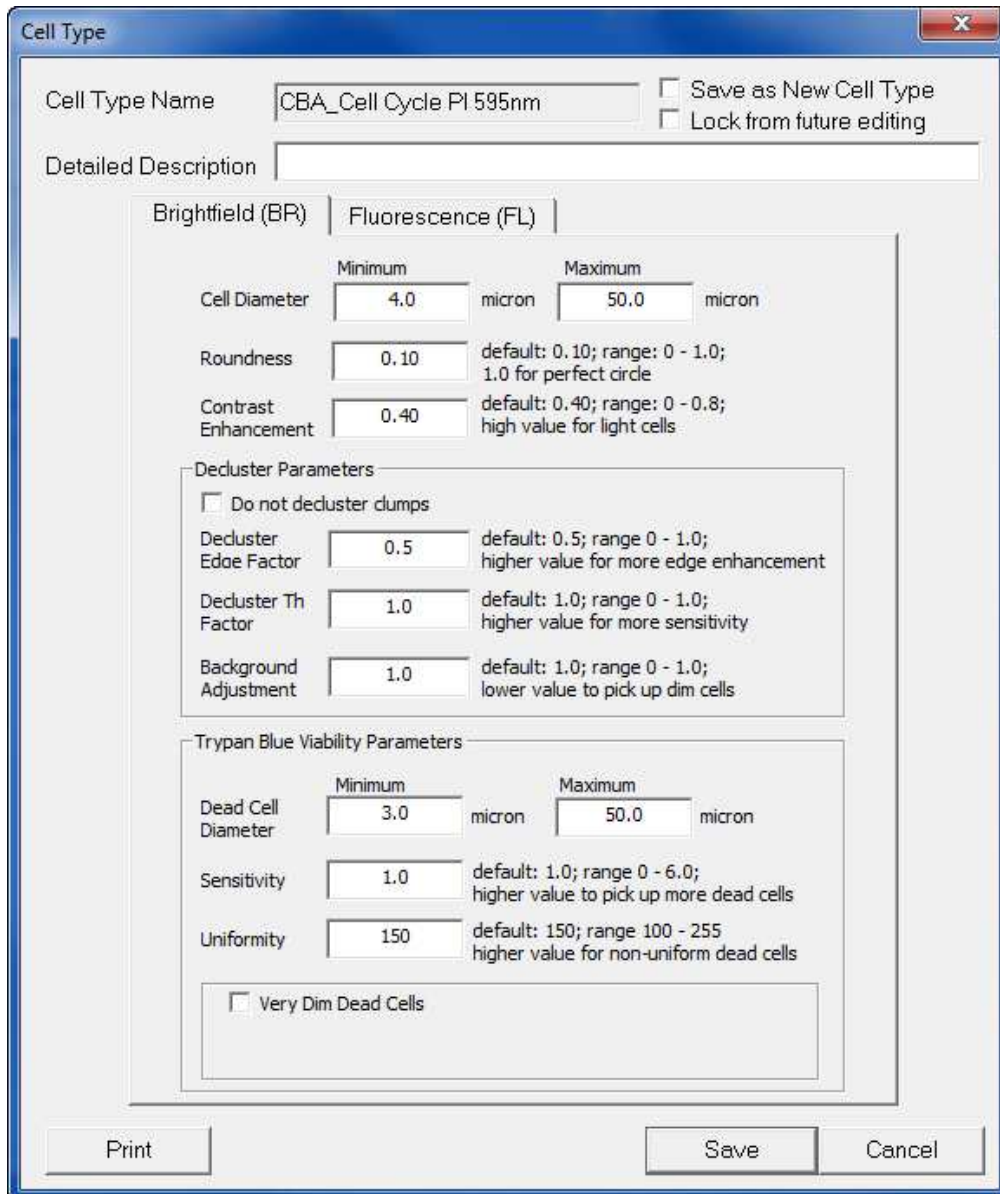


4.2.2 Check Cell Type Settings

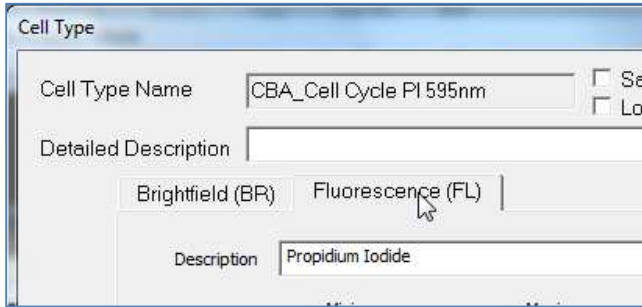
1. Click on the pencil icon under Cell Type on the main Vision CBA software screen.



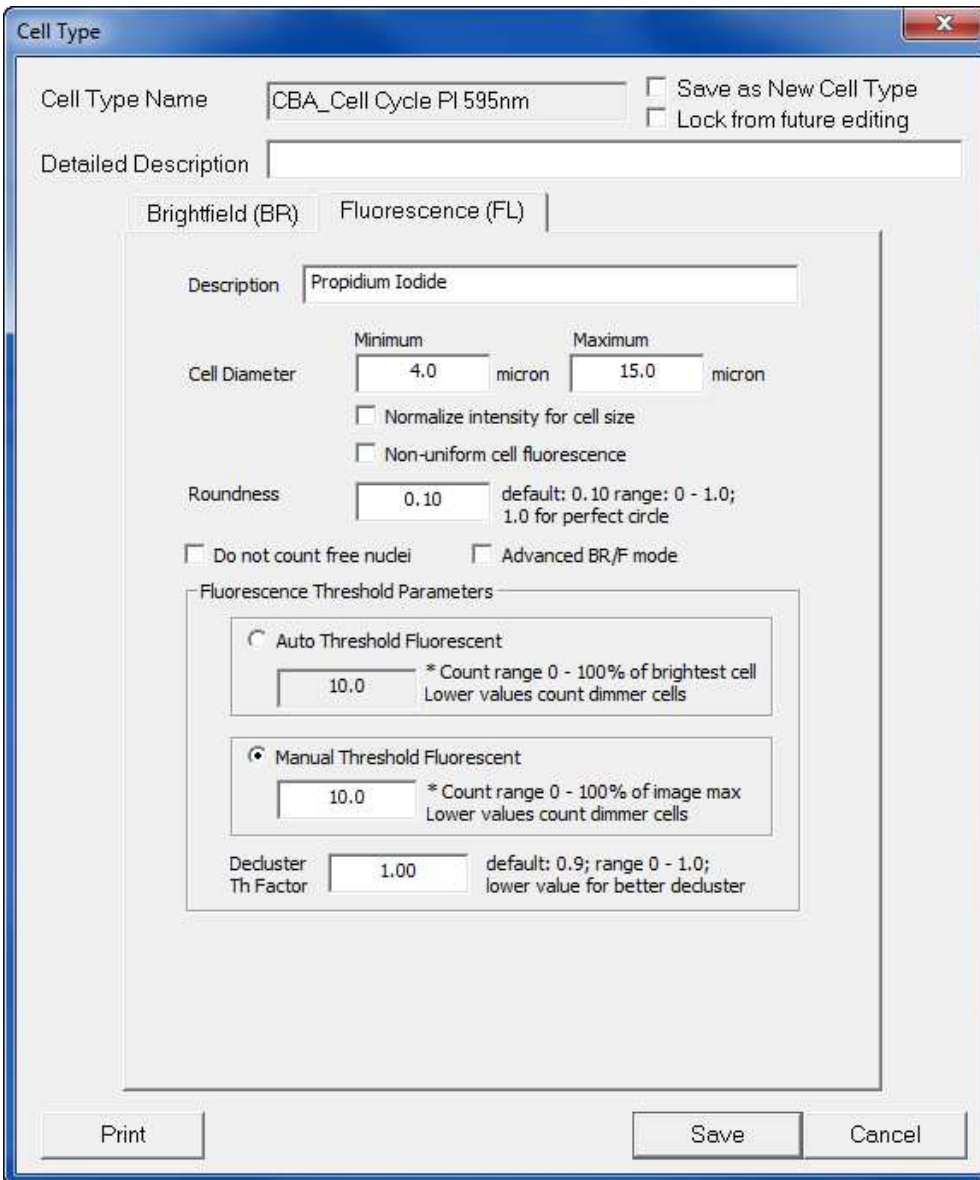
2. Verify that all selections for the bright field (BR) tab on the instrument screen match the default settings below.

A screenshot of the 'Cell Type' configuration dialog box. The 'Brightfield (BR)' tab is selected. The 'Cell Type Name' is 'CBA_Cell Cycle PI 595nm'. There are checkboxes for 'Save as New Cell Type' and 'Lock from future editing'. The 'Detailed Description' field is empty. The 'Brightfield (BR)' section includes: 'Cell Diameter' (Minimum: 4.0, Maximum: 50.0, unit: micron); 'Roundness' (0.10, default: 0.10; range: 0 - 1.0; 1.0 for perfect circle); 'Contrast Enhancement' (0.40, default: 0.40; range: 0 - 0.8; high value for light cells). The 'Deduster Parameters' section includes: 'Do not deduster clumps' (unchecked); 'Deduster Edge Factor' (0.5, default: 0.5; range: 0 - 1.0; higher value for more edge enhancement); 'Deduster Th Factor' (1.0, default: 1.0; range: 0 - 1.0; higher value for more sensitivity); 'Background Adjustment' (1.0, default: 1.0; range: 0 - 1.0; lower value to pick up dim cells). The 'Trypan Blue Viability Parameters' section includes: 'Dead Cell Diameter' (Minimum: 3.0, Maximum: 50.0, unit: micron); 'Sensitivity' (1.0, default: 1.0; range: 0 - 6.0; higher value to pick up more dead cells); 'Uniformity' (150, default: 150; range: 100 - 255; higher value for non-uniform dead cells). There is a checkbox for 'Very Dim Dead Cells' which is unchecked. At the bottom are 'Print', 'Save', and 'Cancel' buttons.

3. Click on the Fluorescence (FL) tab.



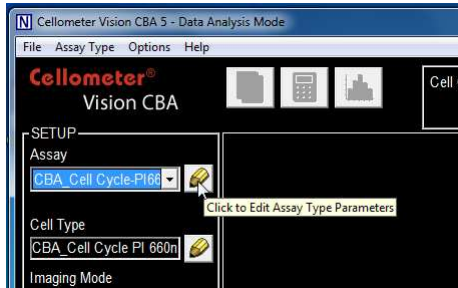
4. Verify that all selections for the Fluorescence (FL) tab on the instrument screen match the default settings below.



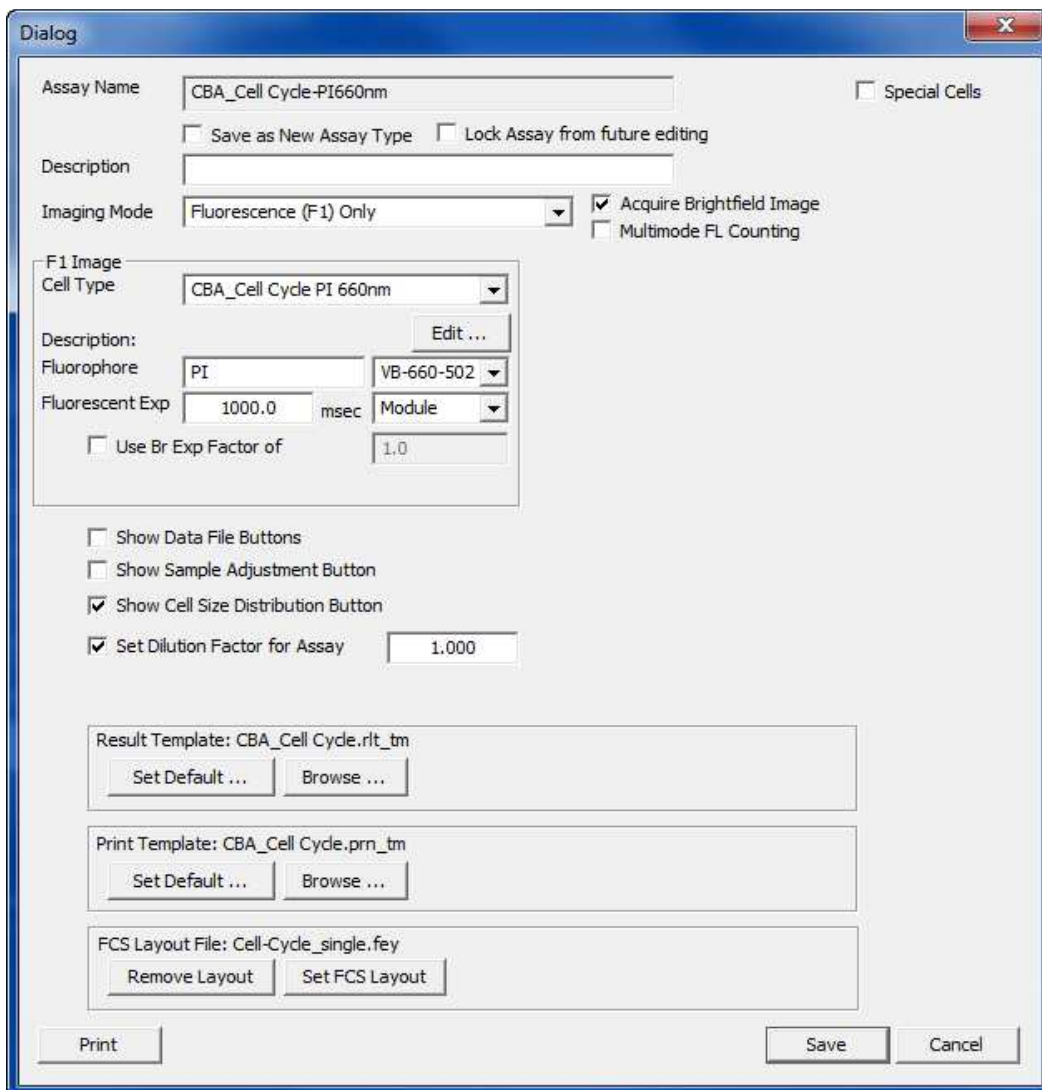
4.3 Default Software Settings for FOM (fluorescence optics module) VB-660-502

4.3.1 Check Dialog Settings

1. Click on the pencil icon under Assay on the main Vision CBA screen.

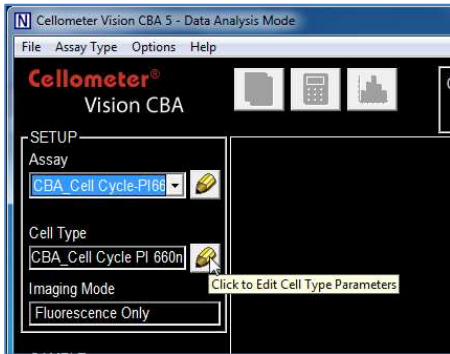


2. Verify that all selections on the instrument screen match the default settings below. If fluorescent exposure time needs to be increased (section 2.3.11). Locate Fluorescent Exp. (1000 msec. default) and change it per recommendation in section 2.3.11.

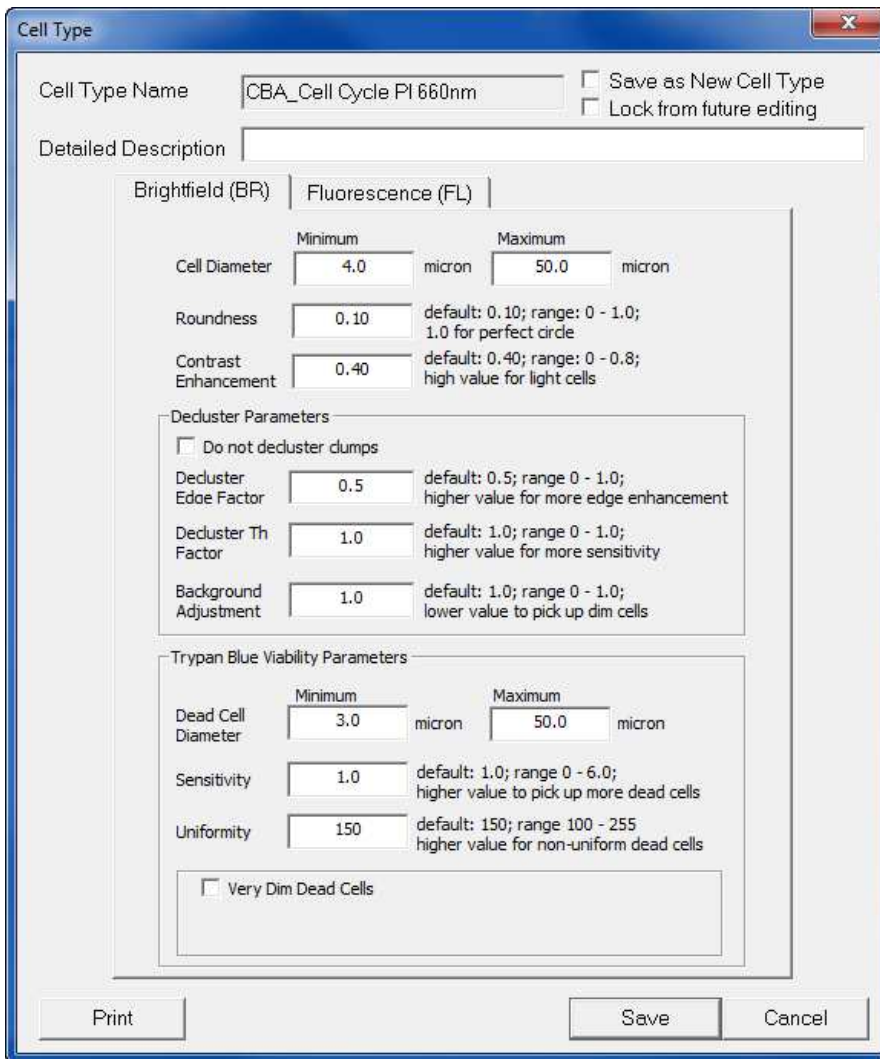


4.3.2 Check Cell Type Settings

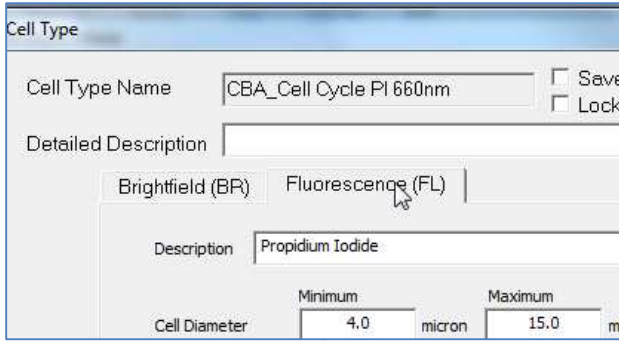
1. Click on the pencil icon under Cell Type on the main Vision CBA software screen.



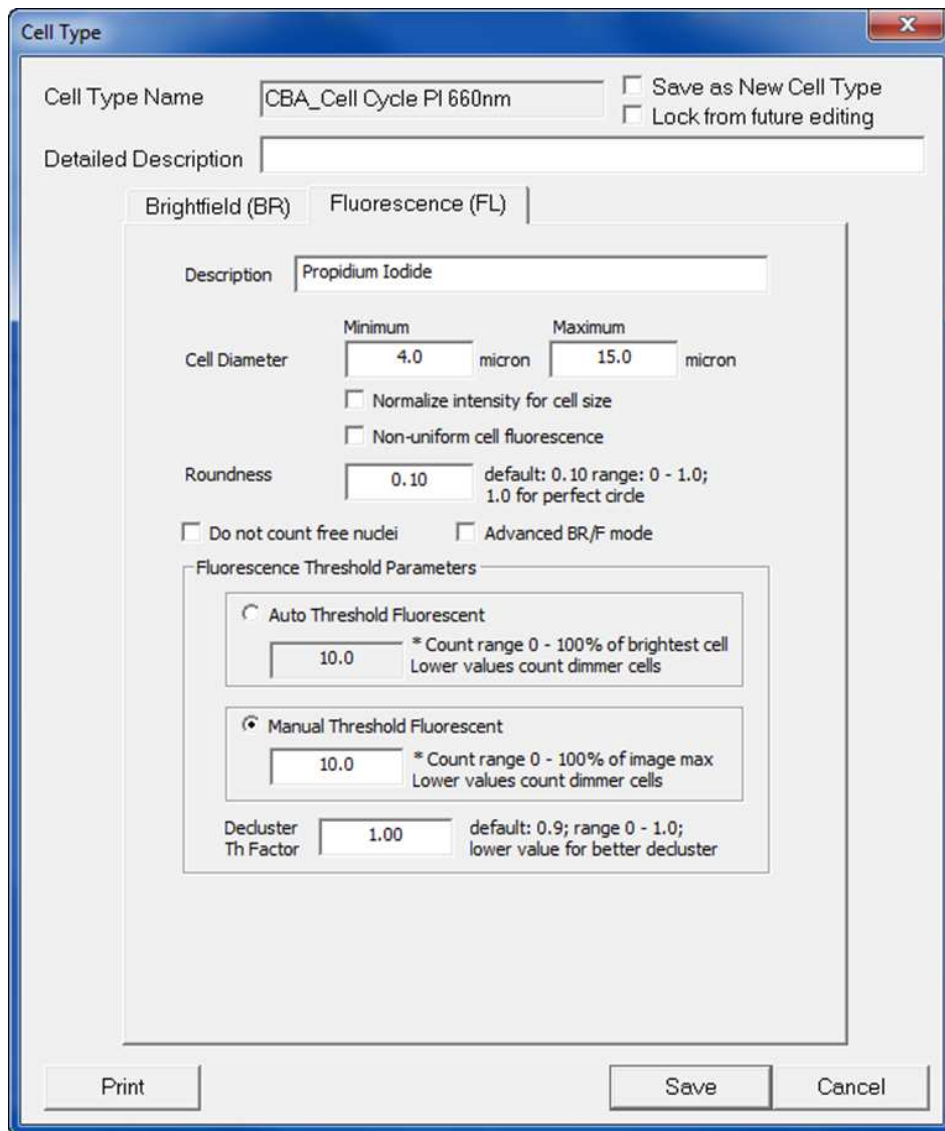
2. Verify that all selections for the Brightfield (BR) tab on the instrument screen match the default settings below.



3. Click on the Fluorescence (FL) tab.



4. Verify that all selections for the Fluorescence (FL) tab on the instrument screen match the default settings below.

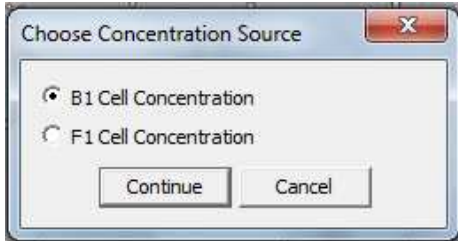


5.0 Using the Sample Adjustment Calculator

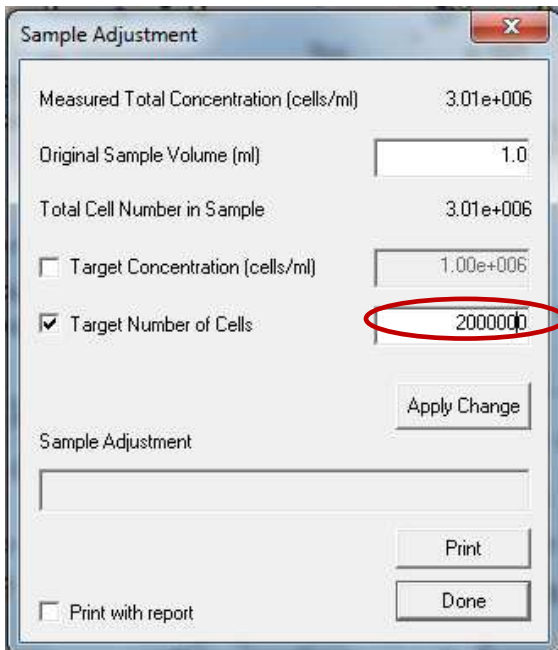
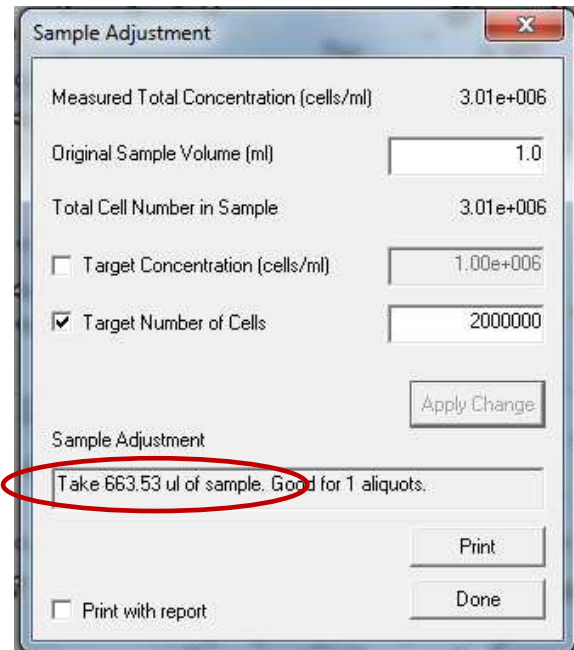
1. Click on the calculator icon at the top of the screen.



2. Select **B1 Cell Concentration**, then click **Continue**.

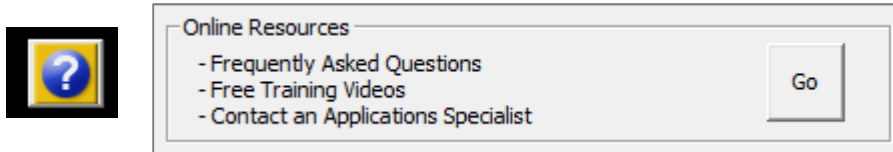
A dialog box titled "Choose Concentration Source" with a close button (X) in the top right corner. It contains two radio button options: "B1 Cell Concentration" (which is selected) and "F1 Cell Concentration". At the bottom, there are two buttons: "Continue" and "Cancel".

3. Enter the target number of cells. For the Cell Cycle assay, it is 2,000,000. Click Apply Change. The required volume of cell sample to be spun down will appear in the Sample Adjustment window.

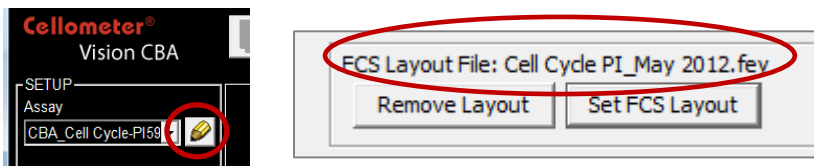
A screenshot of the "Sample Adjustment" dialog box. It displays the following fields: "Measured Total Concentration (cells/ml)" with a value of 3.01e+006; "Original Sample Volume (ml)" with a value of 1.0; "Total Cell Number in Sample" with a value of 3.01e+006; "Target Concentration (cells/ml)" with a value of 1.00e+006 and an unchecked checkbox; and "Target Number of Cells" with a value of 2000000 and a checked checkbox. The "2000000" value is circled in red. Below these fields is an "Apply Change" button. At the bottom, there is a "Sample Adjustment" text box, a "Print" button, and a "Print with report" checkbox. A "Done" button is also present.A screenshot of the "Sample Adjustment" dialog box after the "Apply Change" button has been clicked. The fields are the same as in the previous screenshot, but the "Sample Adjustment" text box now contains the text "Take 663.53 ul of sample. Good for 1 aliquots.", which is circled in red. The "Print" and "Done" buttons remain visible.

6.0 Importing a New FCS Express 4 Layout

1. Click on the Support icon in the bottom right corner of the Vision CBA screen, then click the “Go” box under Online Resources. Click on the Assay Files tab. Select PI Cell Cycle. Compare the date on the PI Cell Cycle layout (.fev file) listed to the version currently saved in your Vision CBA software.



To check the version in your software, click on the pencil icon under Assay on the main Vision CBA screen. The FCS Layout File name (with date) is listed toward the bottom of the Dialog screen.

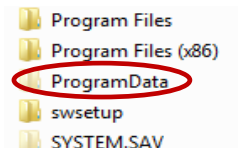


If a newer layout is listed on-line, download the new PI Cell Cycle layout onto your desktop. Copy the layout by right clicking on the layout and selecting copy.

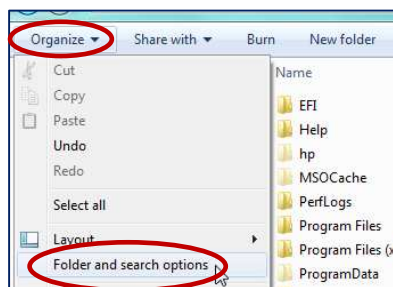
2. Navigate to the START menu and select Computer.



3. Double click on the C: Drive and locate the ProgramData folder (shown at right), then proceed to Step 4. IF the ProgramData folder is not present, it may be hidden. Follow the instructions below to show hidden folders.



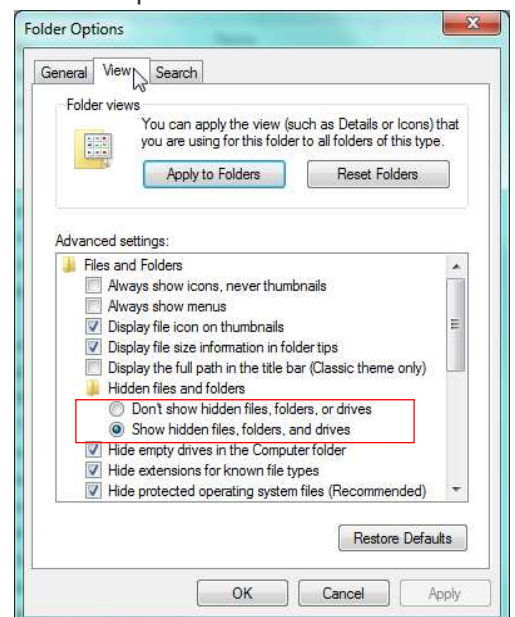
- 3.1 Click on “Organize” (top left of screen) and select “Folder and search options”



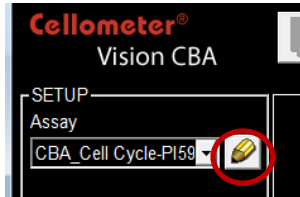
- 3.2 A Folder Options menu will pop up. Select View.

- 3.3 Under the “Hidden files and folders” file, select “Show hidden files, folders, and drives”

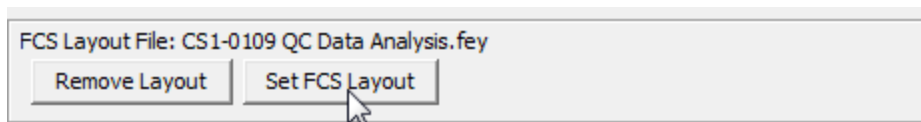
- 3.4 Click OK.



4. Open ProgramData folder, then open the Nexcelom_VisionCBA folder.
5. Open FCS Express Folder, then open the Layouts folder.
6. Copy the PI Cell Cycle layout from your desktop into the folder.
7. Set the new PI Cell Cycle layout as the default layout for the Cell Cycle PI Assay by clicking on the pencil icon under Assay on the main Vision CBA screen.



10. Navigate to the bottom of the page and click on “Set FCS Layout”



11. Select the PI Cell Cycle layout with the most recent date and click Open, then click Save.
12. You have now set the new PI Cell Cycle layout as the default layout for the Cell Cycle PI Assay on your Cellometer Vision CBA instrument.