PRODUCT INSERT

Instrument Compatibility: Cellometer™ Spectrum

ViaStain™ Annexin V-FITC for Cellometer

Part number: CS1-0114-1

Volume: 500 µL

Storage: 2 to 8 °C

Table of Contents

1. Introduction

1.1 Assay Description

Apoptosis, or *programmed cell death*, is a natural process of cellular self-destruction. Apoptosis is a part of routine cell turnover and tissue homeostasis, prevalent in epithelial cells, erythrocytes, and other cell types genetically programmed to have a limited life span. It is also important in embryogenesis, maintenance of immune tolerance, and development of the nervous system. Apoptosis can be induced either by a stimulus, such as irradiation or toxic drugs, or by removal of a repressor agent. The cells disintegrate into membrane-bound particles that are then eliminated by phagocytosis. Problems with the regulation of apoptosis are thought to be linked to many cancers, degenerative diseases, and autoimmune diseases, making apoptosis a key target in many fields of clinical research.

Necrosis is the death of cells or tissues from severe injury or disease, especially in a localized area of the body. Causes of necrosis include inadequate blood supply (as in infarcted tissue), bacterial infection, traumatic injury, and hyperthermia.

Annexin V and propidium iodide are used to measure apoptosis and necrosis. Annexin V is a member of the annexin family of intracellular proteins that binds to phosphatidylserine (PS) in a calcium-dependent manner. PS is normally only found on the intracellular leaflet of the plasma membrane in healthy cells, but during early apoptosis, PS translocates to the external leaflet. Fluorochrome-labeled Annexin V can then be used to specifically target and identify the PS on the surface of apoptotic cells. Annexin V binding alone cannot differentiate between apoptotic and necrotic cells. Propidium Iodide (PI) solution is a membrane-exclusion dye that permeates cells with compromised cell membranes and binds to DNA. Early apoptotic and healthy cells with intact membranes will exclude PI, while late stage apoptotic and necrotic cells with compromised membranes are stained. Use of both Annexin V-FITC and PI allows researchers to characterize a cell population based on % normal, % apoptotic, and % necrotic /very late-stage apoptotic cells.

For additional apoptosis information, please see below reference articles, include usage on Cellometer:

- Elmore S. (2007). Apoptosis: A Review of Programmed Cell Death. *Toxicol. Pathol*. 35(4):495-516.
- Rastogi RP, *et al.* (2009). Apoptosis: Molecular Mechanisms and Pathogenicity. *EXCLI Journal*. 8:155-181.
- Berger EA, *et al*. (2013). HIF-1α Is Essential for Effective PMN Bacterial Killing, Antimicrobial Peptide Production and Apoptosis in Pseudomonas aeruginosa Keratitis. *PLoS Pathogens* 9(7).
- Verma M, *et al*. (2013). Sirtuin-3 Modulates Bak/Bax Dependent Apoptosis. *Journal of Cell Science* 126(1):274-88.

1.2 Materials Supplied

Reagent	Catalog number	Volume
ViaStain™ Annexin V-FITC	CS1-0114-1	500 µL

1.3 Materials Required

- ViaStain™ Annexin V Binding Buffer for Cellometer (Cat. # CS0-0115-1)
- ViaStain™ PI Apoptosis Staining Solution for Cellometer (Cat. # CS1-0116-1)
- Microcentrifuge tubes
- Pipette and pipette tips
- Trypsin EDTA (if working with adherent cells)
- Phosphate Buffered Saline (PBS)
- Cellometer counting slides (Cat. # SD100 or PD100)

1.4 Instrument and Software Requirements

- Cellometer Spectrum Fluorescent Cell Counter with Fluorescence Optics Modules S1-534-470 and S1-655-527
- Operating Computer with:
    - Cellometer Spectrum software
    - FCS Express Software and license (De Novo Software)

2 Assay Protocol

2.1 Preparation of Control Samples

1. A positive control should be used to check exposure time and optimize the y-axis gate for apoptotic and non-apoptotic cells. A positive control may be generated by exposing cells to an apoptosis inducing pharmacological agents such as α-TOS and etoposide. For Cellometer examples, Jurkat cells were incubated overnight with 10 µM α-TOS or Staurosporine.
2. A negative control (untreated cells) should be tested to determine baseline cell concentration, % viability, and % apoptotic cells. The negative control is used to optimize the x-axis gate for live and dead cells.
3. Positive and negative controls should be processed at the same time using the staining and data acquisition procedures outlined below.

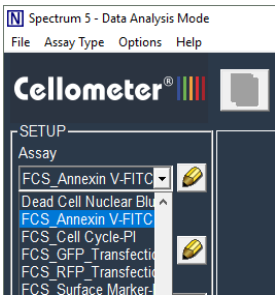
2.2 Preparation of Adherent Cells for Staining

1. Using 1x Trypsin (EDTA), trypsinize cells until they have lifted off the plate.
2. Centrifuge cells at 200 – 400 x g for 5 min.
3. Remove the supernatant carefully and resuspend cells in 1 mL of 1 x PBS.

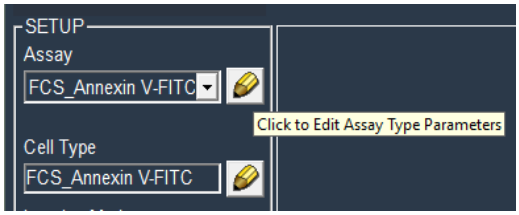
2.3 Staining Procedure

1. Verify the concentration of the positive / negative controls and cell samples using the Cellometer Spectrum Fluorescent Cell Counter.
2. Use the Cellometer Sample Adjustment Calculator to generate a cell sample with a concentration between 2 and 3 million cells per mL (2-3 x 10^6 cells/mL). See section 5.0: Using the Sample Adjustment Calculator.
3. Into a new tube, pipette 50 µL of cells that are at a concentration of 2-3 x 10^6 cells/mL. Spin down cell sample at 200 – 400 x g for 5 min.
4. Remove the supernatant carefully and resuspend cells in 40 µL of Annexin V Binding Buffer (Component C). Mix by pipetting up and down at least 10 times.
5. Add 5 µL of Annexin V-FITC (Component A).
6. Add 5 µL of PI solution (Component B).
7. Gently pipette the cells up and down ten times, then incubate for 15 min at RT (25°C) in the dark.
8. Add 250 µL of 1x PBS.
9. Centrifuge at 200 – 400 x g for 5 min.
10. Remove the supernatant carefully and resuspend cell pellet in 50 µL of Annexin V Binding Buffer (Component C) and mix by pipetting up and down ten times.

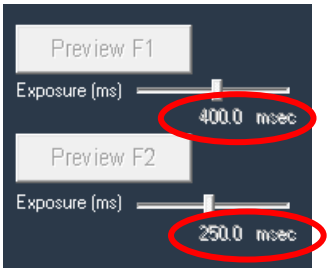
2.4 Data Acquisition



1. Select the **FCS_Annexin V-FITC + PI** or **CBA_Apoptosis Annexin V + PI** assay from the Assay drop-down menu in the upper left corner of the main Spectrum software screen. The apoptosis assay should be run with the default software settings and the exposure times indicated below in step 3. If you suspect that the settings may have been changed, review the default software settings in *Section 4*.
2. If running the **FCS_Annexin V-FITC + PI** or **CBA_Apoptosis Annexin V + PI** assay for the first time, the default exposure time **MUST** be updated. Click on the pencil icon under Assay on the main Spectrum screen.

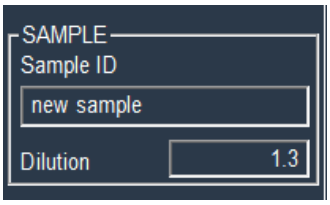


3. Adjust the exposure for the Annexin V FITC (F1) and PI (F2) channels.

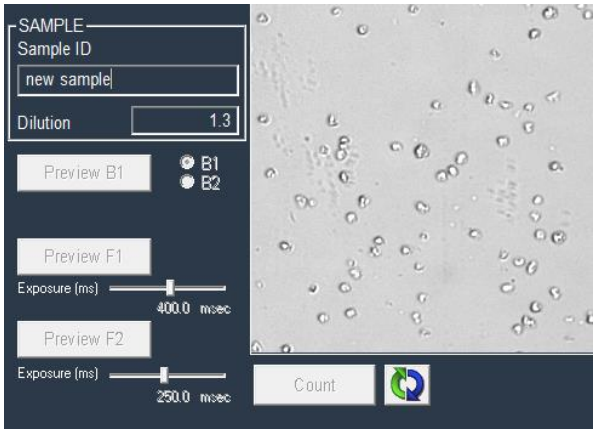


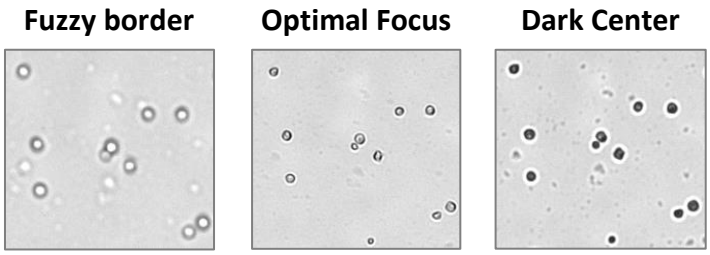
Annexin V FITC (F1) Exposure	PI (F2) Exposure
400 msec	250 msec

4. To import the most current FCS Express Layout for the Annexin V-FITC / PI assay, see *Section 6 Importing a New FCS Express Layout*.
5. Gently mix the cell sample by pipetting up and down at least ten times, then load 20 µL into the Cellometer imaging chamber and insert slide.
6. Wait 60 seconds for the cells to settle in the chamber.
7. Type a name for your sample into the Sample ID text box.
8. Click Preview Brightfield Image at the bottom left of the main Spectrum screen.

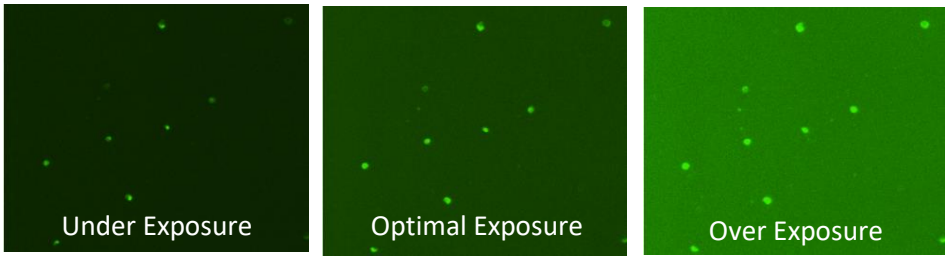


9. Turn the focus knob and adjust focus for the bright field image. Cells in focus for the apoptosis assay will have a bright center and dark outline. There should be a crisp contrast between background and the cell membrane. See Focus Guide below.



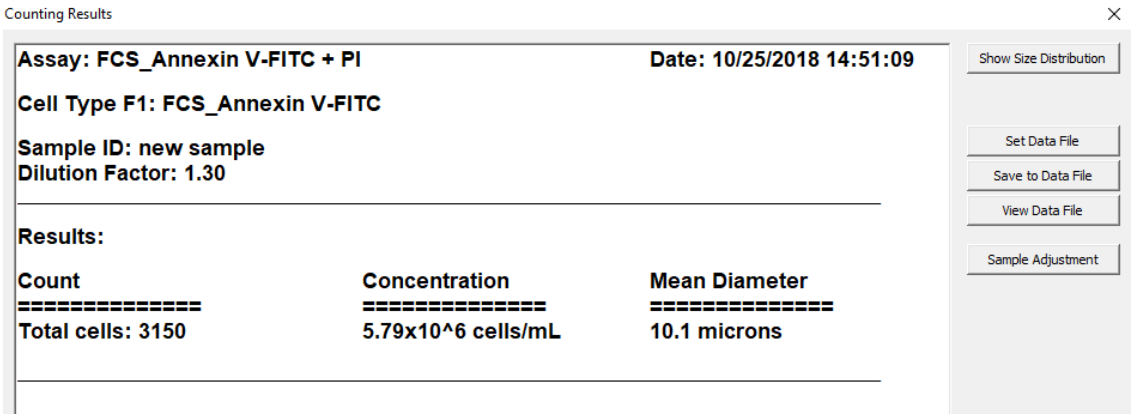


10. Click Stop Preview.
11. Click the Preview F1 Image button (bottom left of screen) and verify that the fluorescence signal displays as 100% of range.

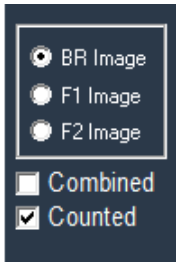


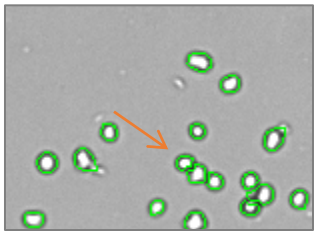
Example images of cells stained with Annexin V-FITC. Optimal exposure time will generate a bright image with well-defined fluorescent spots (middle image). A sample is under exposed when the images are dark with insufficient fluorescent signal, whereas over exposure yields images that are too bright with fluorescent spots that are less distinct from background.

12. Click Preview F2. The F2 (PI) channel should be set to 250 msec exposure for Spectrum.
13. Click the Count button at the bottom of the screen.
14. When counting is complete, an initial Results Table will appear on the screen. For optimal results, the Total Count should be between 2,000 and 8,000 cells (4,000 cells is optimal). If the Total Count is < 2,000 repeat data acquisition with a more concentrated sample. For larger cells (>15 µm in diameter) a minimum of 2,000 cells is acceptable.



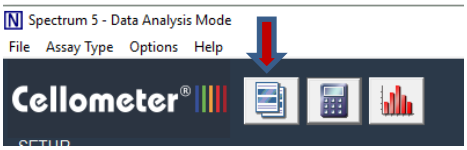
15. Click the **Close** button at the bottom right corner of the Counting Results table. Select the BR Counted image at the right-hand side of the screen.
16. Review the counted image to confirm that cells are being counted correctly. Individual cells within clumps should be circled in green, indicating that they are being counted individually.

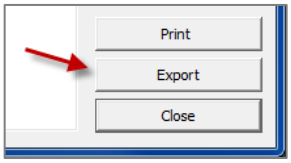




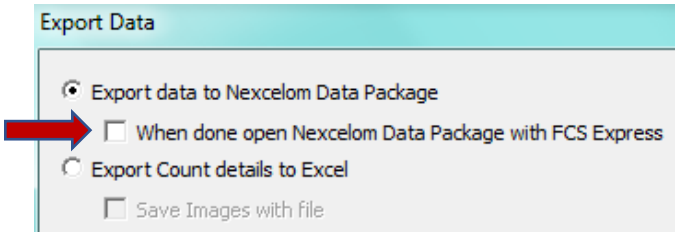
2.5 Exporting and Saving Data Files

1. Once cells are correctly counted, click on the report icon at the top left of the screen, then click the Export button at the bottom right of the Counting Results screen.



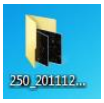


2. Confirm that “Export data to Nexcelom Data Package” is selected, and that “When done open Nexcelom Data Package with FCS Express” is **NOT** selected, then click Continue.



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

Raw Nexcelom data file (.nxdat)



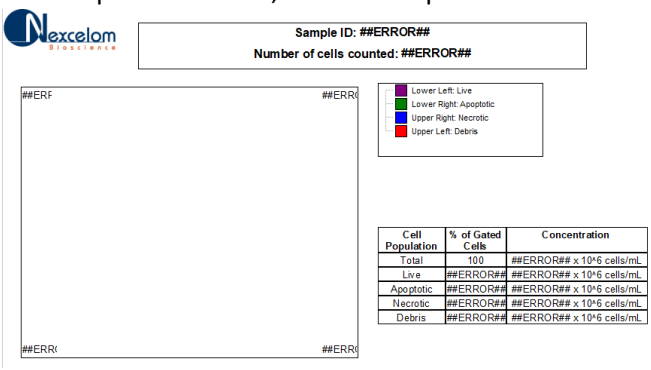
Folder with cell images

3 Data Analysis

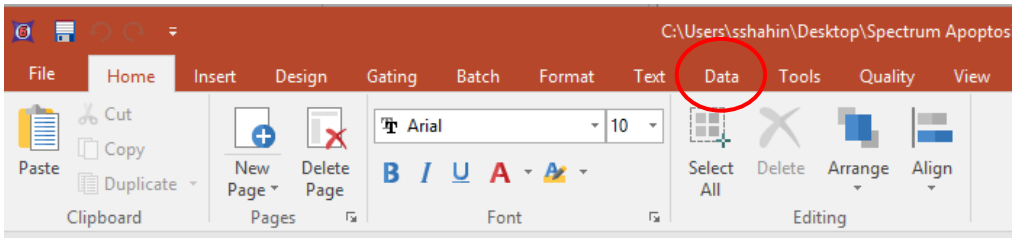
3.1 Opening and Loading Data Files

Double click the launch icon in the designated data package location to open the data file in the FCS Express software.

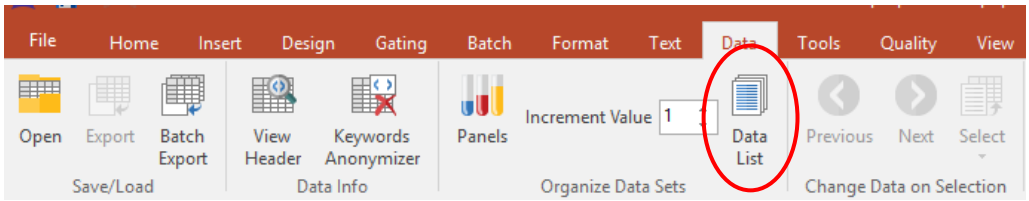
1. Upon opening the FCS Express software, a blank template will automatically load.



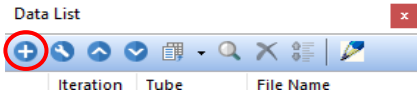
2. To import data, locate and click on the “Data” tab at the top of the page.

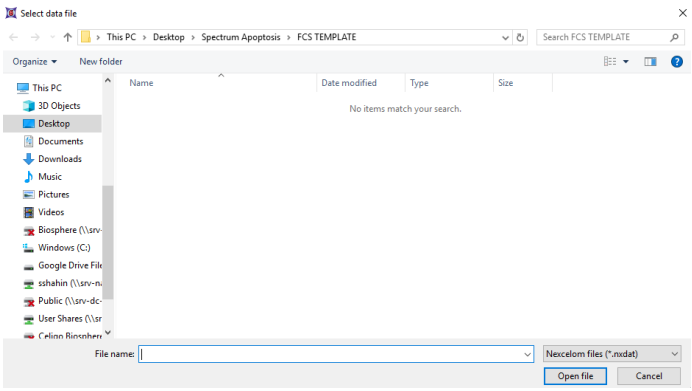


3. Click “Data List” to open the data list window.

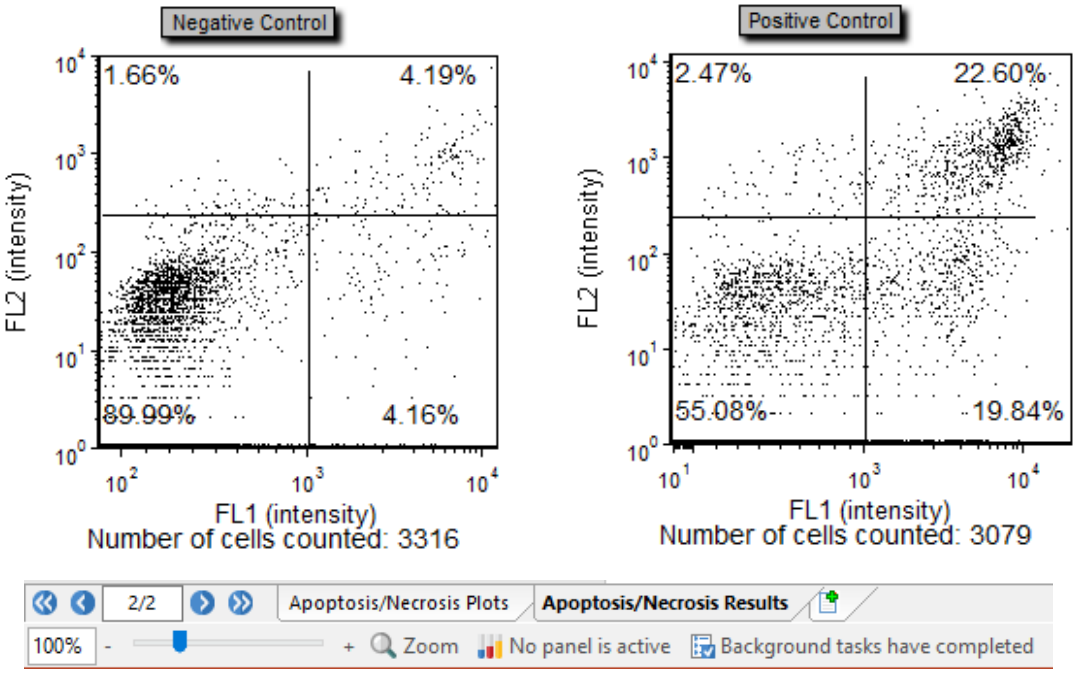


4. First import the **negative control** sample. Click on the blue (**+**) add button. The **Select a Data File** pop-up window will appear. Locate and select your data negative control sample (saved from step 2.5.) and click open.





5. Repeat step 4 to import a positive control and up to four unknown samples. Scatter plots and data tables will auto-populate with the imported data from each sample. The generated data is found on page two of the FSC express layout. Click on “Apoptosis/Necrosis Results” at the bottom of the page to access the data table.



	Cell population (%)	Concentration (x 10^6 cells/ml)
Negative Control	Cells counted: 3316	
Total	100.00	4.68
Live	87.97	4.12
Apoptotic	4.01	0.19
Necrotic	4.98	0.23
Debris	3.05	0.14
Positive Control	Cells counted: 3079	
Total	100.00	4.35
Live	52.71	2.29
Apoptotic	20.95	0.91
Necrotic	23.90	1.04
Debris	2.44	0.11

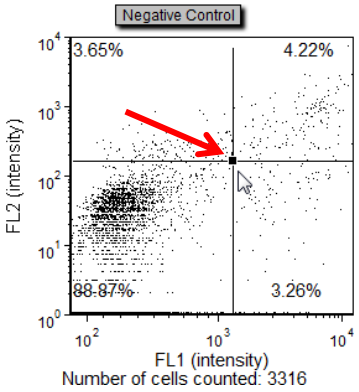
3.2 Gate Optimization

1. Gates may only be adjusted in the display for the negative control sample.

   NOTE: Adjusting the negative control gate will automatically adjust gates in the positive control as well as the other data plots.

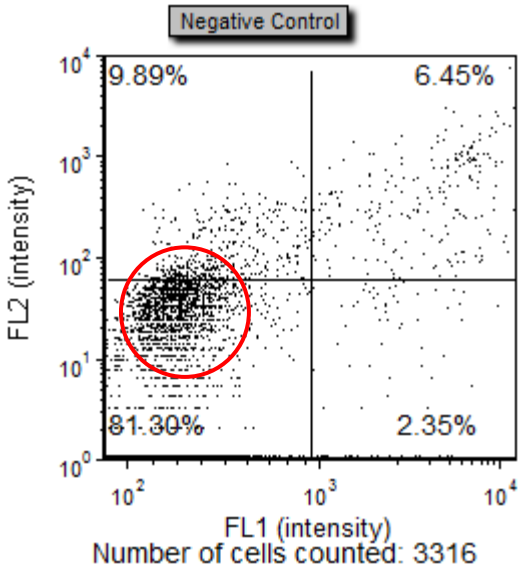
   a. To adjust the gate, select the dot-plot by clicking on it (a green box outline appears around it).

   b. Click on the center of the four quadrant gate and drag it to the desired location.

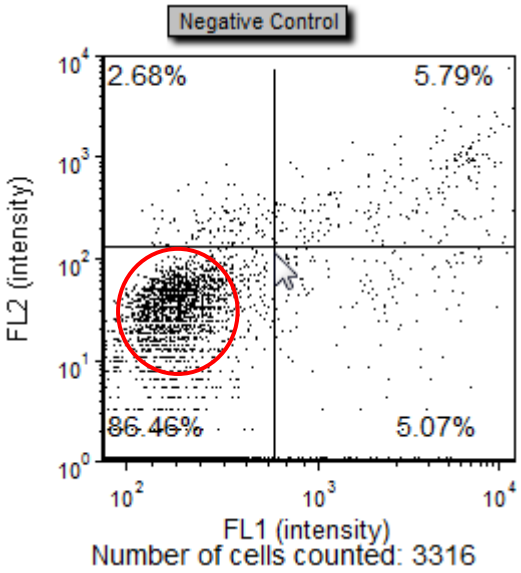


2. Quadrant gating should be adjusted according to data in the negative and positive controls.
    a. In the **negative control** sample, the healthy, live cell population (red circle) should be in the bottom left quadrant.

Incorrect Gating

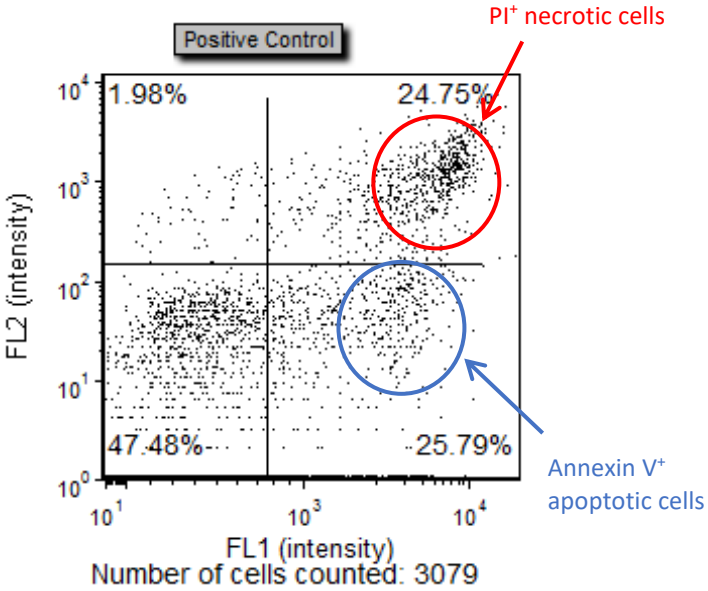


Correct Gating

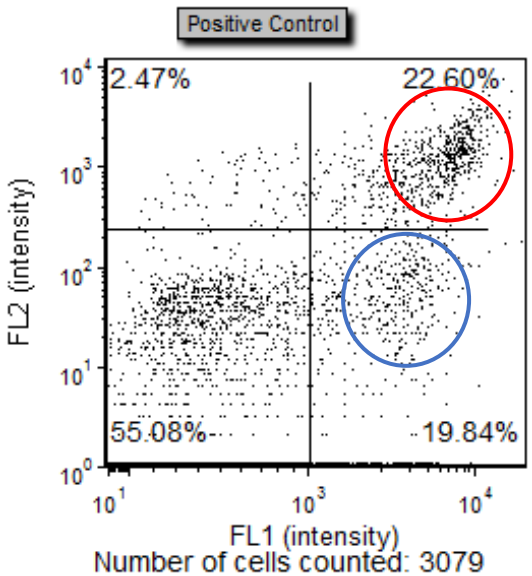


    b. In the **positive control** sample (while moving the gate in the negative control plot), the necrotic or dead cell population (PI +) should be in the top right quadrant, whereas the apoptotic population (Annexin V + only) should be gated in the bottom right quadrant.

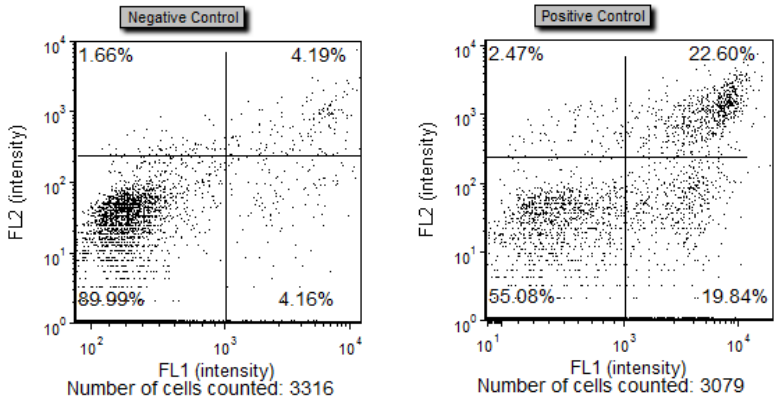
Incorrect Gating



Correct Gating



3. Upon completing gate optimization, distinct cell populations should now be represented individually in each quadrant.

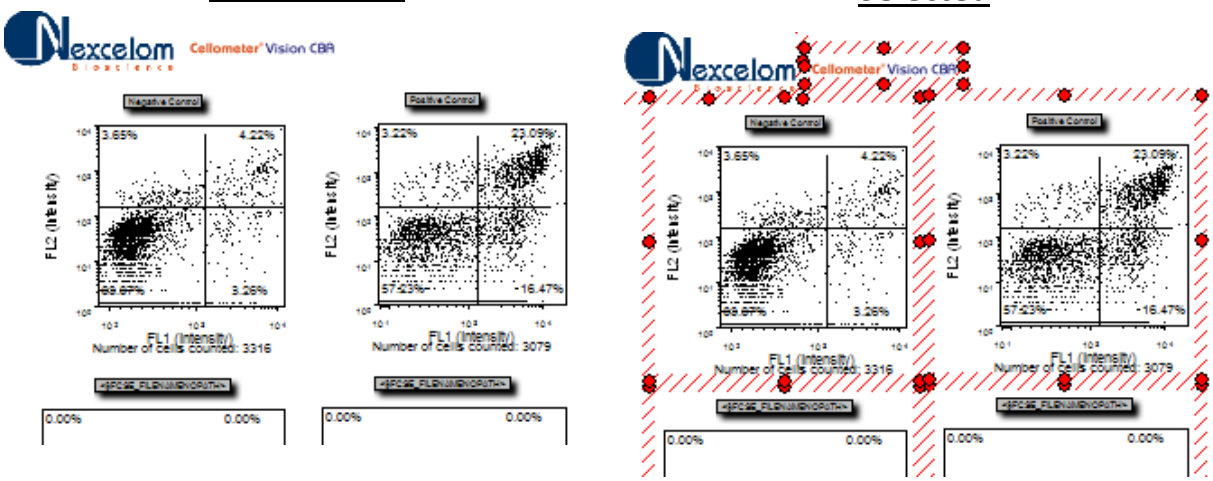


3.3 Axis Optimization for Large Cells

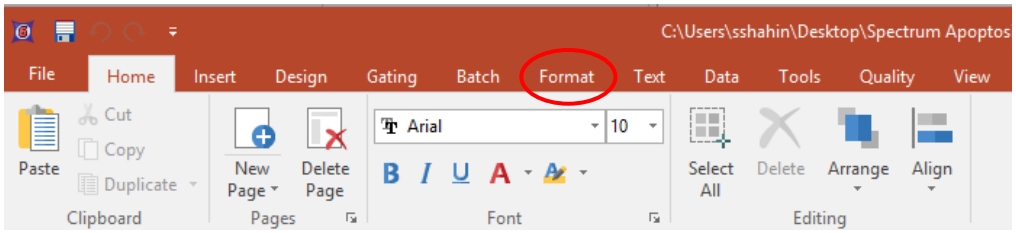
If you have large cells (> 15 µm in diameter), adjust the scatter plot axis as detailed below. If your cells are not large (< 15 µm in diameter), proceed to *Section 3.4 3.4 Saving Optimized Data Files*.

1. Scatter plot parameters may need to be adjusted for all plots in order to compensate for samples containing large cells (> 15 µm in diameter). Select all plots by holding down the "ctrl" (control) button on your keyboard and then pressing the letter "a". Red boxes will appear around all the plots on the sheet (see below).

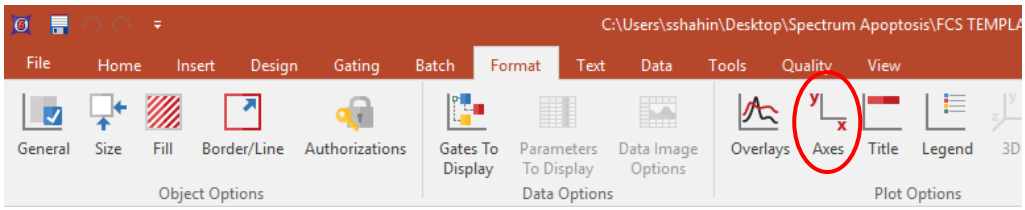
Not selected **Selected**



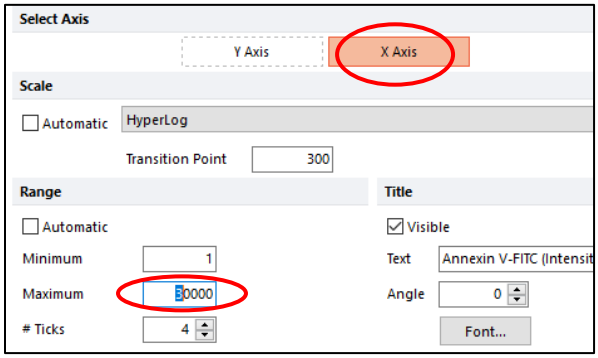
2. Select the "Format" tab at the top of the page.

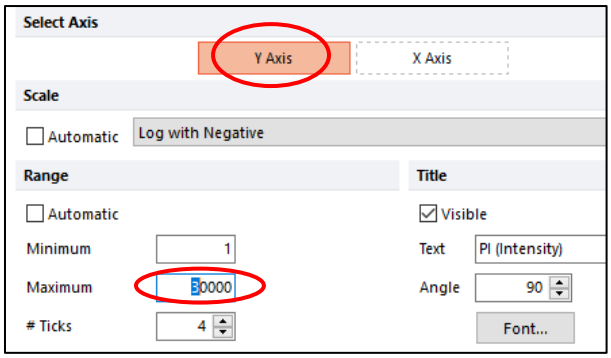


3. Select the “Axes” icon. The “**Formating Axes**” window will pop up.

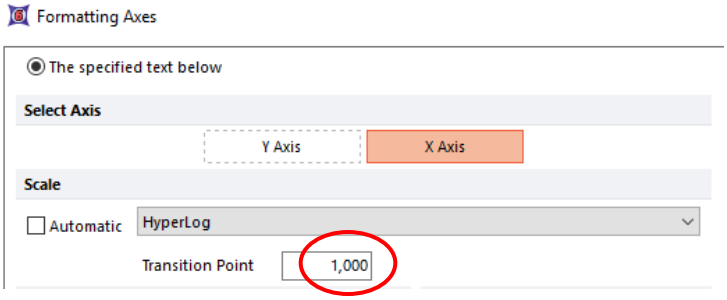


4. Change the scale of the X and Y axis.
    a. Click on the “X axis” button and change the maximum value from 50,000 to 30,000.
    b. Click on the “Y axis” button and change the maximum value from 50,000 to 30,000.





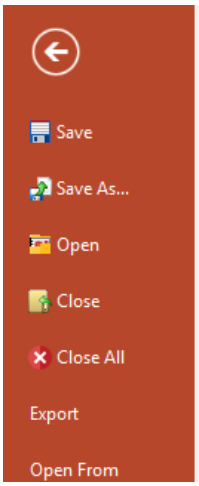
5. Change the “Transition Point” at the bottom of the page from 300 to 1000. Click OK to save the changes.

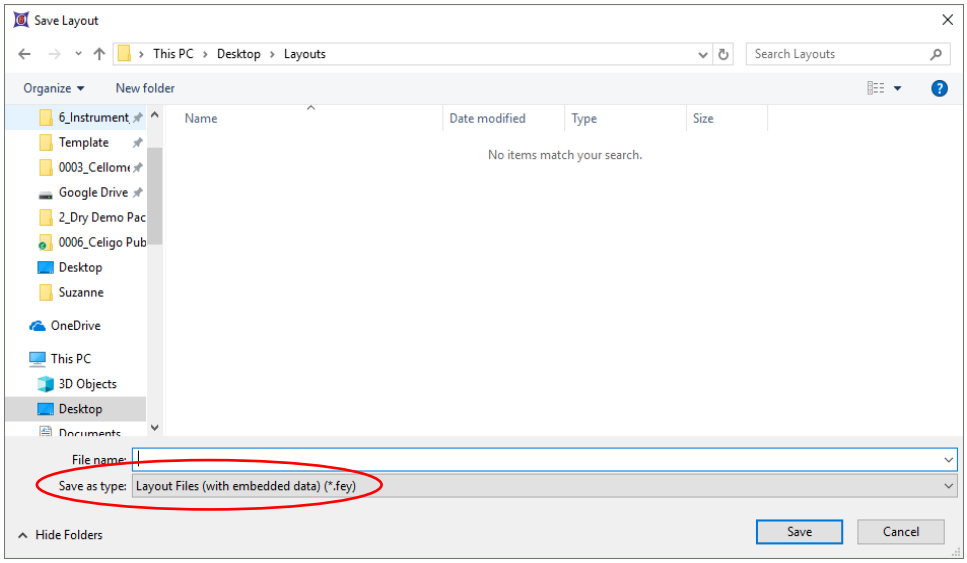


6. Re-adjust gating, if needed, using *Section 3.2 Gate Optimization*.

3.4 Saving Optimized Data Files

1. Once the Data file has the optimized parameters/settings, click Save As.
2. Select save as type file “Layout Files (with embedded data). Enter the desired file name and location, then click Save.

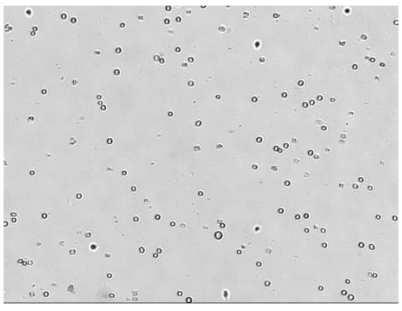


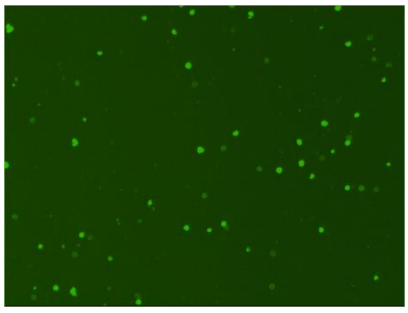


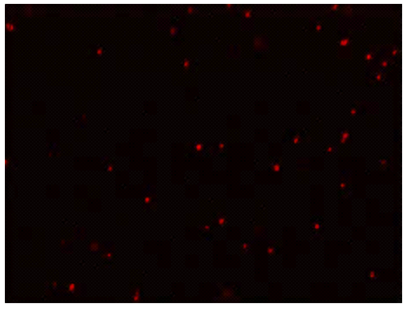
3. The new .fey file will contain the final analyzed data set.

3.5 Viewing Brightfield and Fluorescent Cell Images

To view the brightfield and fluorescent cell images, open the image folder in the original data package location. Brightfield and fluorescent images are captured for the Annexin V-FITC and PI channels for this apoptosis assays.

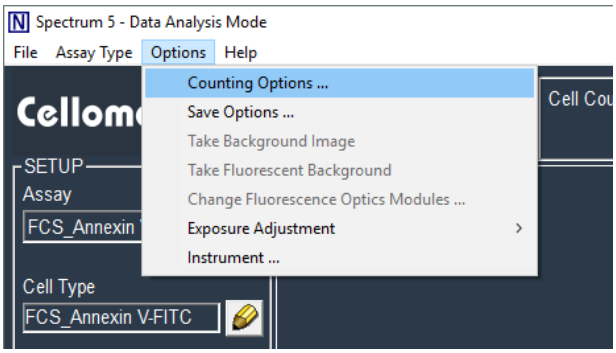


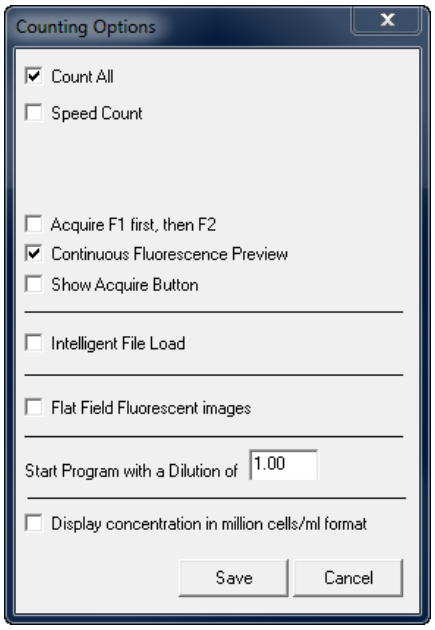




4.1 Default Counting Options Settings

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

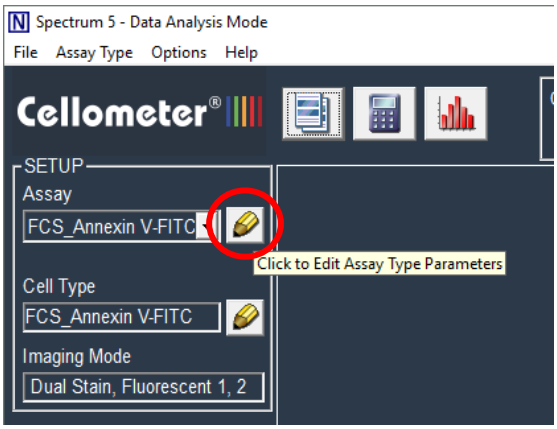




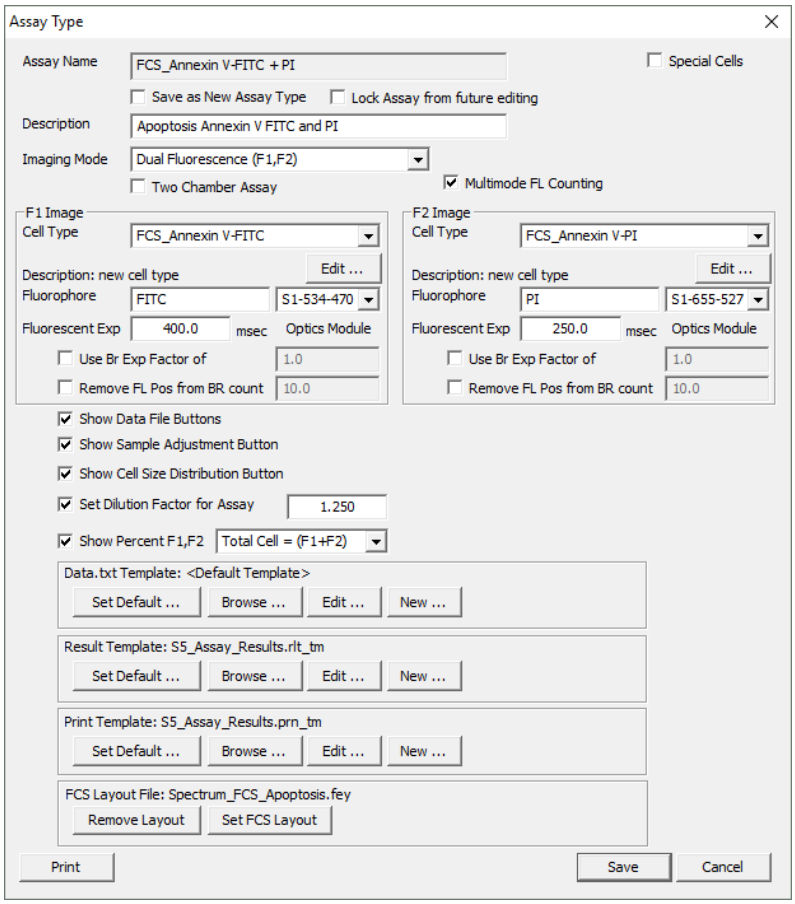
4.2 Default Apoptosis (Annexin V-FITC / PI) Assay and Cell Type Settings

4.2.1 Assay Settings

1. To review the assay settings, click on the pencil icon next to the FCS_Annexin V-FITC + PI Assay on the main Spectrum screen.

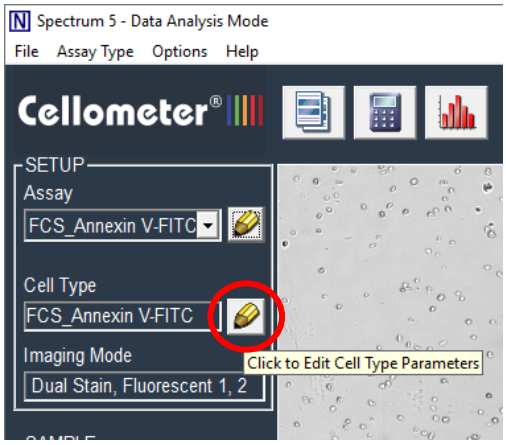


2. Default settings for the FCS_Annexin V-FITC + PI Assay are shown below.

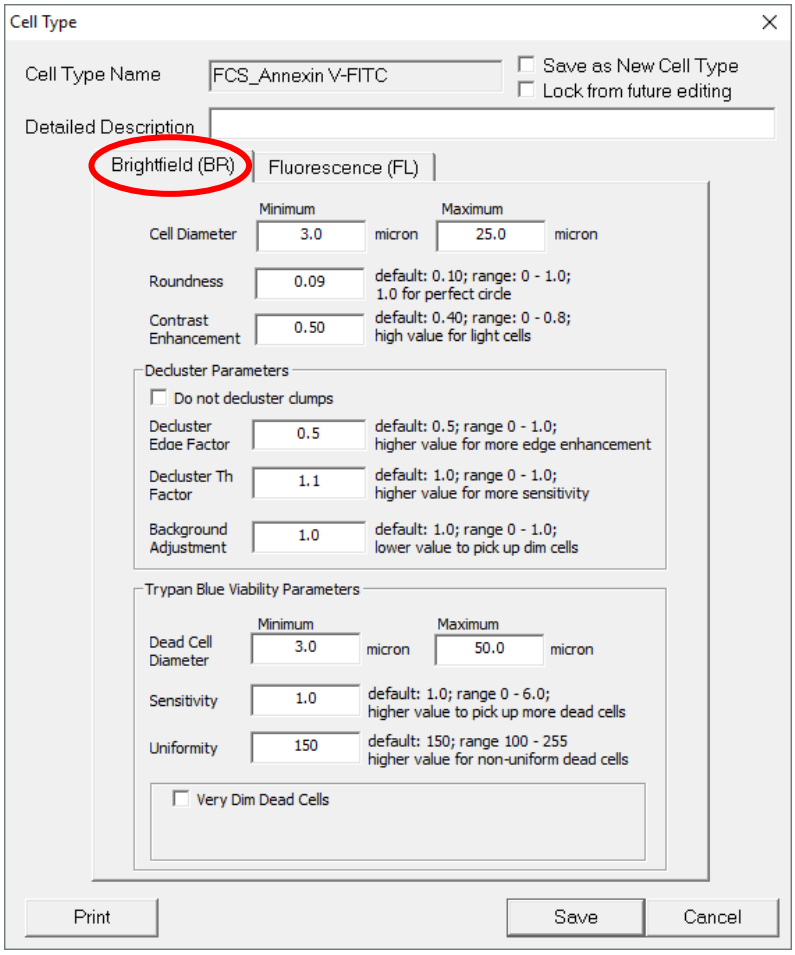


4.2.2 Cell Type Settings

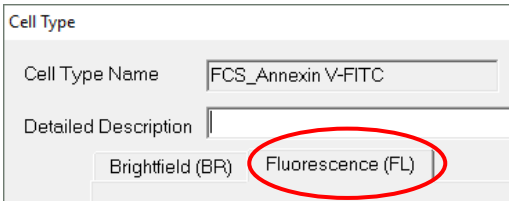
1. To review the cell type settings, click on the pencil icon next to the FCS_Annexin V-FITC Cell Type on the main Spectrum screen.



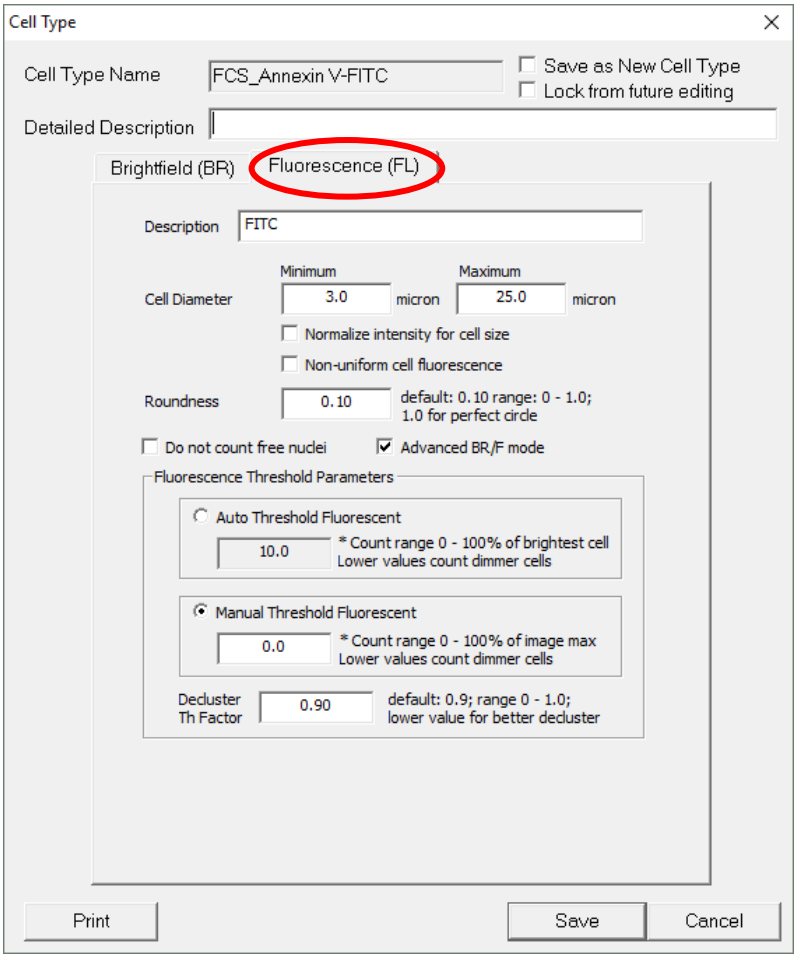
2. Default brightfield (BR) parameters settings for the FCS_Annexin V-FITC Cell Type are shown below.



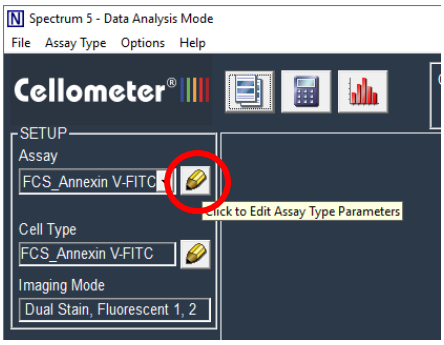
3. Click on the Fluorescence (FL) tab.

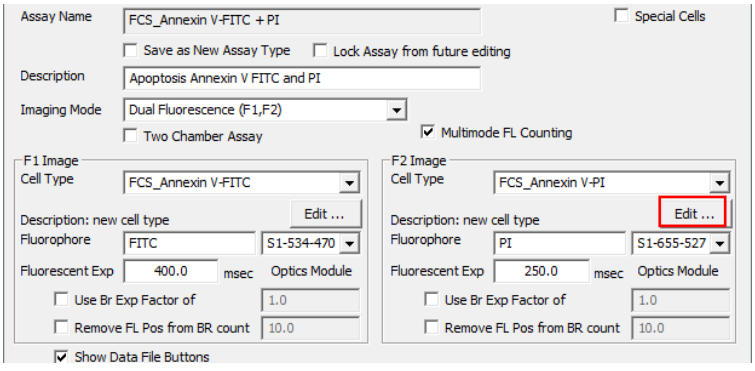


4. Default Fluorescence (FL) parameters settings for the FCS_Annexin V-FITC Cell Type are shown below.

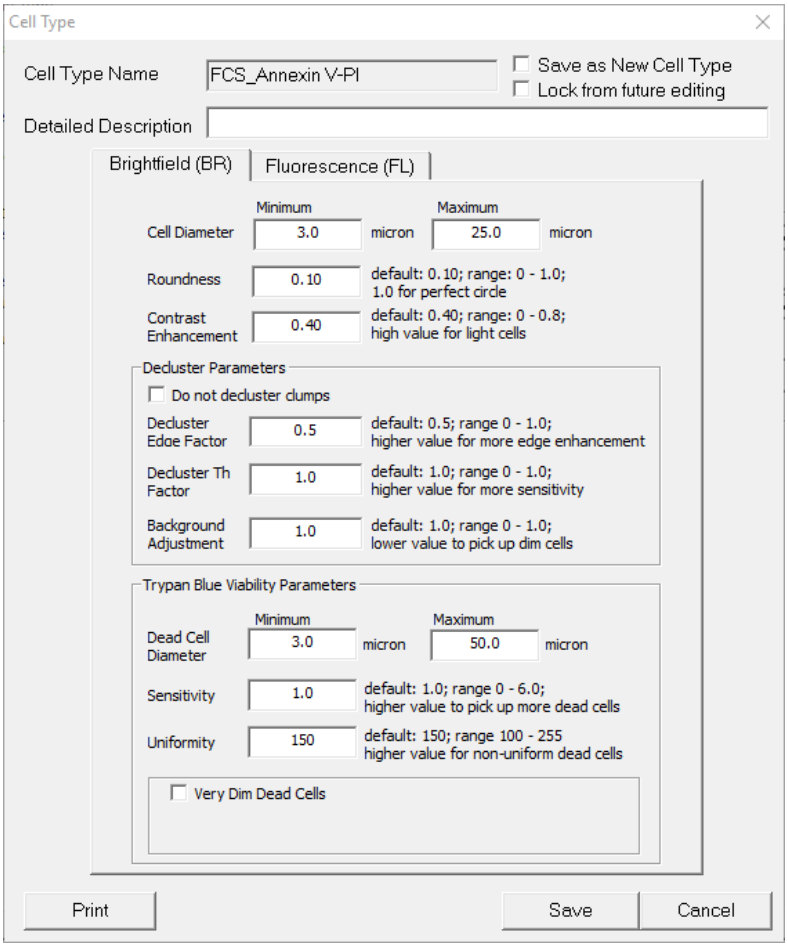


5. Click on the pencil icon under Assay on the main Spectrum screen, then click the Edit button for the F2 Image Cell Type on the right-hand side of the dialog box.

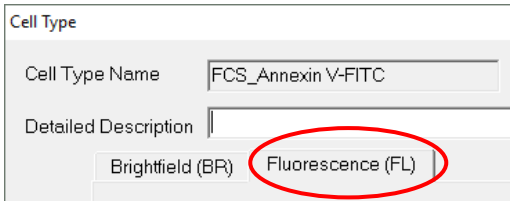




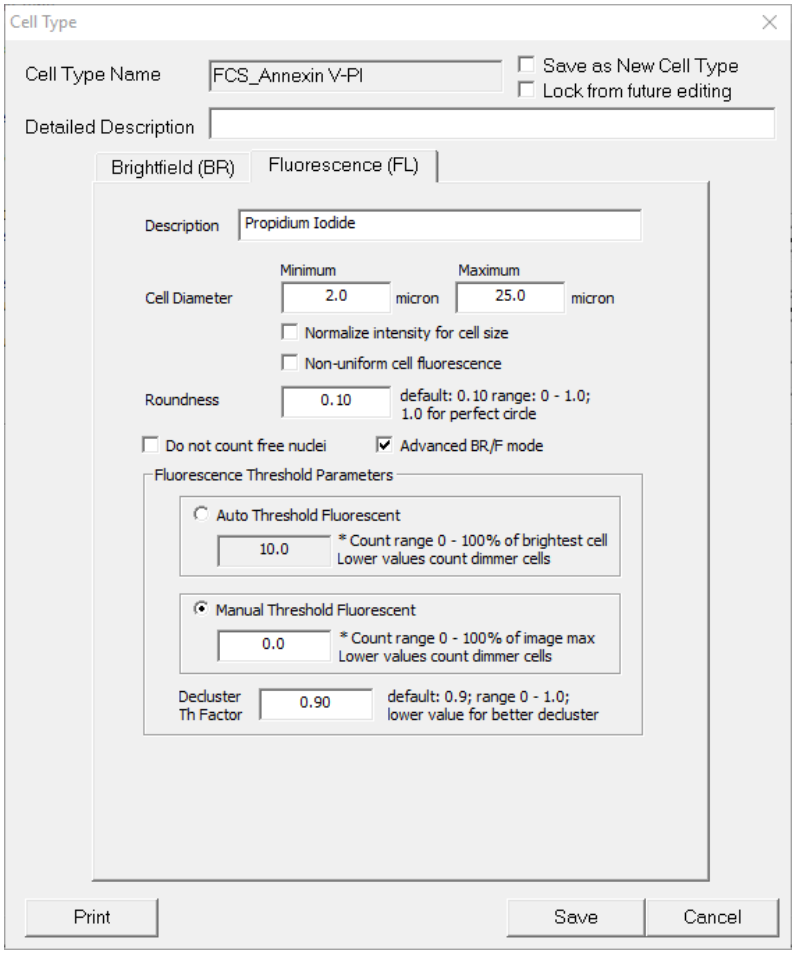
6. Default Brightfield (BR) parameters settings for the FCS_Annexin V-PI Cell Type are shown below.



7. Click on the Fluorescence (FL) tab.

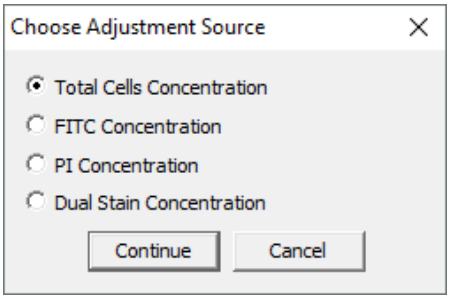


8. Default Fluorescence (FL) parameters settings for the FCS_Annexin V-PI Cell Type are shown below.

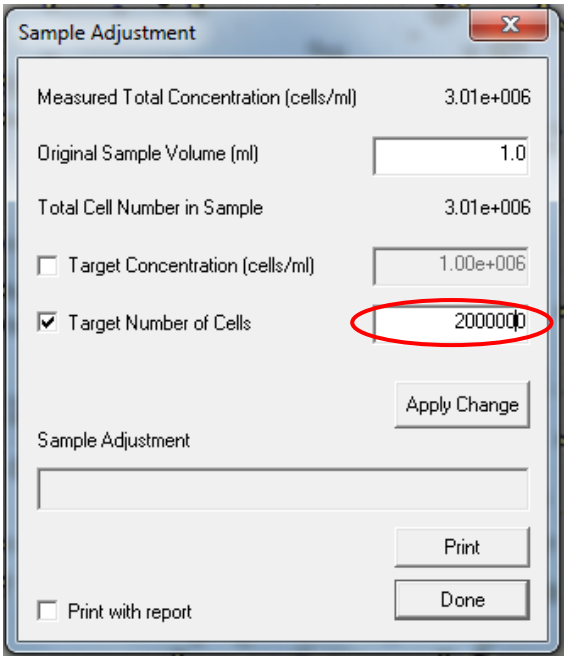


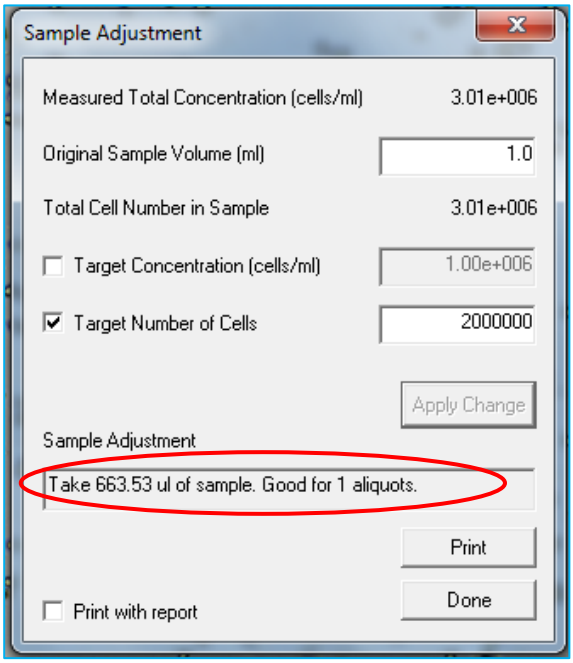
5 Sample Adjustment Calculator

1. Click on the calculator icon at the top of the screen.
2. Select **B1 Cell Concentration** and click **Continue.**



3. Enter the target number of cells. For the Apoptosis 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.

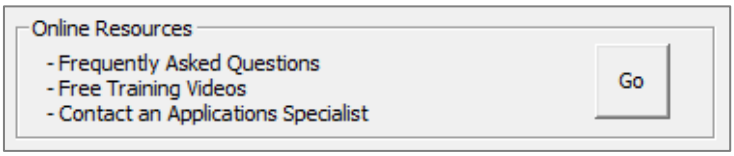




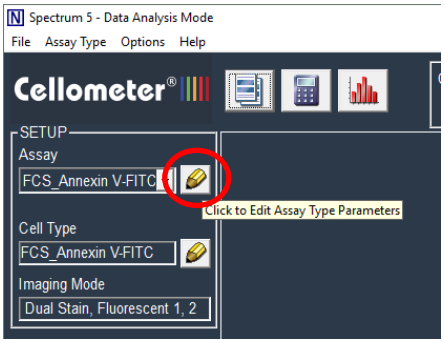
6 Importing a New FCS Express Layout

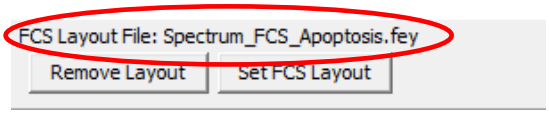
If you are unable to locate the needed FCS Express Layout or need help importing, please contact your local Field Application Specialist or e-mail Revvity Support.

1. Click on the Support icon in the bottom right corner of the Spectrum screen, then click the "Go" box under Online Resources. Click on the Assay Files tab. Select Annexin V-FITC / PI. Compare the date on the Annexin V-FITC / PI layout (.fey file) listed to the version currently saved in your Spectrum software.



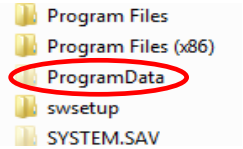
***NOTE**: To check the version in your software, click the pencil icon under Assay on the main Spectrum screen. The FCS Layout File name (with date) is listed toward the bottom of the Dialog screen.*

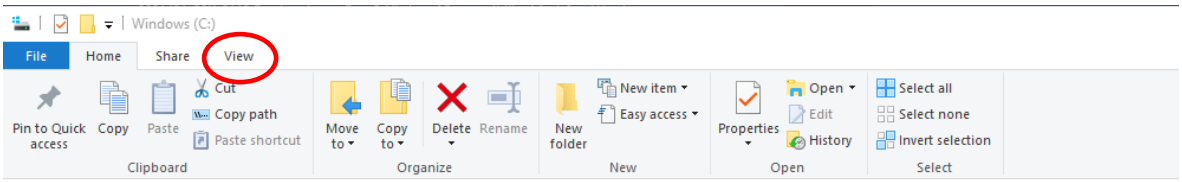




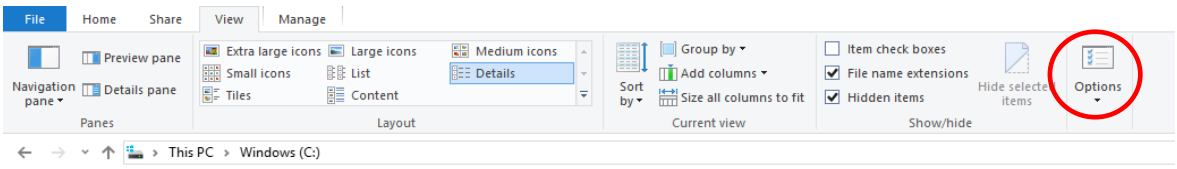
***NOTE**: If a newer layout is listed on-line, download the new PI Cell Cycle layout onto your desktop. Copy the layout by right clicking on the layout and selecting copy.*

2. Navigate to the START menu and select Computer.
3. Double click the C:\ProgramData folder to open it.
   NOTE: If ProgramData folder is not present, it may be hidden. Follow the instructions below to show hidden folders.
   a. Click the "View" tab.

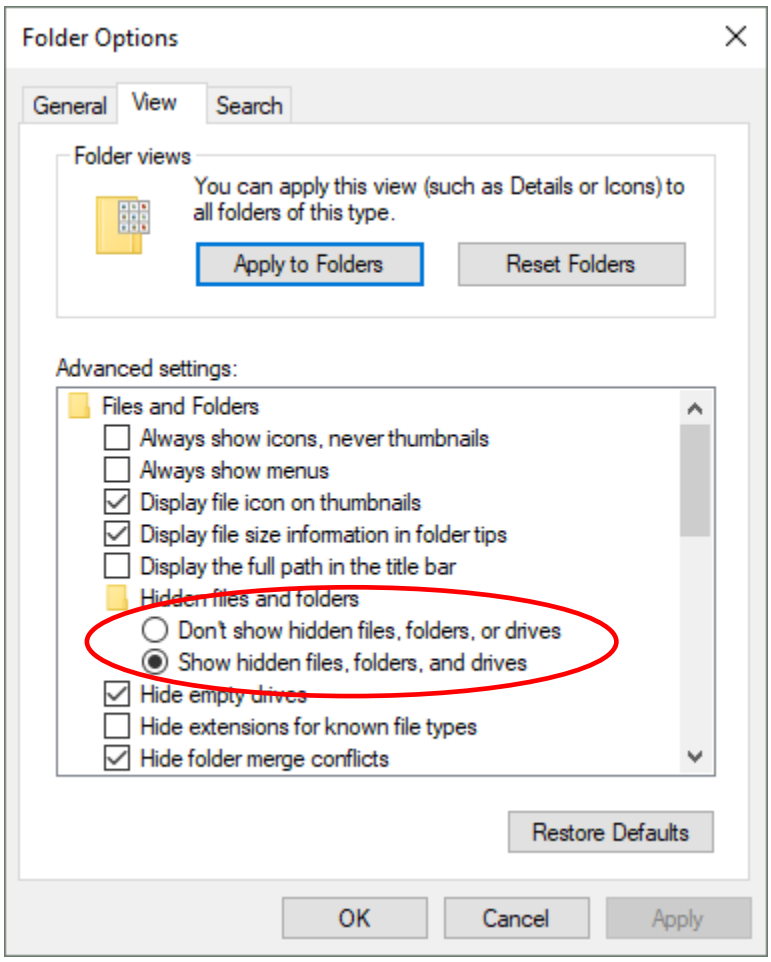




b. Click “Options”.



c. A Folder Options menu will pop up. Select View.



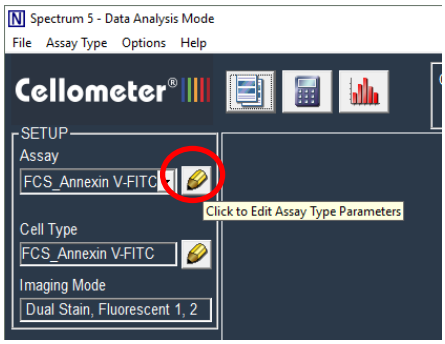
d. Under the “Hidden files and folders” file, select “Show hidden files, folders, and drives”.

e. Click OK.

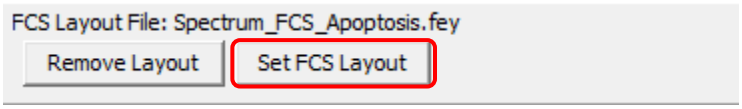
f. The C:\ProgramData folder will now appear. Double-click to open.

4. Open the Nexcelom Spectrum folder.
5. Open FCS Express folder.
6. Open Layouts folder.
7. Save/Paste new layout to the Layouts folder.

8. Set the new Annexin V-FITC / PI layout as the default layout for the Annexin V-FITC / PI Assay by clicking on the pencil icon under Assay on the main Spectrum screen.



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



10. Select the Annexin V-FITC / PI layout with the most recent date and click Open, then click Save.
11. You have now set the new Annexin V-FITC / PI layout as the default layout for the Annexin V-FITC / PI Assay on your Spectrum instrument.

7 Storage and Handling

Store the Annexin V-FITC at 2 to 8 °C. Safety precautions must be taken when handling the solution. Please consult the Safety Data Sheet for more safety information, found on **www.revvity.com/cellcountingreagents**.

8 Warranty

This product is for RESEARCH USE ONLY and is not approved for diagnostic or therapeutic use. The product is warranted to meet the specifications outlined in the Certificate of Analysis when stored and used according to the manufacturer's instructions. No other warranty, expressed or implied (such as merchantability, fitness for a particular purpose, or non-infringement), is granted. Warranty is valid until the expiration date stated on the product label.

Warranty will be void if product is stored incorrectly, the recommended protocol is not followed, or the product is used for a different application.

9 Ordering Information / Support

When ordering with a Purchase Order:

E-mail a copy of the order to **Cellc-sales@revvity.com**

For online orders, please visit:

https://www.revvity.com/cellcountingreagents

For support, e-mail **USCAN.service@revvity.com**

www.revvity.com

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