Cellometer®

ViaStain™ Annexin V-FITC Kit for Cellometer

Reagent Product Numbers: CSK-0117-1

Sample Kit: CSK-0117-S (Not available for purchase)

Cell Type Markers

Apoptosis

Cell Cycle

Proliferation

Metabolism

Viability

Nexcelom Bioscience

Table of Contents

1.0 Introduction

1.1 Description of Assay

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 more information on apoptosis, several reference articles are available, including the those where Cellometer is referenced:

- 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 and Reagents

- CSK-0117-1 kit:
    - **Component A**: ViaStain™ Annexin V-FITC
    - **Component B**: ViaStain™ Propidium Iodide Staining Solution
    - **Component C**: ViaStain™ Annexin V Binding Buffer
- Trypsin EDTA (if working with adherent cells)
- Phosphate Buffered Saline (PBS)

1.3 Instrument and Software Requirements

Spectrum:

- FCS Express Flow Cytometry software is a product of De Novo Software
- Spectrum instrument
- Spectrum software
- Fluorescence Optics Module S1-534-470 and S1-655-527

OR

Vision CBA:

- FCS Express Flow Cytometry software is a product of De Novo Software
- Vision CBA instrument
- Vision CBA software
- Fluorescence Optics Module VB-535-402 and VB-660-502

2.0 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

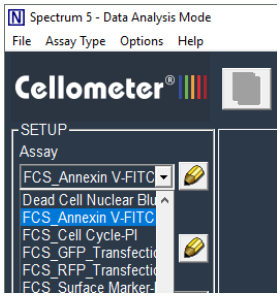
1. Using 1 x Trypsin-Versene (EDTA), trypsinize cells until they have lifted off the plate.
2. Spin down cells at 1,000 to 2,000 rpm for five minutes.
3. Decant the supernatant and re-suspend cells in 1 ml of 1 x PBS.

2.3 Staining Procedure

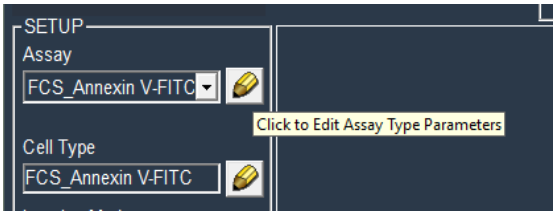
1. Verify the concentration of the positive / negative controls and cell samples using the Spectrum or Vision CBA Analysis System.
2. Use the Cellometer Sample Adjustment Calculator to generate a cell sample with a concentration between 2 and 3 million cells per mL (2-3 x 10^6 cells/mL). See section 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 (~1,000 to 2,000 rpm) for 5 minutes, aspirate medium, then re-suspend cells in 40 µl of Annexin V Binding Buffer (Component C). Mix by pipetting up and down at least 10 times.
4. Add 5 µl of Annexin V-FITC (Component A).
5. Add 5 µl of PI solution (Component B).
6. Gently pipette the cells up and down ten times, then incubate for 15 min at RT (25°C) in the dark.
7. Add 250 µl of 1x PBS to the sample and spin down the cell sample at 200 - 400 x g (~1,000 to 2,000 rpm) for 5 minutes.
8. Carefully aspirate off the medium and re-suspend cell pellet in 50 µl of Annexin V Binding Buffer (Component C). 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 or Vision CBA software screen. The apoptosis assay should be run with the default software settings and the exposure times indicated below (2.4.3). If you suspect that the settings may have been changed, review the default software settings in section 4.0.



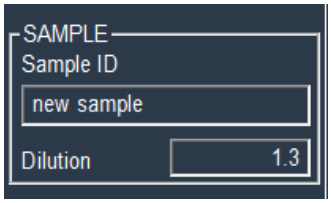
2. If running the **FCS_Annexin V-FITC + PI** or **CBA_Apoptosis Annexin V + PI** assay for the first time, the default exposure time **MUST** be updated. Click on the pencil icon under Assay on the main Spectrum or Vision CBA screen.



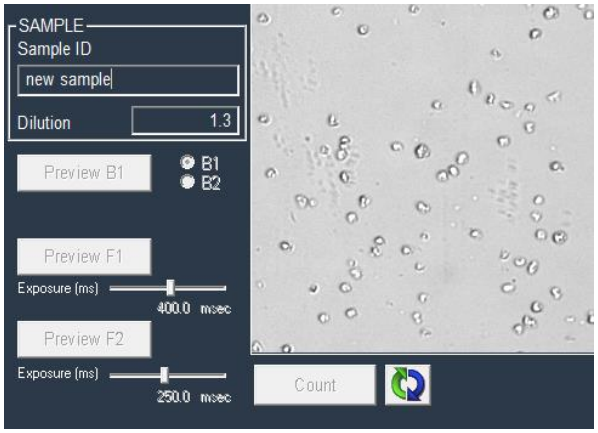
3. Adjust the exposure for the Annexin V FITC (F1) and PI (F2) channels based on the instrument

Instrument	Annexin V FITC (F1) Exposure	PI (F2) Exposure
Spectrum	400 msec	250 msec
Vision CBA	4000 msec	2000 msec

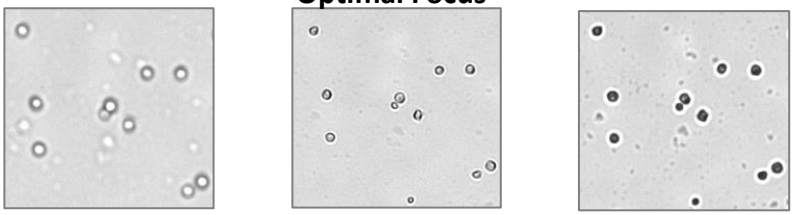
4. To import the most current FCS Express Layout for the Annexin V-FITC / PI assay, see section 6.0 Importing a New FCS Express Layout
5. Gently mix the cell sample by pipetting up and down at least ten times, then load 20 µL into the Cellometer imaging chamber and insert slide.
6. Wait 60 seconds for 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 or Vision CBA screen.



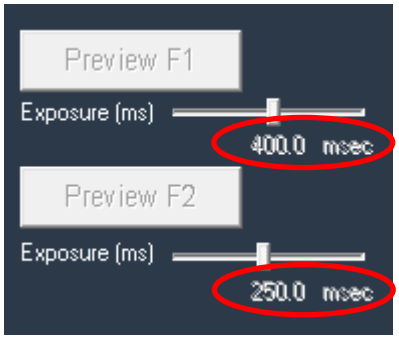
9. Turn the focus knob and adjust focus for the bright field image. Cells in focus for the apoptosis assay will have a bright center and dark outline. There should be a crisp contrast between background and the cell membrane. See Focus Guide below.



Optimal Focus



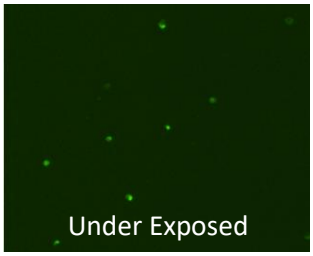
10. Click Stop Preview
11. Click the Preview F1 Image button (bottom left of screen) and verify that the fluorescence signal displays as 100% of range.

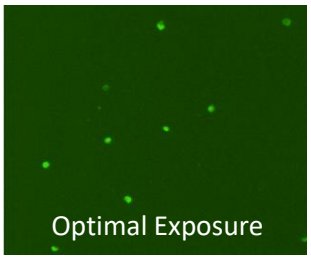


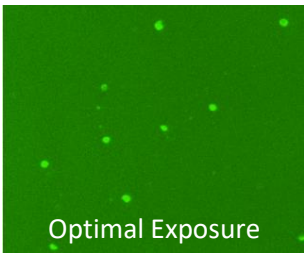
The default exposure is 400 milliseconds for Spectrum and 4000 milliseconds for Vision CBA for F1.

Below shows example images of cell stained with Annexin V FITC + PI. Optimal exposure time will generate a bright image with well-defined fluorescent spots.

Under-exposure will yield dark images with insufficient fluorescent signal. Over-exposure will yield images that are too bright with fluorescent spots that are less distinct from background.



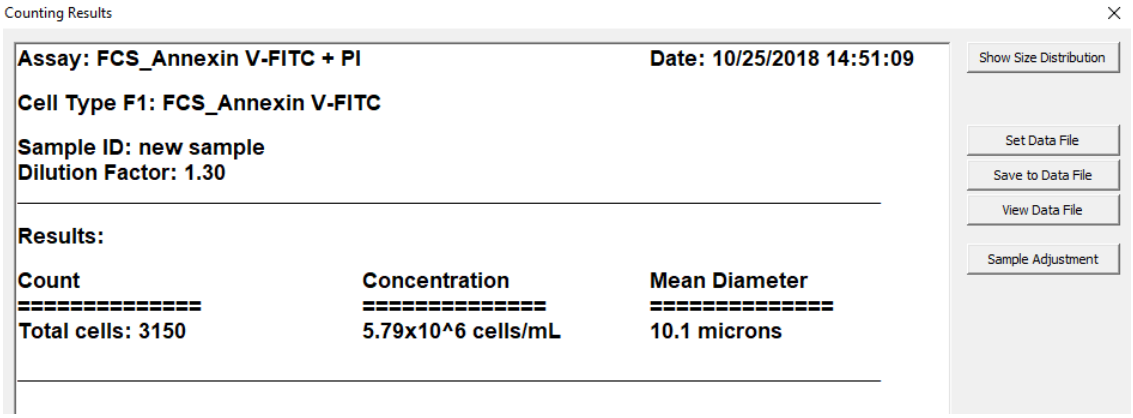




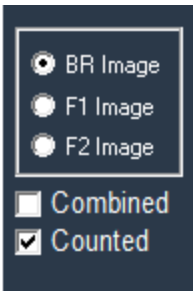
12. Click Preview F2. The F2 (PI) channel should be set to 250 or 2000 msec exposure for Spectrum and Vision CBA respectively.

13. Click the Count button at the bottom of the screen

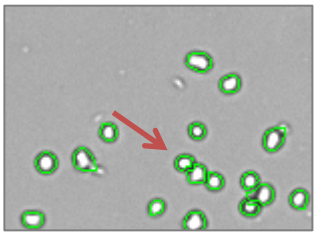
14. When counting is complete, an initial Results Table will appear on the screen. For optimal results, the Total Count should be between 2,000 and 8,000 cells (4,000 cells is optimal). If the Total Count is < 2,000 repeat data acquisition with a more concentrated sample. For larger cells (>15 microns 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.

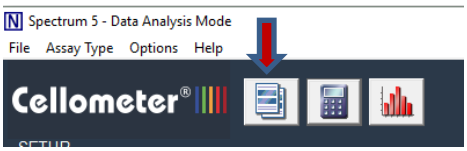


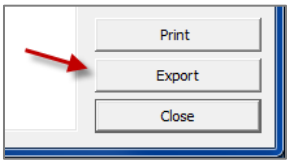
16. Review the counted image to confirm that cells are being counted correctly. Individual cells within clumps should be circled in green, indicating that they are being counted individually.



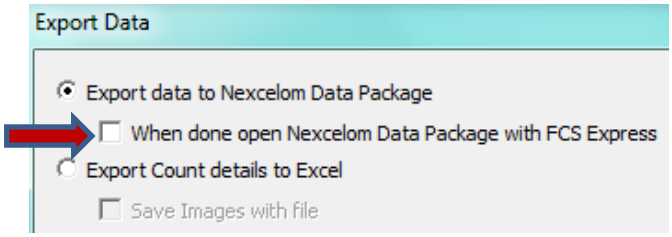
2.5 Exporting and Saving Data Files

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)

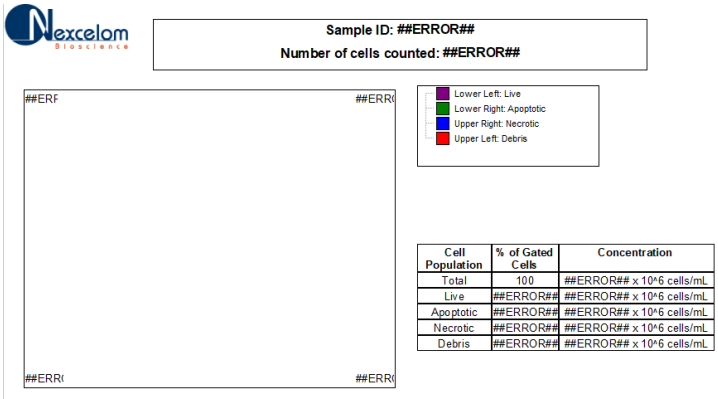
Folder with cell images

3.0 Data Analysis

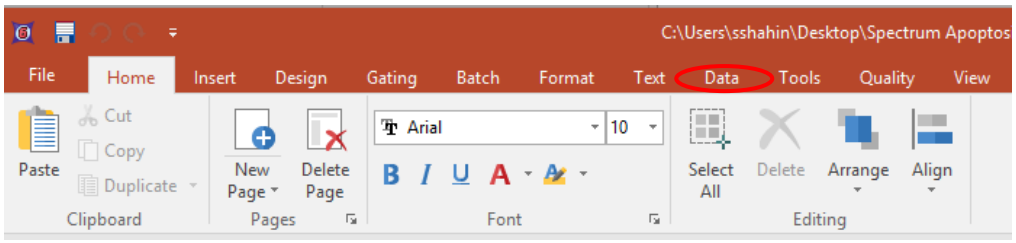
3.1 Opening and Loading Data Files

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

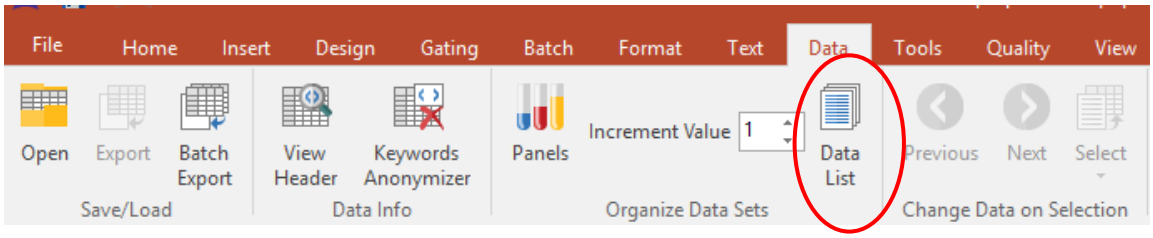
1. Upon opening the FCS Express software, a blank template will automatically load.



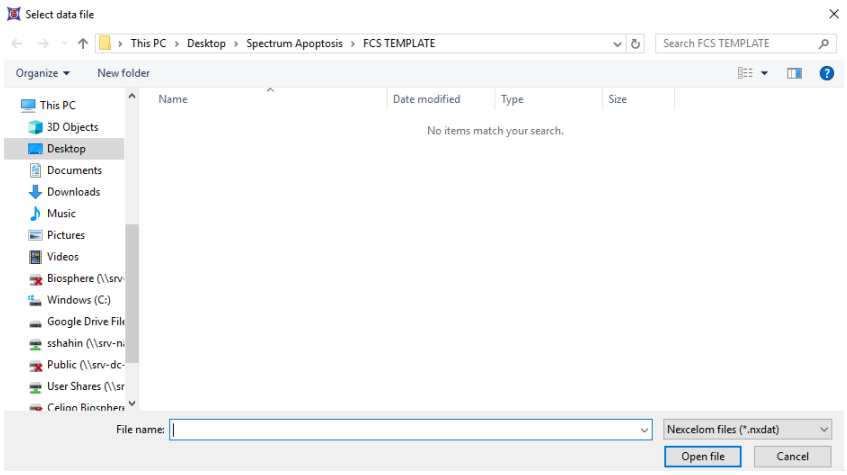
2. To import data, locate and click on the “Data” tab at the top of the page.



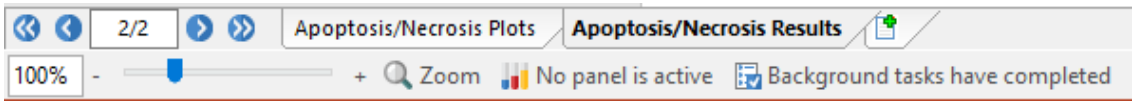
3. Locate and click on the folder labeled “Data List”

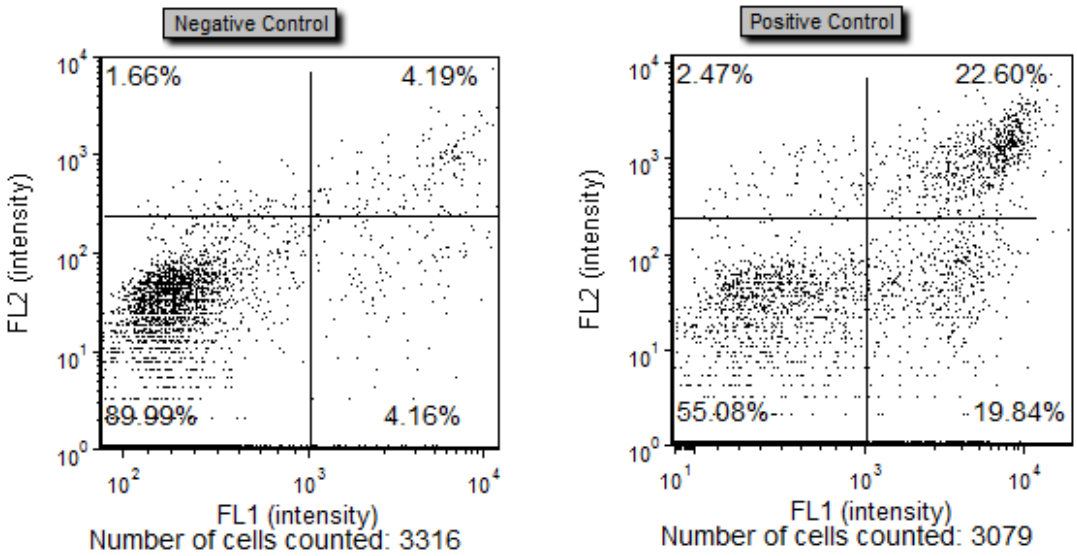


4. A data list pop-up window has now appeared. To insert the **negative control** sample first, click on the blue (**+**) shown in the red circle (below). The **Select a Data File** pop-up window will appear (see example at right). Locate and select your data negative control sample (saved in step 2.4.3) and click open.



5. Repeat steps 2 and 3 to load positive control and up to four unknown samples. A scatter plot and data table generated using default gating parameters are automatically displayed for 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.

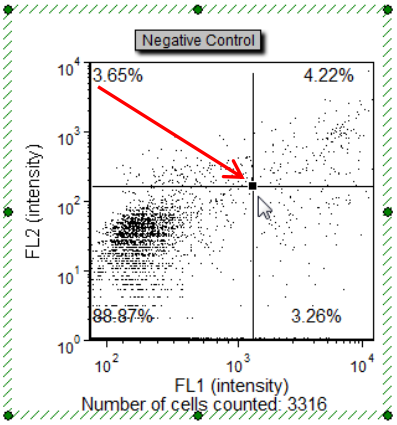




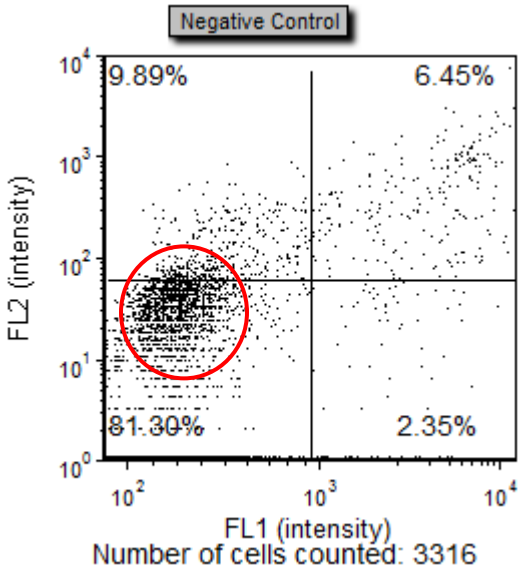
	Cell population (%)	Concentration (x 10^6 cells/ml)
Negative Control	Cells counted: 3316	
Total	100.00	4.68
Live	87.97	4.12
Apoptotic	4.01	0.19
Necrotic	4.98	0.23
Debris	3.05	0.14
Positive Control	Cells counted: 3079	
Total	100.00	4.35
Live	52.71	2.29
Apoptotic	20.95	0.91
Necrotic	23.90	1.04
Debris	2.44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

3.2 Gate Optimization

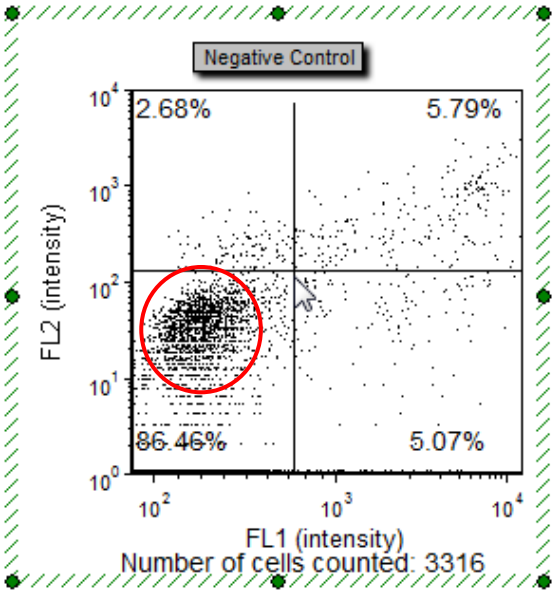
1. You may adjust the gate in the display for the negative control sample only. Adjusting the negative control gates will automatically and proportionally adjust the gates in the positive control as well as the other data plots. To adjust the gate, first click on the dot-plot. A green box appears around the dot-plot singnaling that it is selected. Next, click on the center of the four quadrants. A small black box appears in the center (see arrow). You may now click-on and drag the gate to a desired location.



2. The quadrant gating should be adjusted according to data in the negative and positive controls. First, the gate should be set based on the **negative control**. The healthy, live cell population (red circle) should be in the bottom left quadrant.



Incorrect Gating

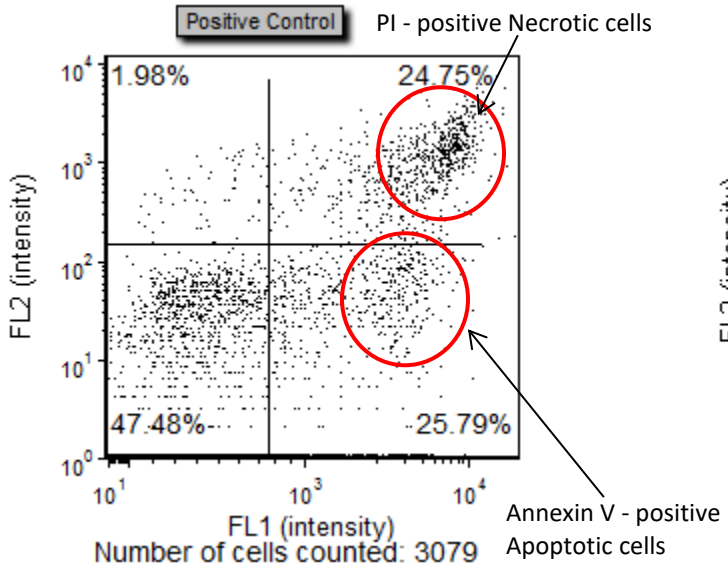


Correct Gating

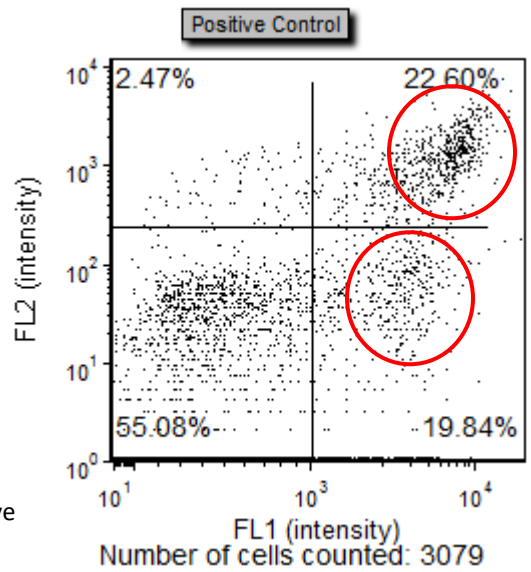
3. The next gating parameters should be based on the **positive control** or drug-treated sample. Remember that gate adjustments are performed on the Negative Control sample and are automatically applied to all plots. Move the gate in the Negative Control while monitoring the gate adjustments that are takng place in the Positive Control sample. The necrotic or dead cell

population should be in the top right quadrant. The apoptotic cell population should be gated into the bottom right quadrant.

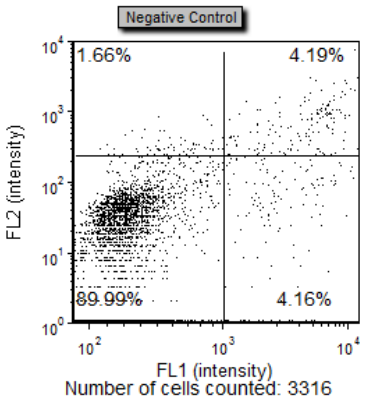
Incorrect Gating

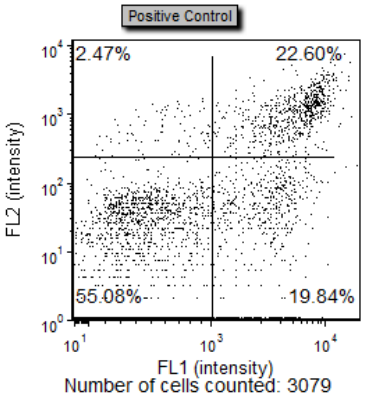


Correct Gating



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

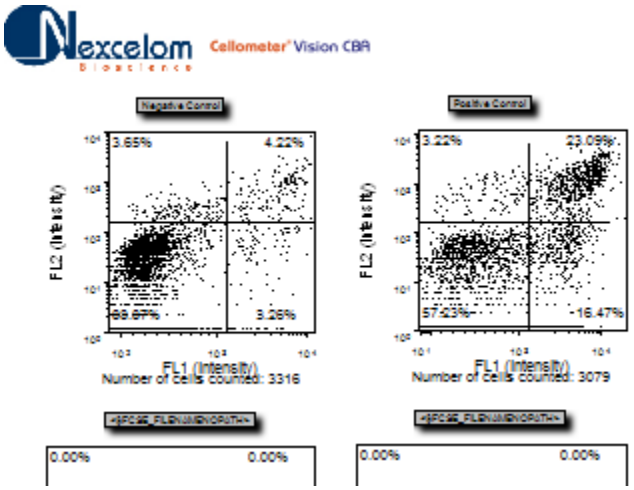




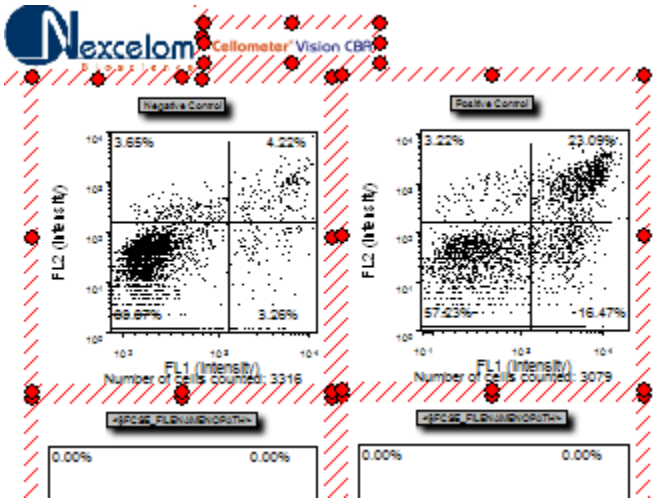
3.3 Gate Optimization for Large Cells

1. Dot-plot parameters may need to be adjusted in order to compensate for samples containing large cells (typically > 15 microns in diameter). Changes to the dot-plot parameters and gating adjustments must be made silmultaneously to all of the plots. To select the plots, press and hold down the “ctrl” (control) button on your keyboard and then press the letter “a”. This will select all the plots on the sheet. You will notice that red boxes have appeared around all the plots on the sheet (see below).

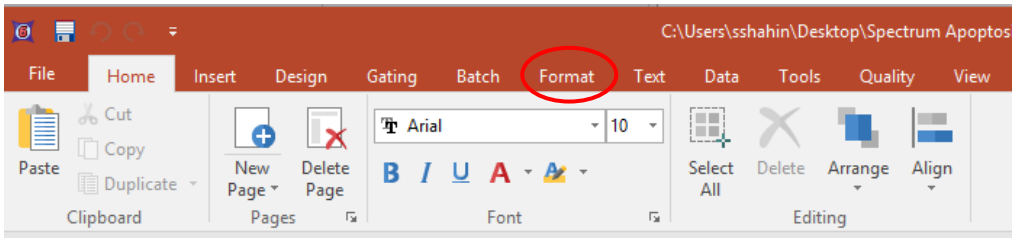
Not selected



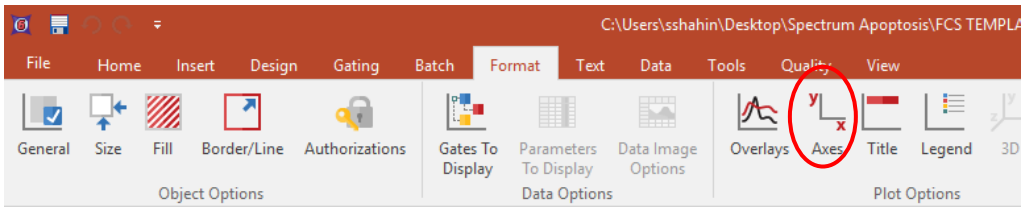
Selected



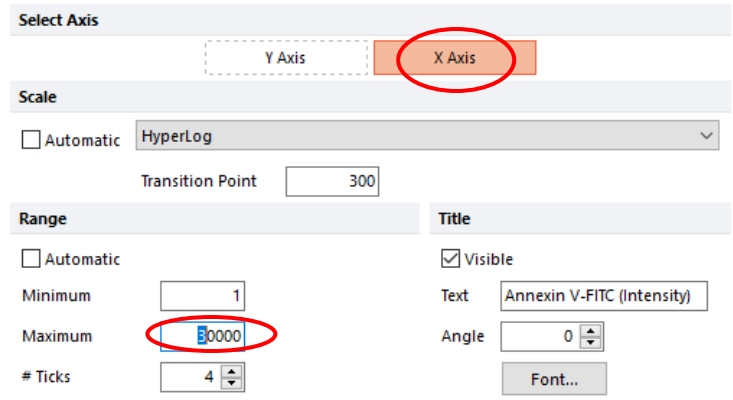
2. Navigate to and click-on the "Format" tab at the top of the page.

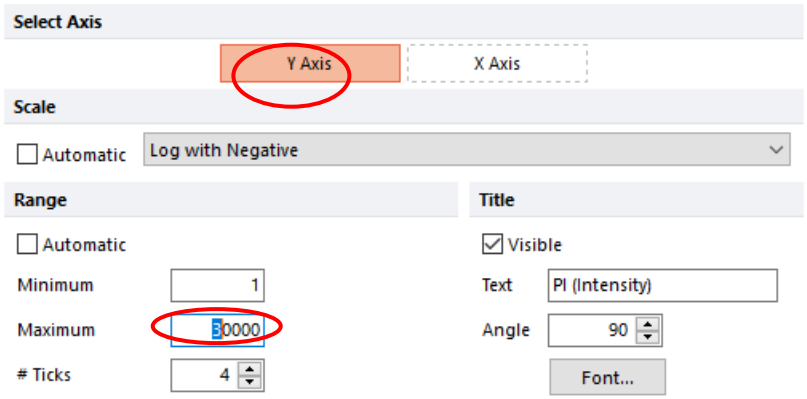


4. Under the Format menu, locate and click on "Axes".

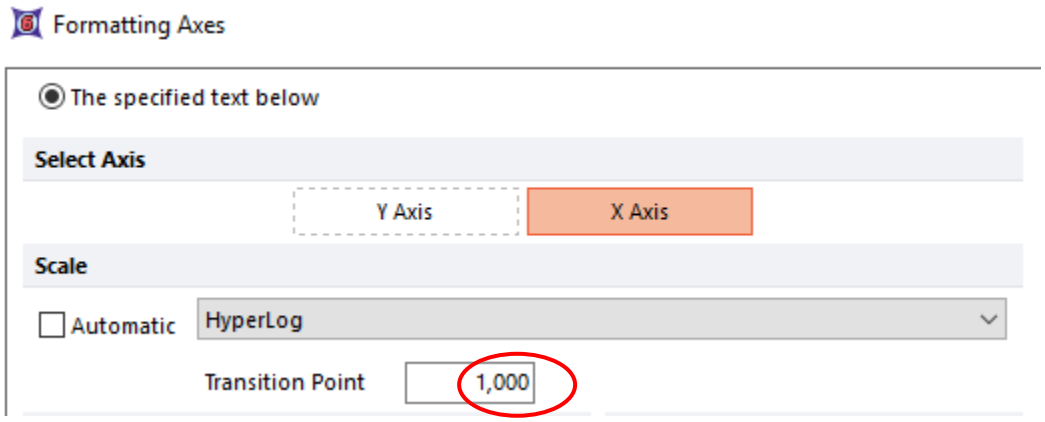


5. The "**Formating Axes**" window will pop up. Notice that the default maximum value is 50,000. You can now change the scale of the X and Y axis. Change the "maximum" value from 50,000 to 30,000. To change the X axis, click on "X axis" and also change the value from 50,000 to 30,000.





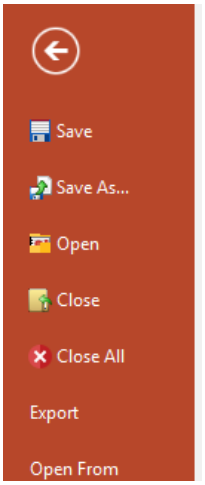
6. Change the "Transition Point" at the bottom of the page from 300 to 1000. Click OK to save the changes. Adjust gating based on the instructions in section 3.2.



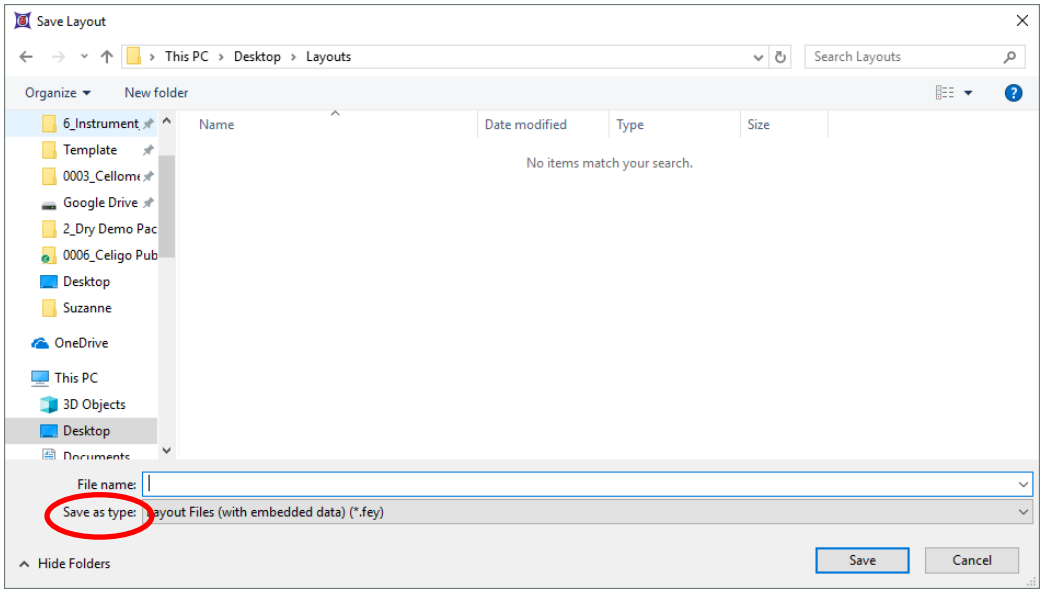
3.4 Saving Optimized Data Files

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

2. Click Save As



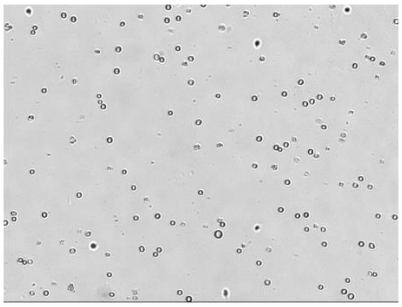
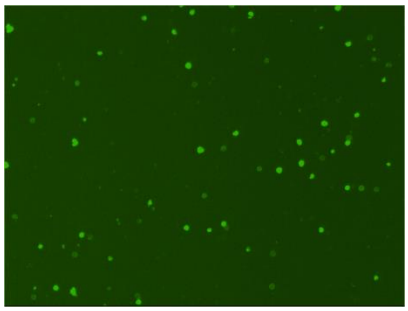
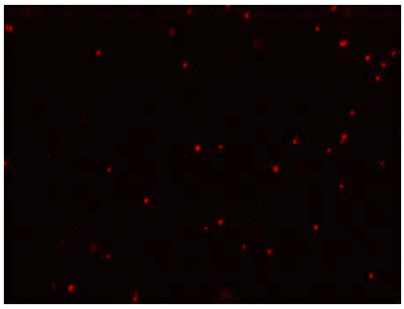
3. Click “Embed” in the bottom right of the Save Layout window under Data Files. Enter the desired file name and location, then click Save.



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

3.5 Viewing Image Report

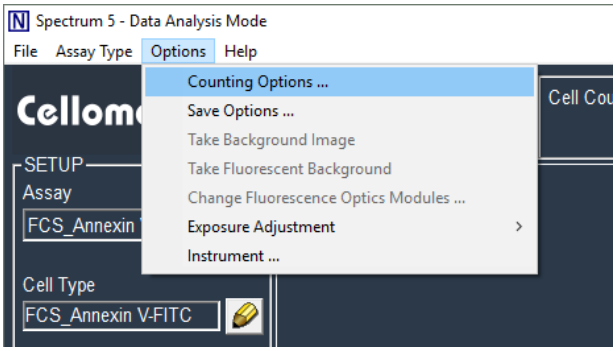
To view the bright field and fluorescent cell images, open the image folder in the original data package location. Bright field and fluorescent images are captured for apoptosis (Annexin V-FITC / PI) assays.

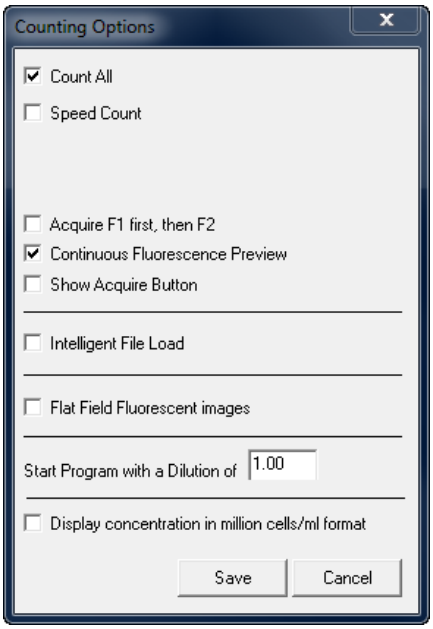




4.0 Software Settings

4.1 Review Apoptosis Counting Options Screen

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

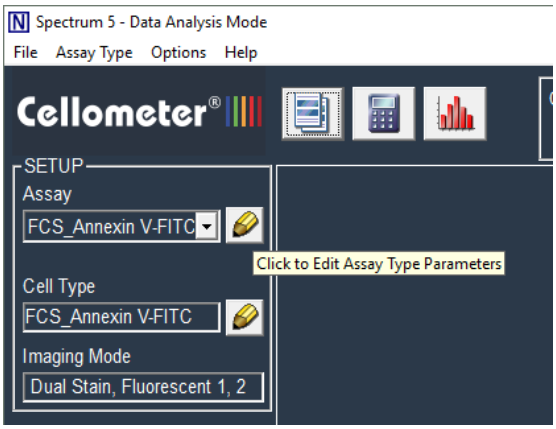




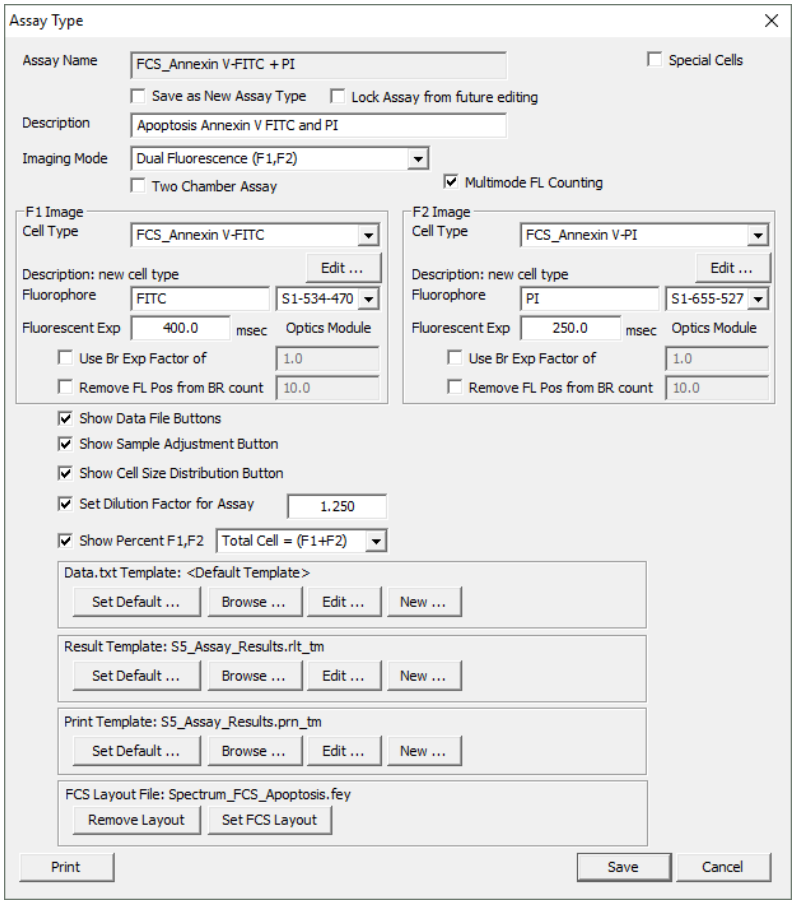
4.2 Default Apoptosis (Annexin V-FITC / PI) Software Settings

4.2.1 Check Dialog Screen Settings

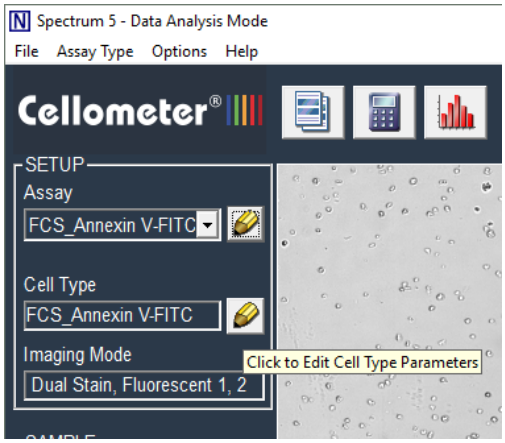
1. Click on the pencil icon under Assay on the main Spectrum or Vision CBA screen.



2. Verify that all selections on the instrument screen match the default settings below.

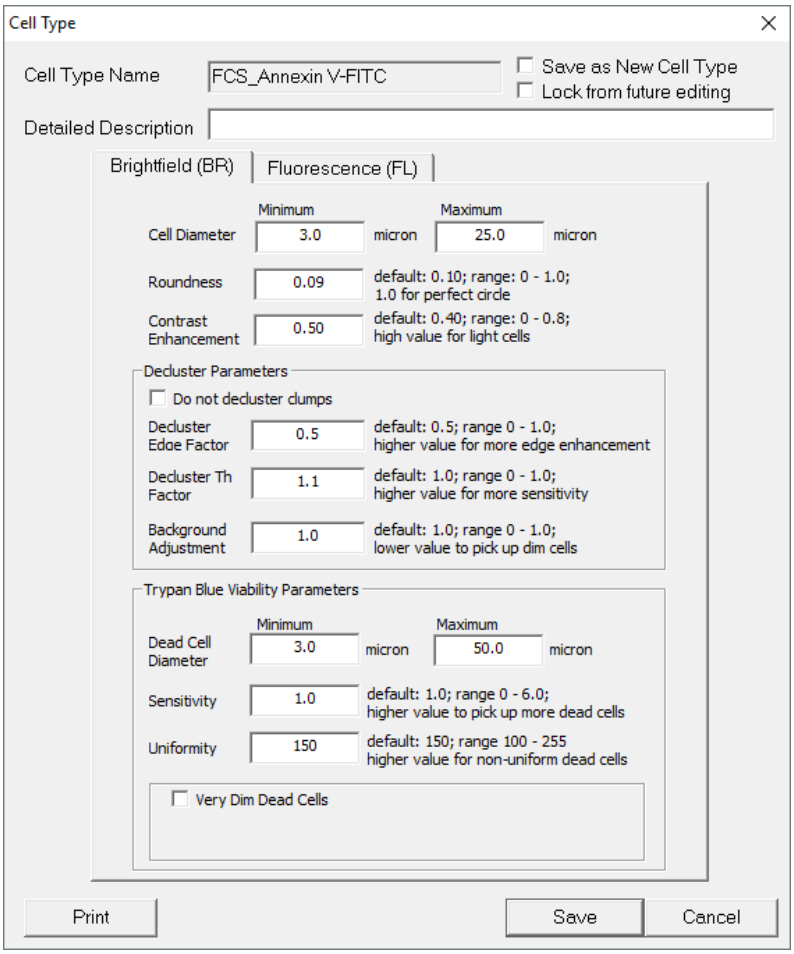


4.2.2 Check Cell Type Settings

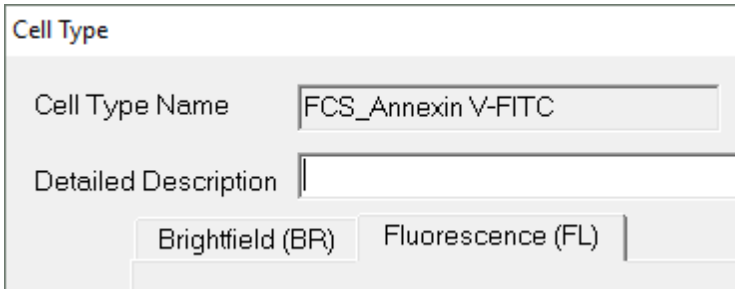


1. Click on the pencil icon under Cell Type on the main Spectrum or Vision CBA software screen.

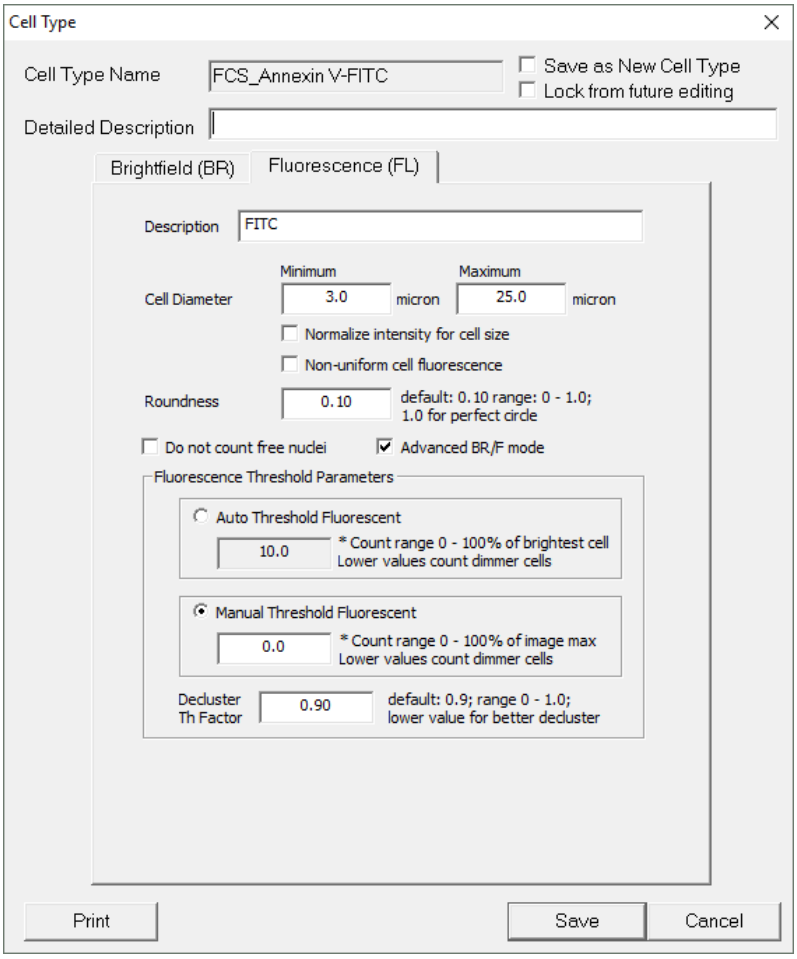
2. Verify that all selections for the bright field (BR) tab on the instrument screen match the default settings below.



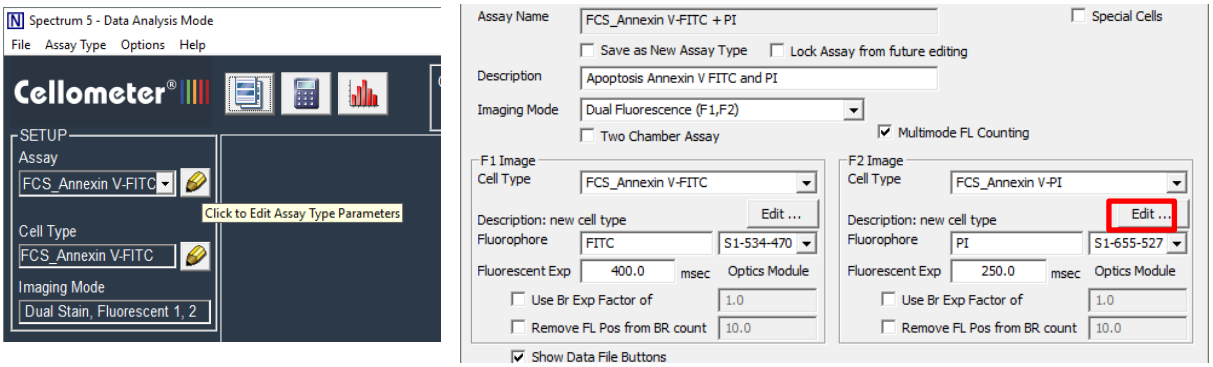
3. Click on the Fluorescence (FL) tab.



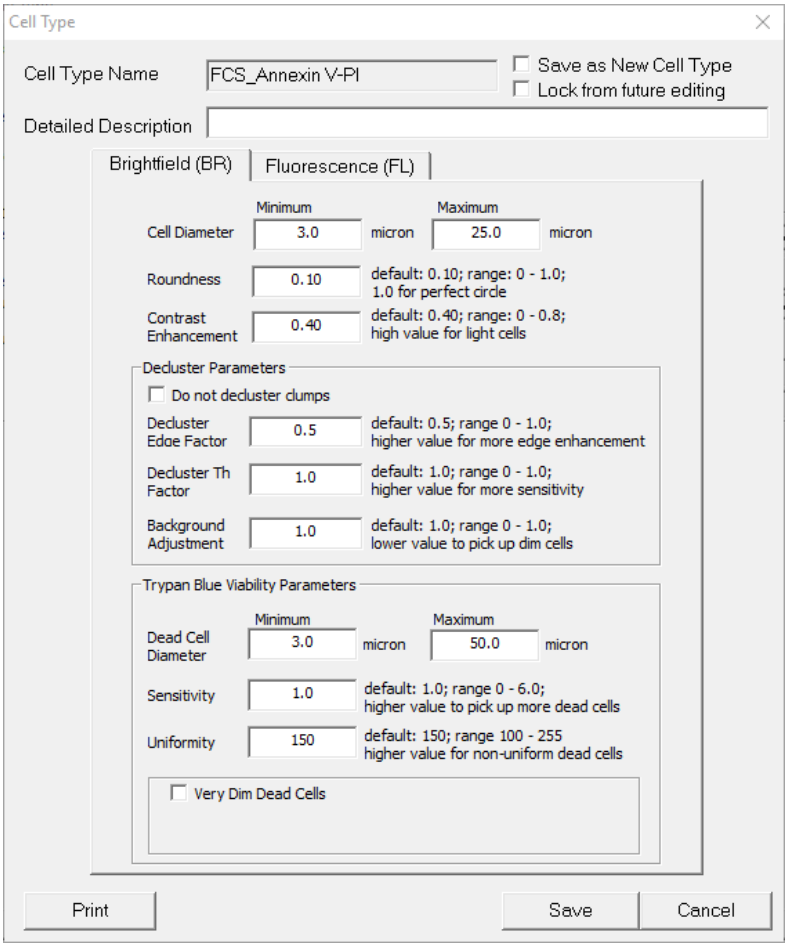
4. Verify that all selections for the Fluorescence (FL) tab on the instrument screen match the default settings below.



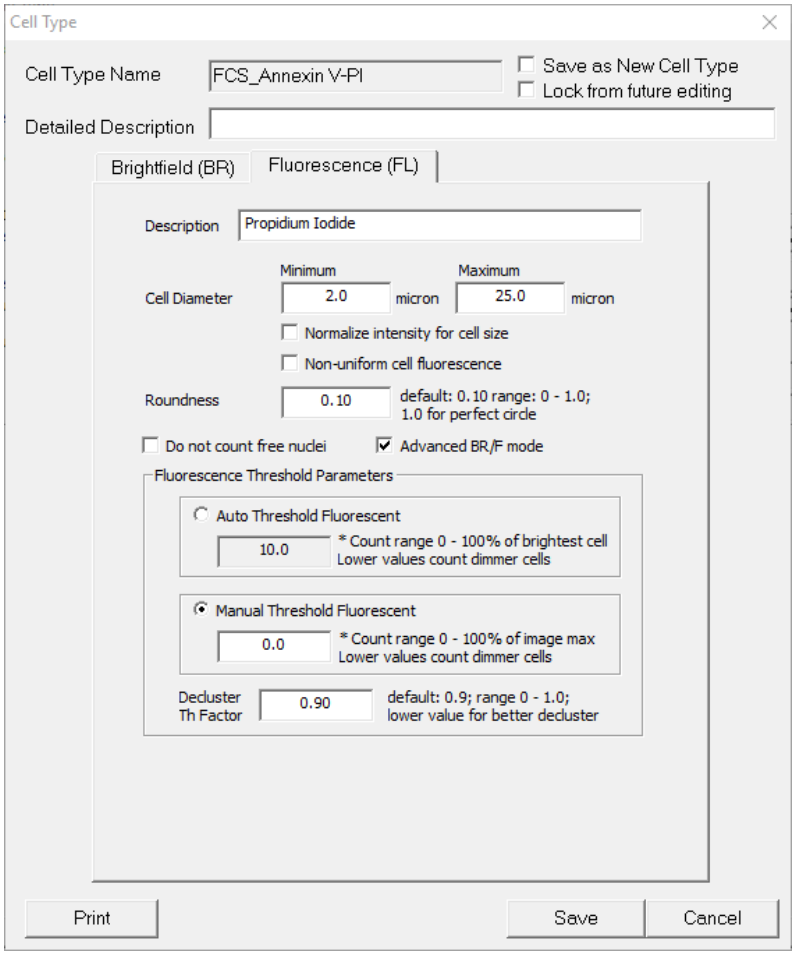
5. Click on the pencil icon under Assay on the main Spectrum or Vision CBA screen, then click the Edit button on the right-hand side of the dialog box.



6. Verify the Bright field settings for the F2 image.



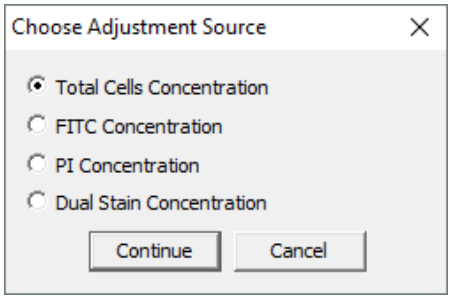
7. Verify the fluorescent settings for the F2 image



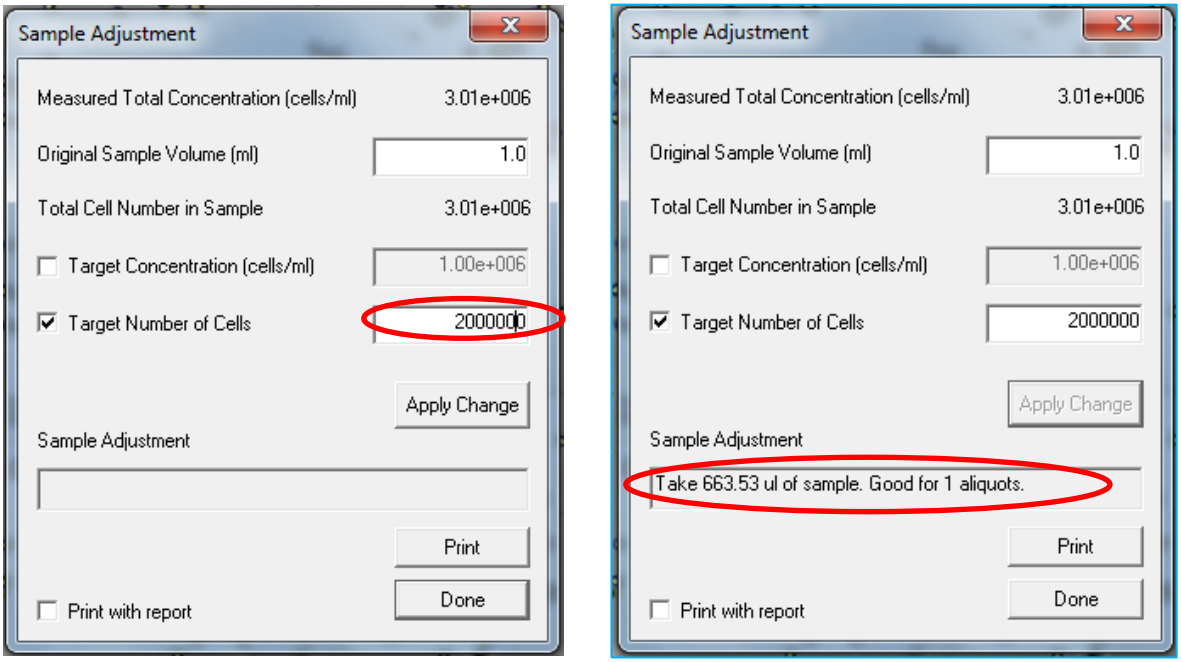
5.0 Using the Sample Adjustment Calculator

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

2. Select **B1 Cell Concentration** and click **Continue**.

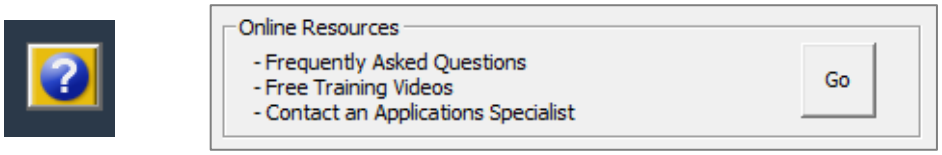


3. Enter the target number of cells. For the Apoptosis assay, it is 2,000,000. Click Apply Change. The required volume of cell sample to be spun down will appear in the Sample Adjustment window.

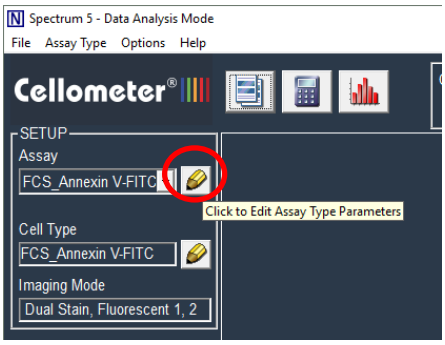


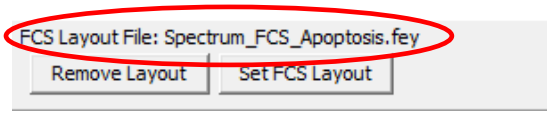
6.0 Importing a New FCS Express Layout

1. Click on the Support icon in the bottom right corner of the Spectrum or Vision CBA 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 or Vision CBA software.



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

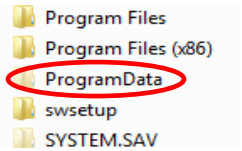




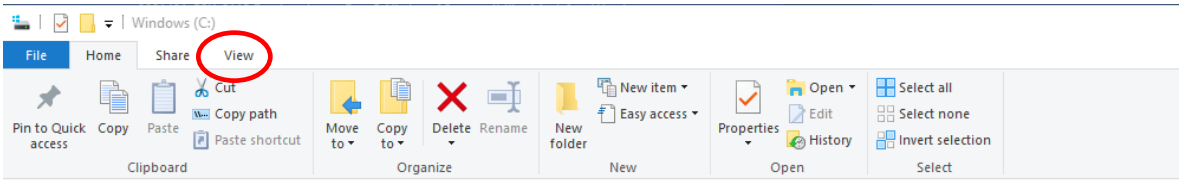
If a newer layout is listed on-line, download the new PI Cell Cycle layout onto your desktop. Copy the layout by right clicking on the layout and selecting copy.

2. Navigate to the START menu and select Computer.

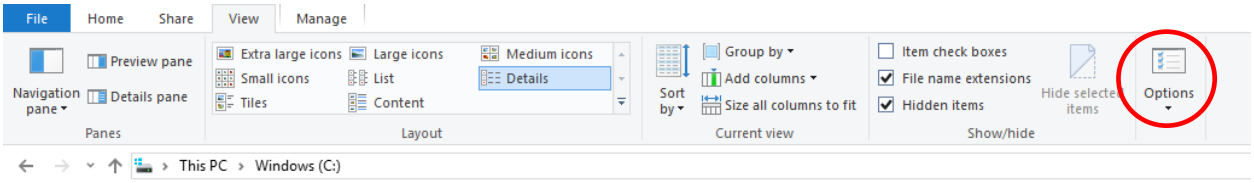
3. Double click on the C: Drive and locate the ProgramData folder (shown at right), then proceed to Step 4. IF the ProgramData folder is not present, it may be hidden. Follow the instructions below to show hidden folders.



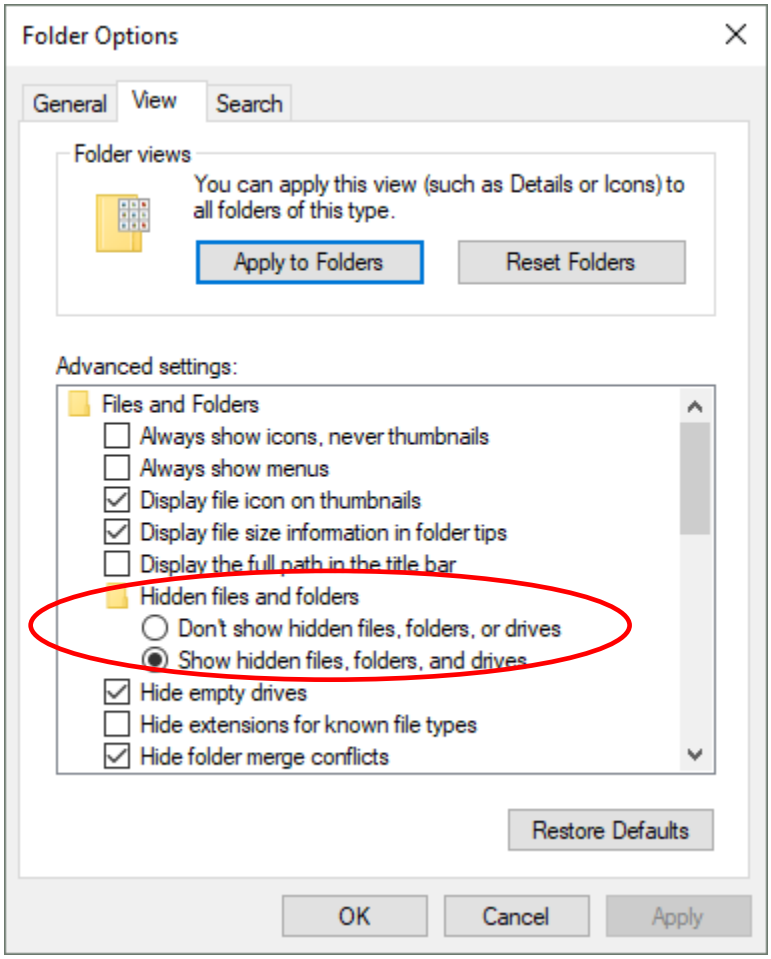
3.1 Click the "View" tab (top of screen).



3.2 Click on "Options" (top left of screen).



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

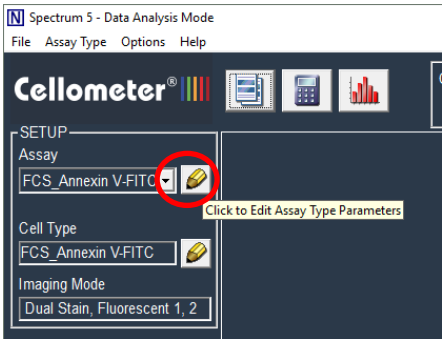


3.3 Under the “Hidden files and folders” file, select “Show hidden files, folders, and drives”

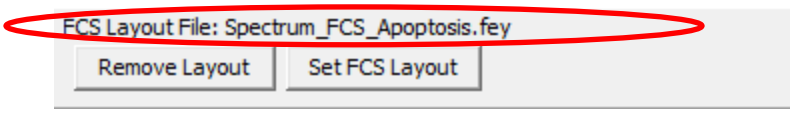
3.4 Click OK.

4. Open ProgramData folder, then open the Nexcelom Spectrum or Vision CBA folder.
5. Open FCS Express Folder, then open the Layouts folder.
6. Copy the Annexin V-FITC / PI layout from your desktop into the folder.

7. 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 or Vision CBA screen.



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



9. Select the Annexin V-FITC / PI layout with the most recent date and click Open, then click Save.

10. 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 or Vision CBA instrument.

7.0 Support

Warranty

This product is for RESEARCH USE ONLY and is not approved for diagnostic or therapeutic use. 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. If no expiration is listed, the warranty is valid for 12 months from the date of product receipt.

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

Ordering Information

For orders shipping to destinations in the United States:

- When ordering with a Purchase order
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  - Email a copy of your order to sales@nexcelom.com
- When ordering with a Credit Card
  - Visit www.shop.nexcelom.com and place your order

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- Contact your local distributor or Nexcelom Representative to place your order