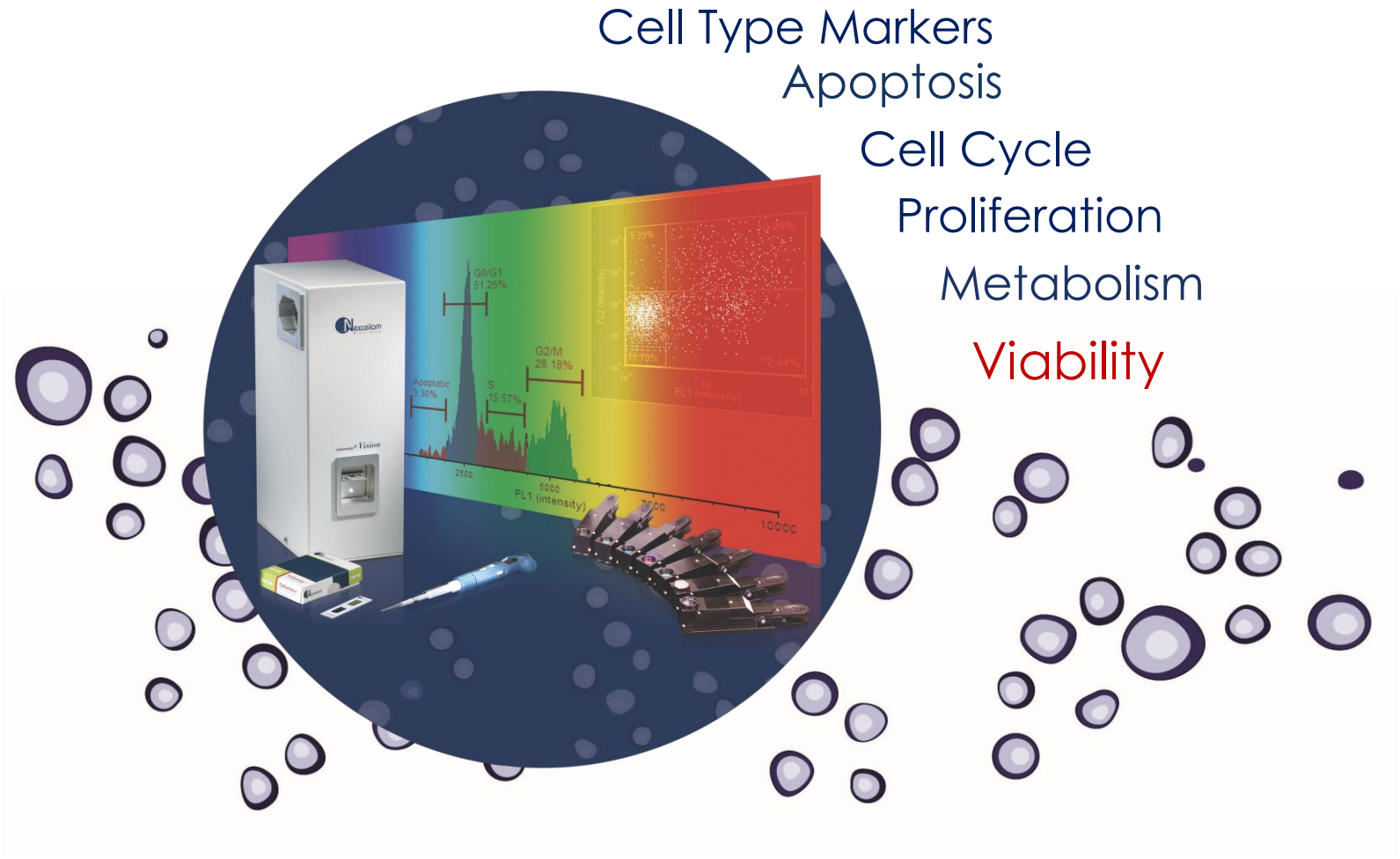
Cellometer®

Calcein-AM/PI Vitality and Viability Kit

Product Number: CSK-0118



Nexcelom Bioscience

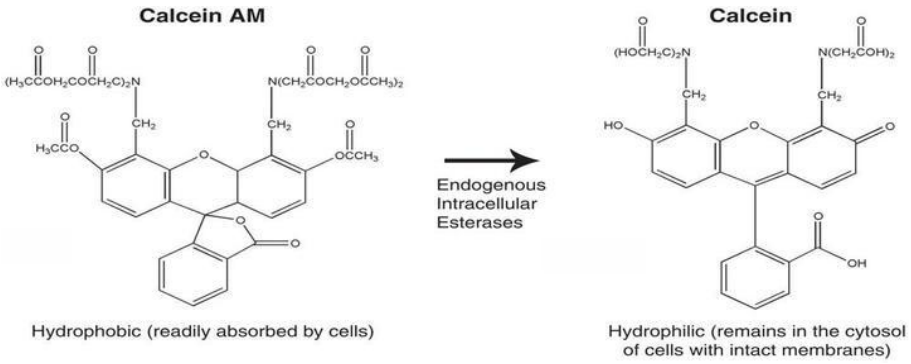
This product is for RESEARCH USE ONLY and is not approved for diagnostic or therapeutic use.

Table of Contents

1.0 Introduction

1.1 Description of Assay

Calcein AM (Calcein acetoxymethyl ester) is a cell permeable, non-fluorescent compound. Upon crossing the cell membrane, Calcein AM is rapidly hydrolyzed by cellular esterases inside live cells. The hydrolysis cleaves the AM group, converting the non-fluorescent Calcein AM to a strongly green fluorescing Calcein. The more hydrophilic Calcein is trapped inside the cell (1). Cells that do not possess active cytoplasmic esterases are unable to convert Calcein AM to Calcein, and therefore do not fluoresce green. This allows for a quick and easy detection of metabolically-active cells in a sample.



Stains such as propidium iodide (PI), 7-AAD, and ethidium bromide (EB), are membrane exclusion dyes that are frequently used to stain non-viable nucleated cells with compromised membranes. Acridine orange freely diffuses across the cell membrane and stains DNA in all nucleated cells. When AO and PI are combined it is possible to determine % viability for nucleated cells. When Calcein AM is used in conjunction with PI, it is possible to determine % vitality / viability based on the number of metabolically-active (green fluorescent) and non-viable (red fluorescent) cells in a sample.

Since Calcein AM does not require DNA binding, it stains all metabolically-active cells and can be used to measure metabolic activity in non-nucleated cells, such as platelets (4). Calcein-AM is also a good alternative for analysis of adipocytes, as the AO dye has shown some non-specific binding of lipid droplets that does not occur with Calcein AM (5). Because Calcein AM is photostable, shows low cytotoxicity, does not affect cellular functions, and requires cellular esterases for conversion to green-fluorescing Calcein, it is a popular stain for the examination of cell vitality and viability. (1,2,3).

1. Braut-Boucher, F. et al. *Journal of Immunological Methods.* Vol. 178, Issue 41 (1995).
2. Luc S. De Clerck. et al. *Journal of Immunological Methods*. Vol. 172, Issue 1, (1994).
3. Parish, CR. *Immunology and Cell Biology*. Vol. 77 (1999)
4. Verheul , HW. et al. *Blood*. Vol. 96 No. 13 (2000)
5. Kilroy, G. et al. *PLoS One*. Vol.4, Issue 9 (2009)

1.2 Materials and Reagents

- Cellometer Calcien-AM Staining Solution (Nexcelom Part# CS1-0119)
- Cellometer Propidium Iodide Staining Solution (Nexcelom Part# CS1-0116)
- dH_20
- Trypsin EDTA (if working with adherent cells)

1.3 Instrument and Software Requirements

- Cellometer Vision or Vision CBA Image Cytometry System
- Cellometer Vision or Vision CBA Software
- Fluorescence Optics Module VB-535-402 and VB-660-502

2.0 Assay Protocol

2.1 Preparation of Adherent Cells for Staining

1. Using 1 x Trypsin-Versene (EDTA), trypsinize cells until they have lifted off the plate (approximately 15 minutes).
2. Use the Cellometer Sample Adjustment Calculator to determine the sample volume required to obtain a concentration of 2-3 x 10^6 cells/ mL.
3. Spin down cells at 1,000 to 2,000 rpm for five minutes.
4. Decant the supernatant and re-suspend cells in 1ml of 1 x PBS or culture media in which the cells were grown.

2.2 Preparation of Calcein AM Reagent

1. Pipette 2 µl Calcein-AM (Nexcelom Part# CS1-0119) into 18 µl of dH_2O. This is now **Calcein-AM Solution A**. Mix by pipetting up and down at least 15 times or vortex.

2.3 Staining Procedure for Cultured Cells

1. Add 5 µl of **Calcein-AM Solution A** and 5 µl of PI Staining Solution to 40 µl of cell sample.
2. Gently pipette the sample up and down ten times, then incubate for 20 min at 37°C in the dark.
3. After the 20 minute incubation, the sample is ready for analysis. Proceed to step 2.5.

2.4 Staining Procedure for Whole Blood, Cord Blood, and Bone Marrow Clinical Samples.

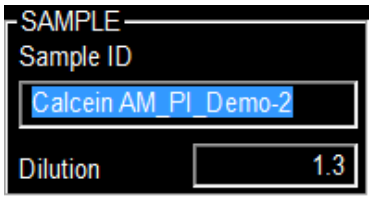
1. Pipette 10 µl of fresh blood sample into 70 µl of 1 x PBS
2. Pipette 40 µl of the diluted blood sample into a new eppendorf tube.
3. Add 5 µl of **Calcein-AM Solution A** and 5 µl of PI Staining Solution to 40 µl of cell sample.
4. Gently pipette the sample up and down ten times, then incubate for 20 min at 37°C in the dark.
5. After the 20 minute incubation, the sample is ready for analysis. Proceed to step 2.5.

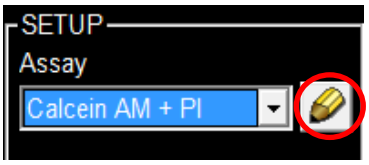
2.5 Data Acquisition

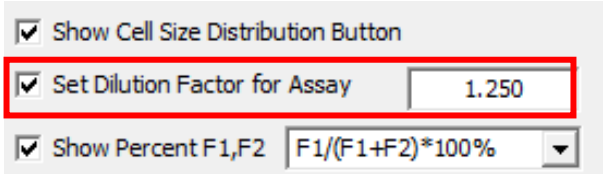
NOTE: Please review the auto save set-up to make sure the acquired data is properly saved (See step 16).

1. Gently mix the cell sample by pipetting up and down at least ten times, then load 20 µL into the Cellometer counting chamber and insert into the Cellometer instrument.
2. Wait 60 seconds for the cells to settle in the chamber
3. Type a name for your sample into the Sample ID text box

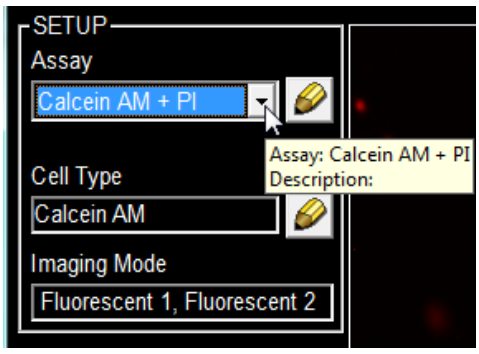
4. Verify that the default dilution factor for the Calcein AM + PI (For cultured cells) assay is 1.25 by clicking on the pencil icon and locating the "Set Dilution Factor for Assay" in the dialog pop-up screen. The default dilution factor for the Calcein AM + PI CS (**C**linical **S**amples) assay is 10. If a higher sample dilution was performed in step 2.4, or the cell culture sample was pre-diluted in step 2.3, adjust the dilution factor accordingly. Please note that dilution factor on the main screen is rounded up for display purposes and will read as 1.3.





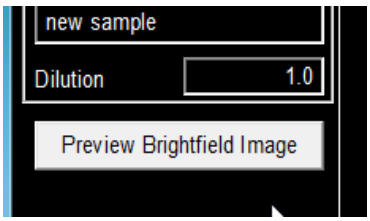


5. Select the **Calcein AM + PI or Calcein AM + CS Assay** from the Assay drop-down menu in the upper left corner of the main Vision CBA software screen. If this assay is not present in your drop-down menu, import the Calcein AM + PI assay files using the instructions in section 5.0.

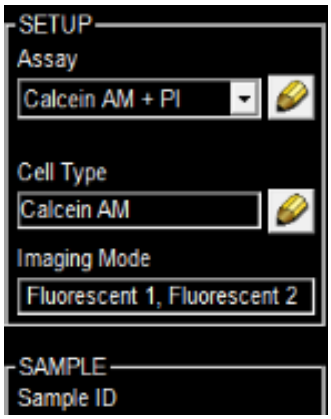


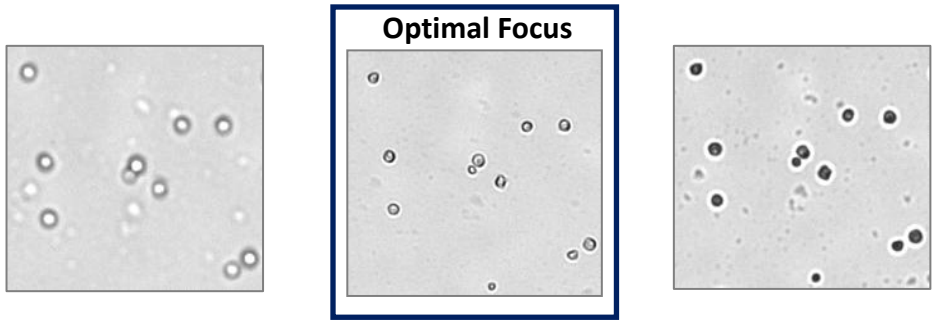
6. Unless you are testing clinical samples, the Calcein AM + PI assay should be run with the default software settings. If you suspect that the settings may have been changed, review the default software settings in section 4.0. To update the Calcein AM + PI assay to the Calcein AM + PI CS assay for whole blood, cord blood, and bone marrow clinical samples see section 4.3.

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



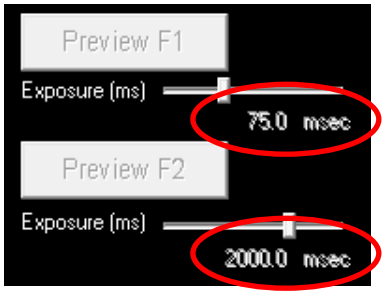
8. Turn the focus knob and adjust focus for the bright field image. Cells in focus for the Calcein-AM/PI assay will have a bright center and dark outline. There should be a crisp contrast between background and the cell membrane. See Focus Guide below.





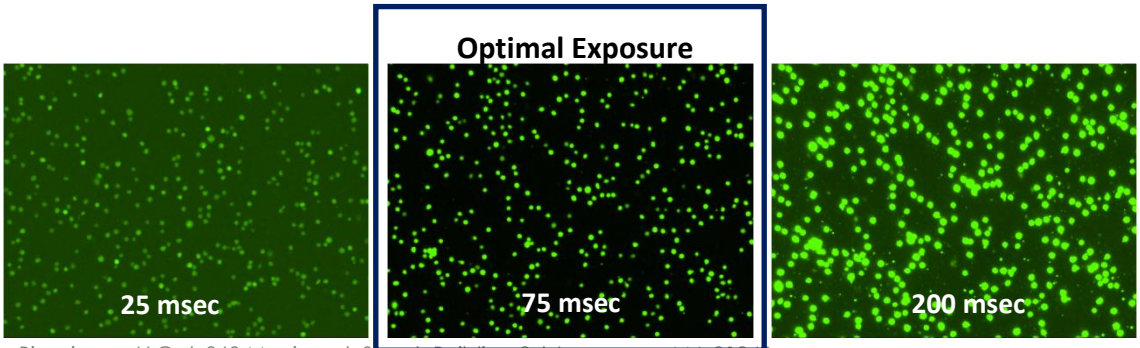
9. Click Stop Preview

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

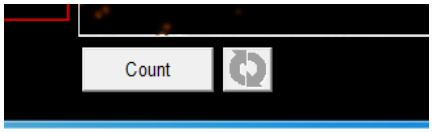


For the Calcein AM + PI Assay the default exposure time is 75 milliseconds (msec). For the Calcein AM + PI CS Assay the optimal exposure is 200 milliseconds (msec). Optimal exposure time will generate a bright image with well-defined fluorescent spots.

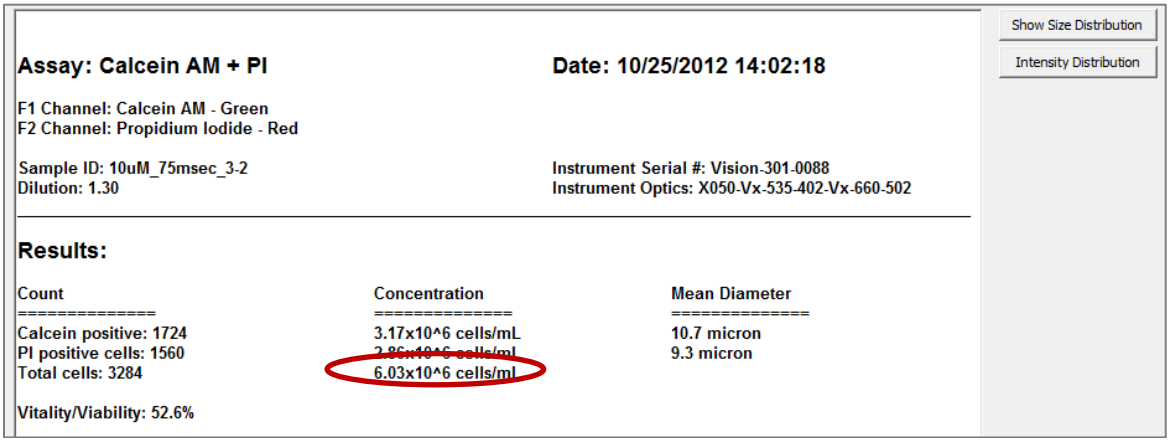
Under-exposure will yield dark images with weak spots, like the 25 msec image below, with insufficient fluorescent signal. Over-exposure will yield images that are too bright with fluorescent spots that are large and sometimes overlapping, as shown in the 200 ms image below. Spots are also less distinct from background.



11. Click Preview F2. The F2 (PI) channel should be set to 2000 msec exposure for the Calcein AM + PI assay and 4000 msec for the Calcein AM + PI SC assay.
12. Click the Count button at the bottom of the screen



13. When counting is complete, an initial Results Table will appear on the screen. For optimal counting results, the total cell concentration should be between 3 x 10^5 cells/mL and 1 x 10^7 cells/mL (see circled value in the report below). Recount a concentrated or diluted cell sample if necessary.

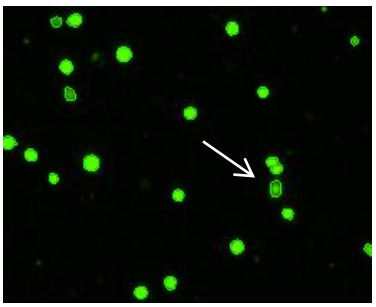


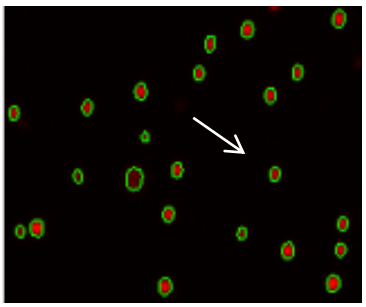
The default report displays:

- **Calcein-positive (metabolically-active) cells** counted, cell concentration, and mean cell diameter
- **PI-positive (non-viable) cells** counted, cell concentration, and mean cell diameter
- **Total cells** counted and total cell concentration
- **% Vitality/Viability** for the cell sample: Calcein-positive cells / (Calcein-positive cells + PI-positive cells)

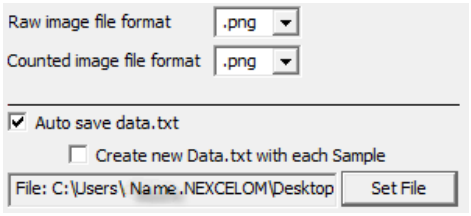
14. Click the **Close** button at the bottom right corner of the Counting Results table. Select the F1 Image and check Counted at the right-hand side of the screen.

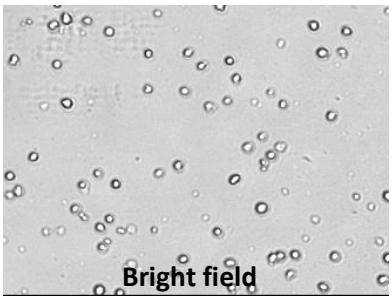
15. Review the counted image to confirm that the Calcein-positive cells are being counted correctly. Individual cells within clumps should be circled in green, indicating that they are being counted individually. Click F2 Image to confirm that the PI stained cells are being counted correctly as well. If cells are not being counted correctly, please contact Nexcelom Technical Support for assistance with optimization of counting parameters (see Section 3.0).

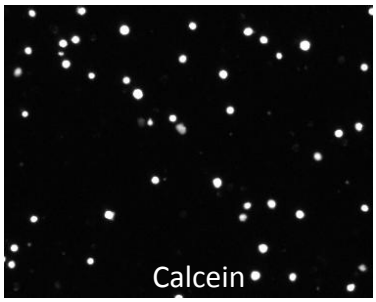


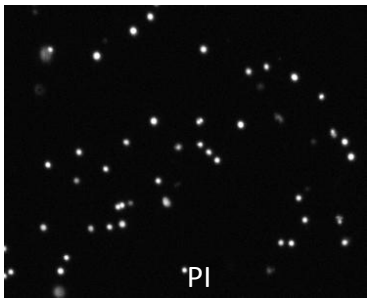


16. To view bright field and fluorescent cell images that have been saved, open the image folder where the data has been saved. Bright field and fluorescent images are captured for the Calcein AM / PI assay and may be saved automatically. Saved **Raw** images may be opened in the Cellometer software for re-analysis. Images are only saved if "Auto save data.txt" is selected in the save options menu. The options menu is found by selecting options, then "save options" at the top of the main screen.









A screen-capture software may be used to save both uncounted and counted colorized fluorescent images for presentation and publication.

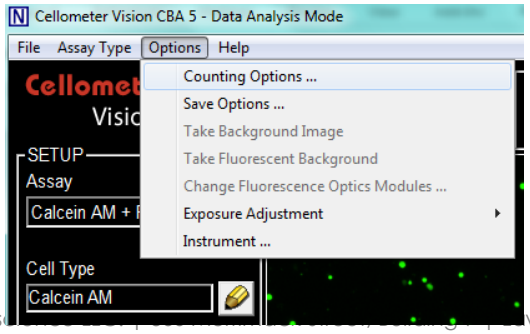
3.0 Technical Support

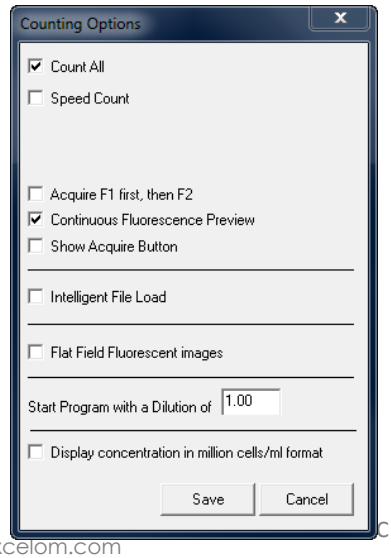
Nexcelom Technical Support is available from 9am to 5pm EST.
E-mail: support@nexcelom.com
Phone: 978-327-5340

4.0 Software Settings

4.1 Review Calcein AM/PI Counting Options Screen

Click on the Options Page and select Counting Options. If you suspect that default settings may have been changed, verify that all selections on the instrument screen match the default settings below.

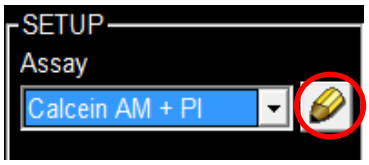




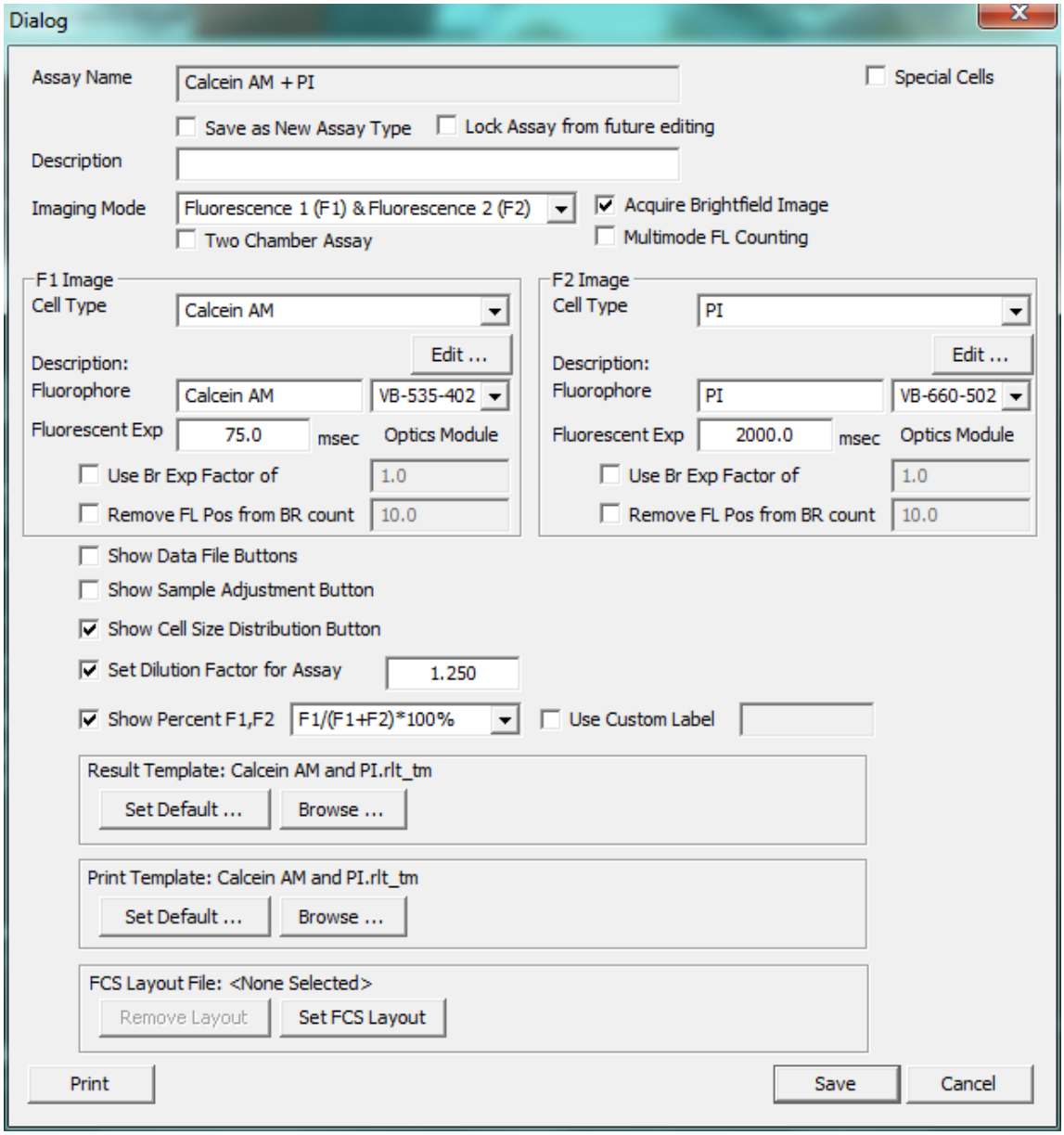
4.2 Default (Calcein AM / PI) Software Settings

4.2.1 Check Dialog Screen Settings

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

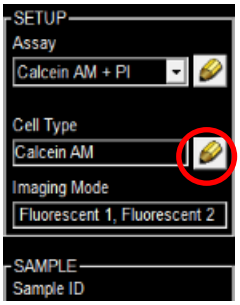


2. Verify that all selections on the instrument screen match the default settings below.

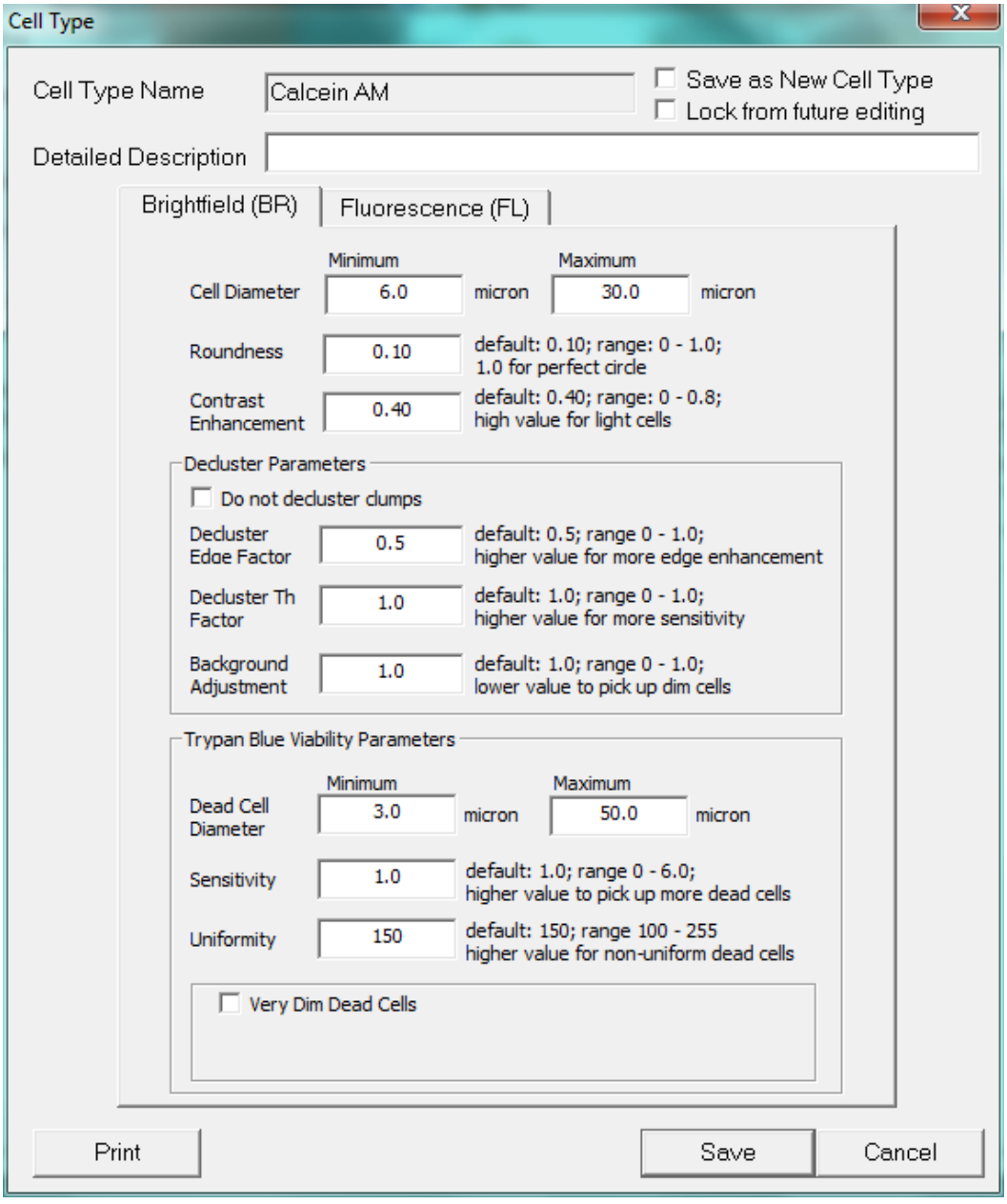


4.2.2 Check Cell Type Settings

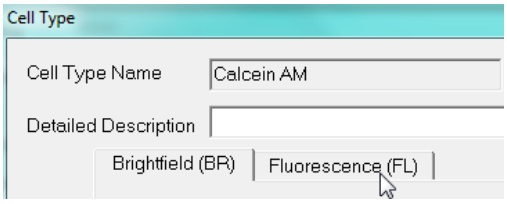
1. Click on the pencil icon under Cell Type on the main Vision CBA software screen to check the Calcein AM cell type settings.



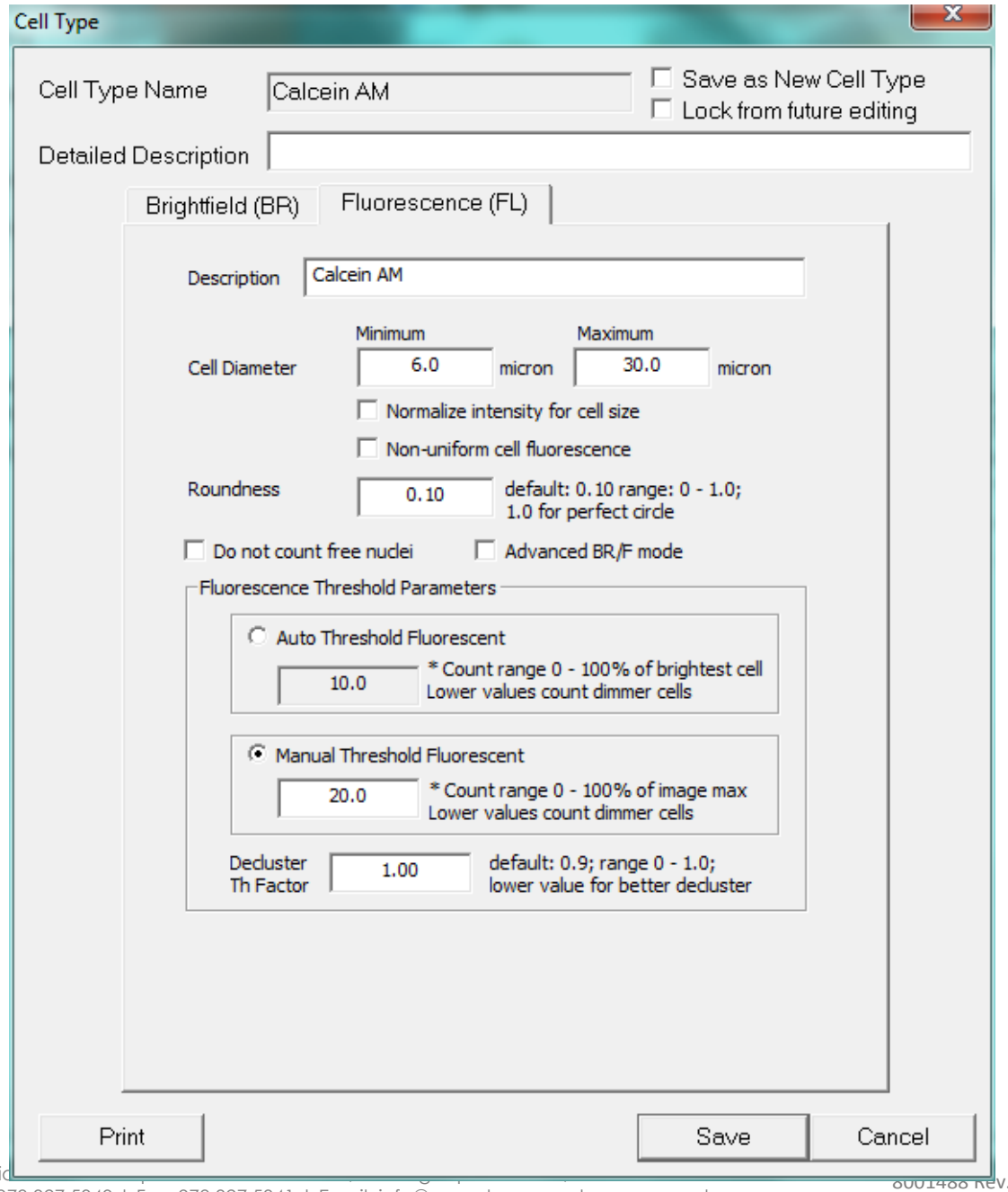
2. Verify that all selections for the bright field (BR) tab on the instrument screen match the default settings below.



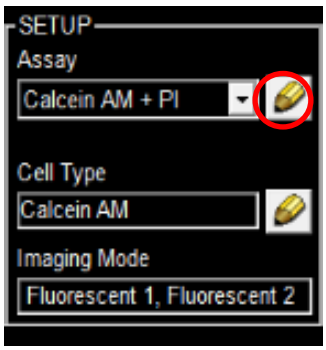
3. Click on the Fluorescence (FL) tab.

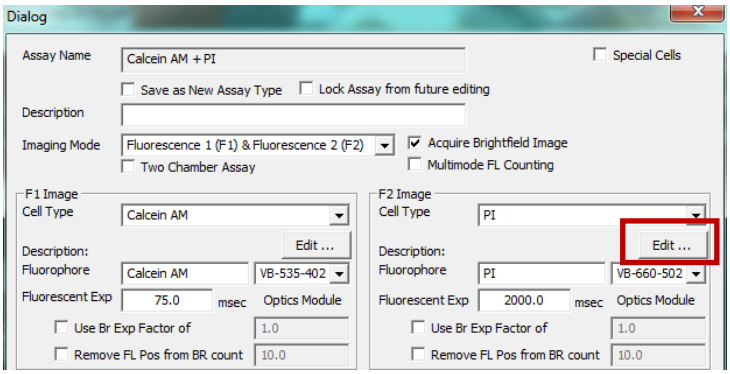


4. Verify that all selections for the Fluorescence (FL) tab on the instrument screen match the default settings below.

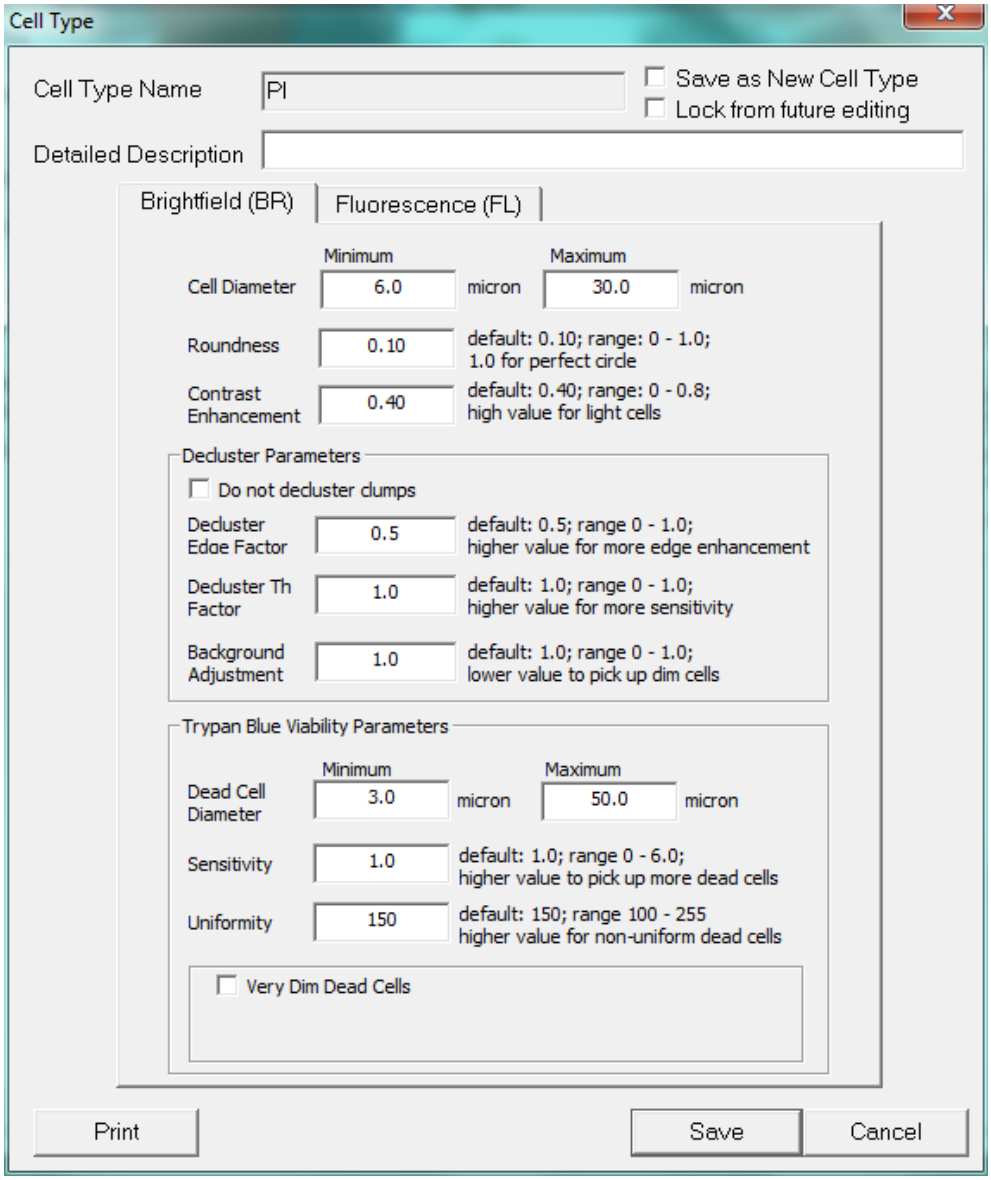


5. Click on the pencil icon under Assay on the main Vision CBA screen, then click the Edit button on the right hand side of the dialog box.

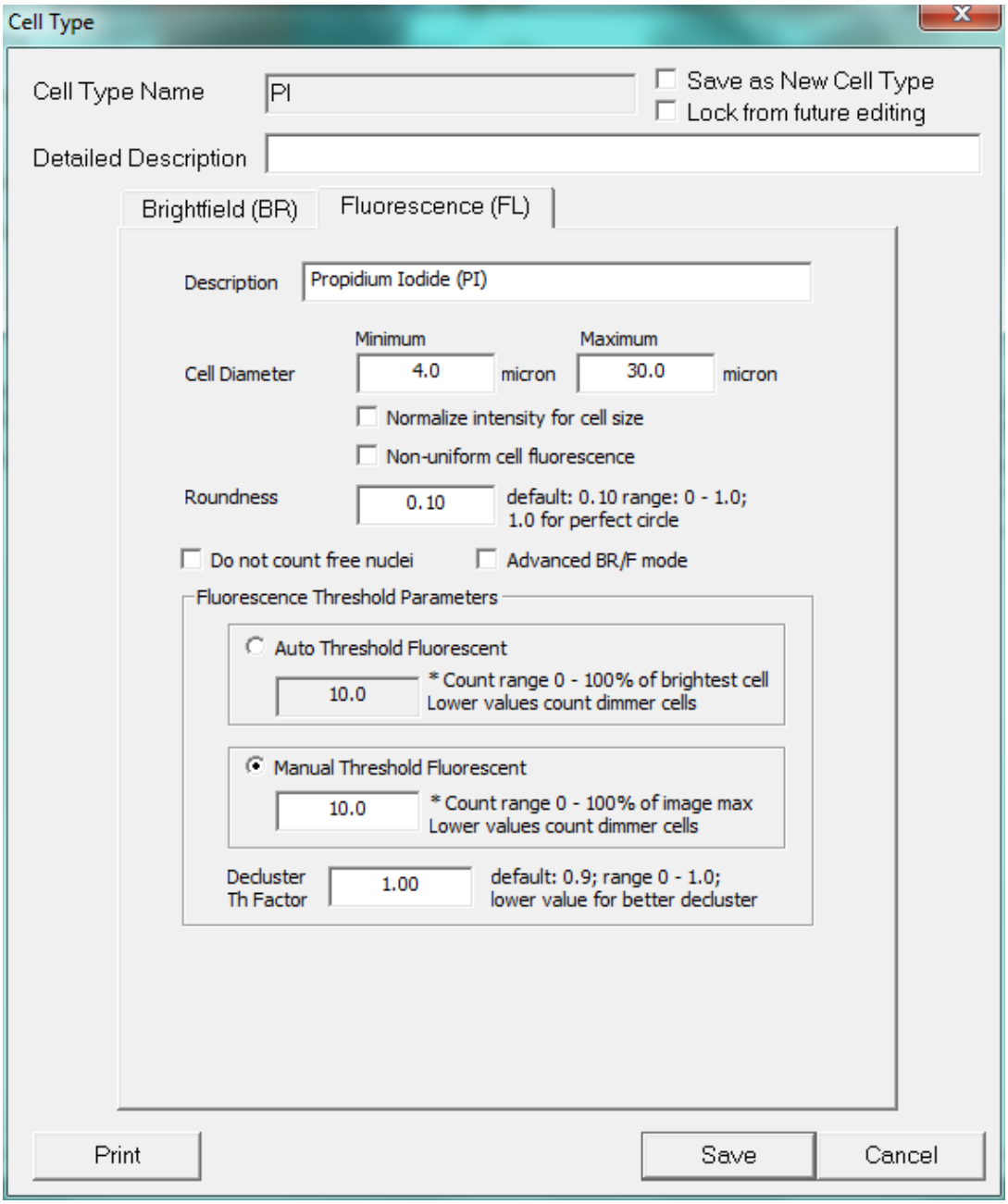




6. Verify the Brightfield (BR) settings for the F2 image (PI).

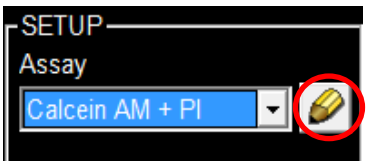


7. Verify the fluorescent settings for the F2 image, by selecting the Fluorescence (FL) tab.



4.3 Software Settings for Whole Blood, Cord Blood, and Bone Marrow Clinical Samples (Calcein AM + PI CS)

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



2. Adjust the current settings.

   2.1 Check the Save as New Assay Type box

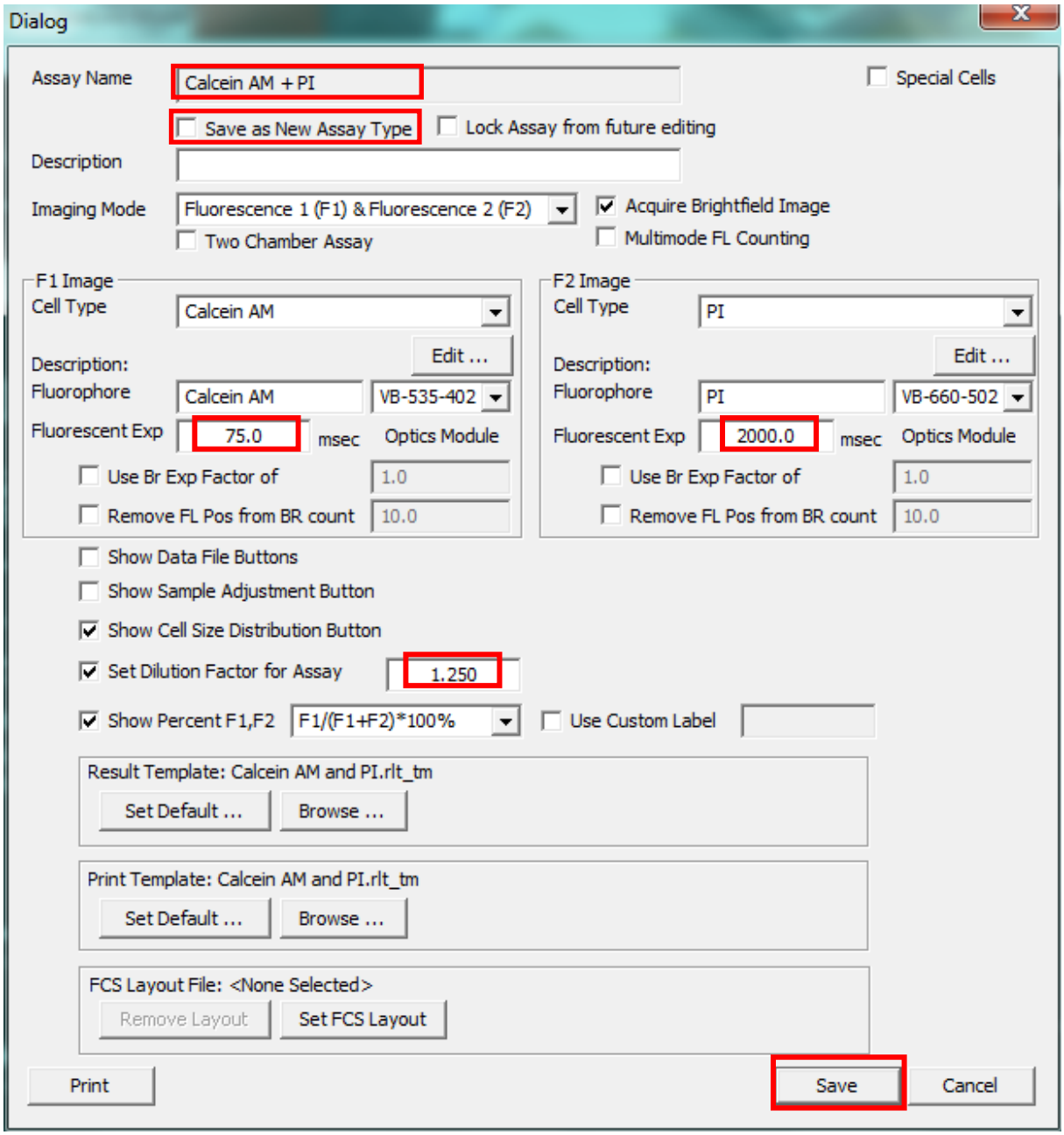
   2.2 Rename Assay to Calcein AM + PI CS

   2.3 Change the Fluorescent Exp for Calcein AM from 75.0 to 200.0

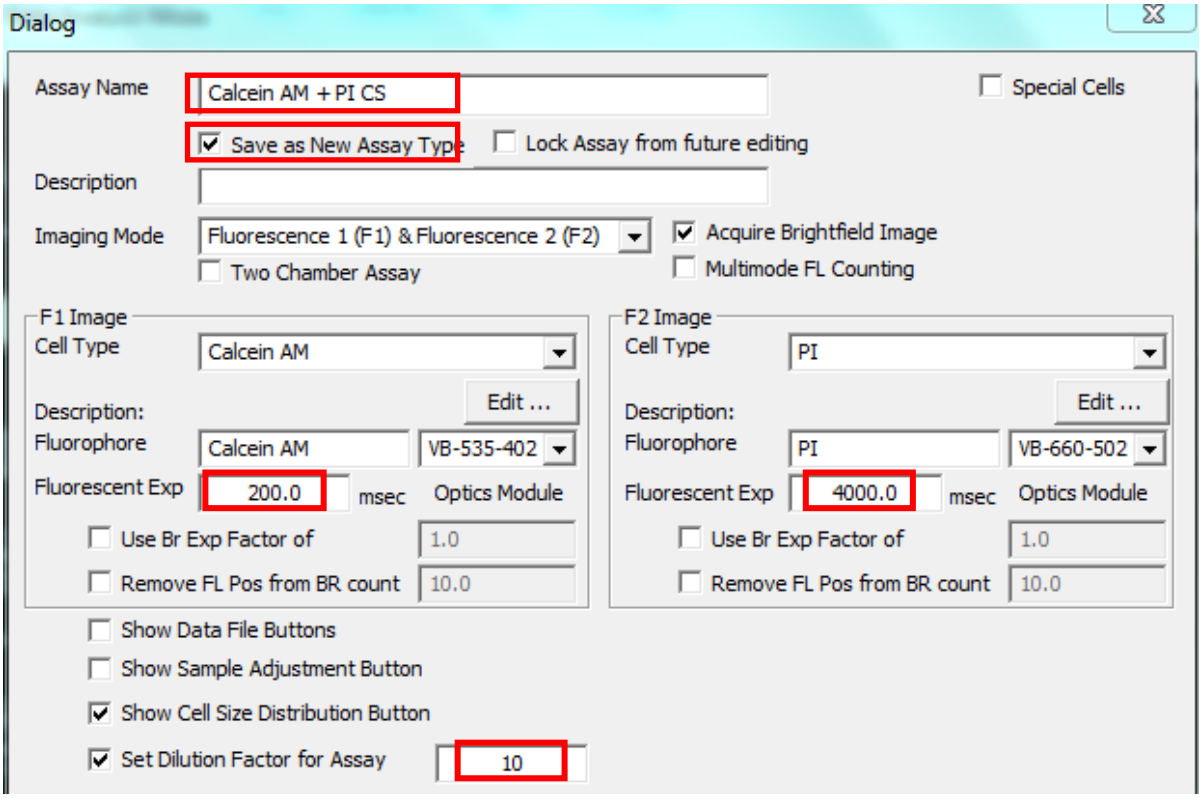
   2.4 Change the Fluorescent Exp for PI from 2000.0 to 4000.0

   2.5 Change the Dilution Factor for Assay from 1.25 to 10

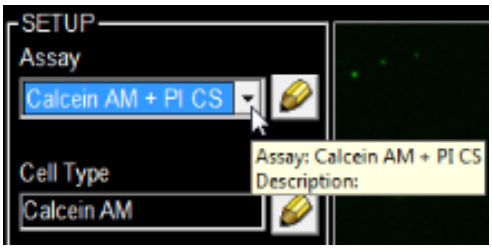
   2.6 Click Save



3. The updated setting should match those shown below.

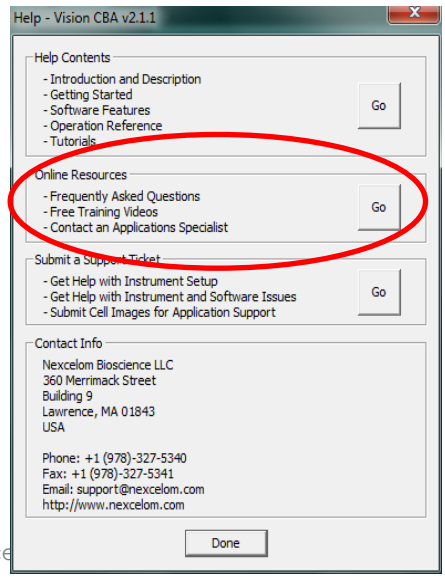


4. You may now select the Calcein AM + PI CS assay from the drop down menu and proceed with data aquisition in section 2.5.



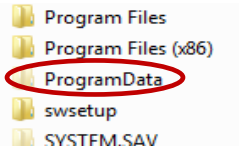
5.0 Importing a New Cell Type, Assay Type, and Templates

1. Click the question mark in the right hand corner to access the help menu.
2. Click on the "Go" button in the "Online Resources" section. This will automatically load the Cellometer Vision CBA Online Resources webpage.
3. Under the "Assay Files" tab, locate and download the Calcein AM + PI files onto the desktop.

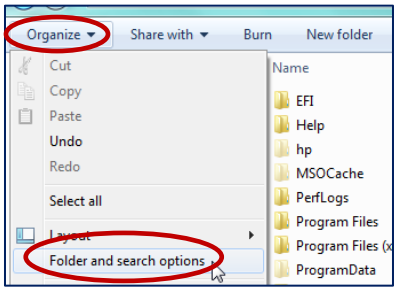


5.1 Import Result and Print templates

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



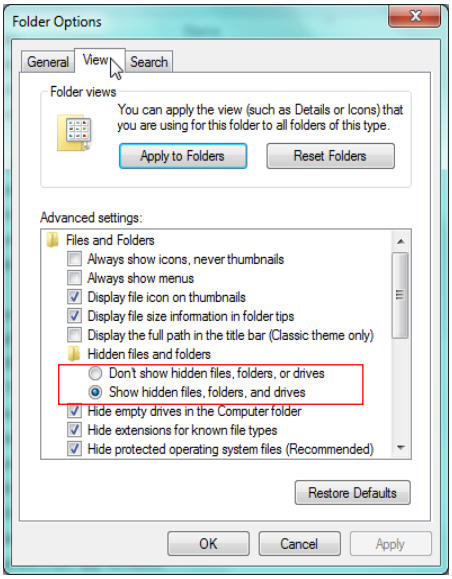
2.1 Click on “Organize” (top left of screen) and select “Folder and search options”



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

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

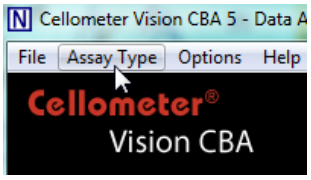
2.4 Click OK.

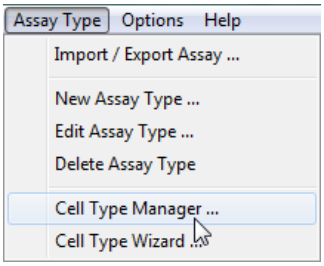


3. Open ProgramData folder, then open the Nexcelom_VisionCBA folder.
4. Open the Template folder. Copy the result template (Calcein AM and PI.rlt_tm) and print template (Calcein AM and PI .prn_tm) files from the new folder on the desktop and paste them into the Template folder.

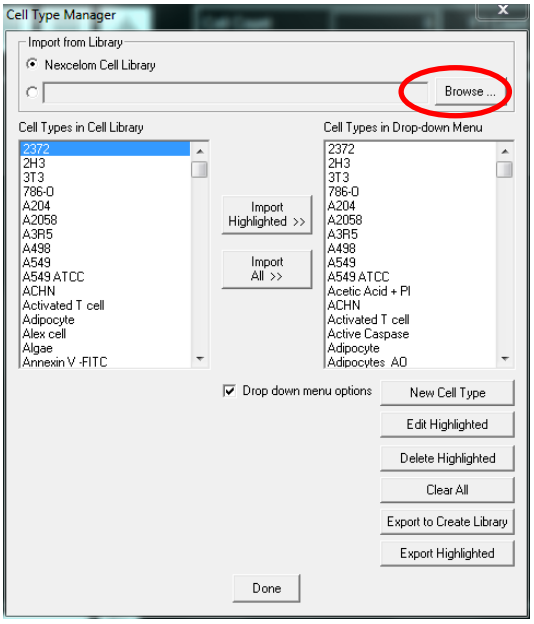
5.2 Import Cell Type

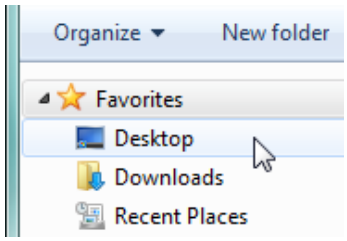
1. Locate and click on the “Assay Type” at the top of the screen. Followed by Cell Type Manager.



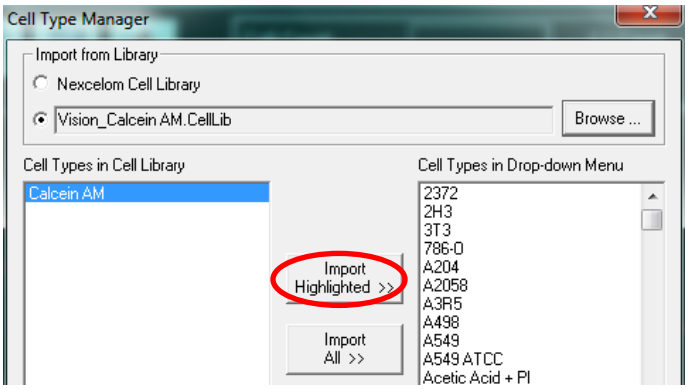


2. Once the Cell Type Manager appears, click on Browse, navigate to the desktop and locate the "Vision_Calcein AM.CellLib" file that has been downloaded onto your desktop.





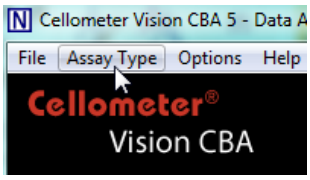
3. Select Vision_Calcein AM.CellLib on the desktop, and click Import Highlighted.

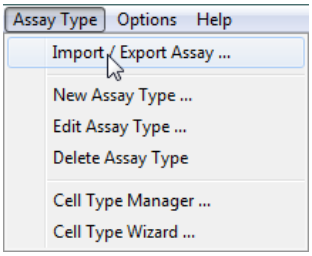


4. Repeat step 1-4 to download "Vision_PI.CellLib"

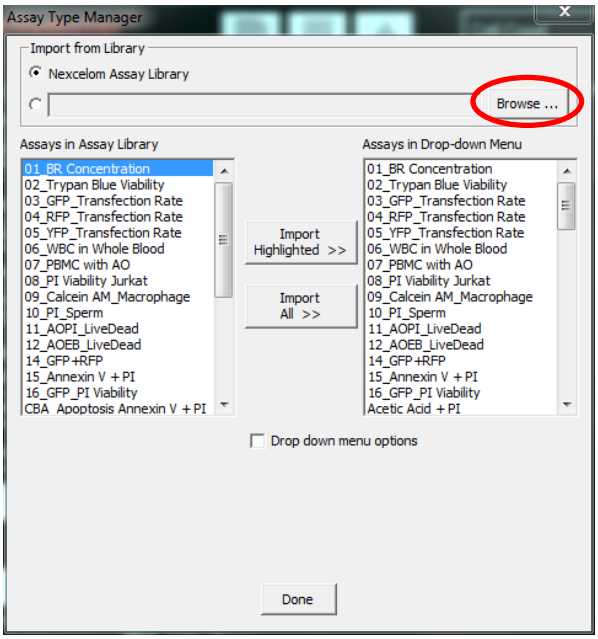
5.3 Import Assay Type

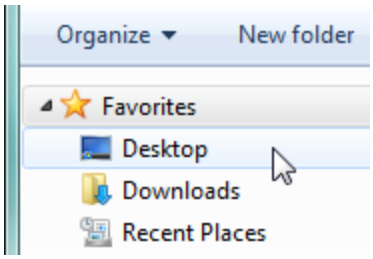
1. Locate and click on the "Assay Type" at the top of the screen. Followed by Import / Export Assay.



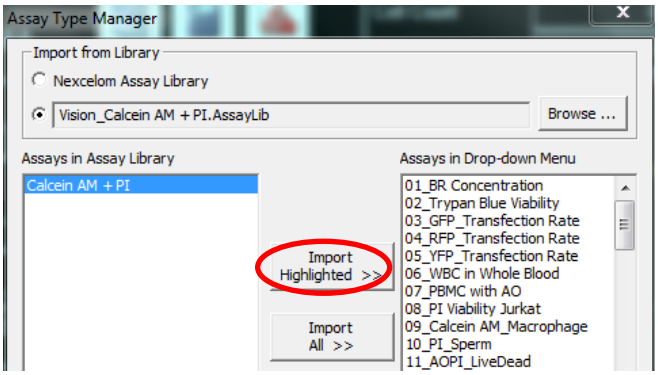


2. Once the Assay Type Manager appears, click on Browse, navigate to the desktop and locate the "Vision_Calcein AM.CellLib" file that has been downloaded onto your desktop.





3. Select "Vision_Calcein AM + PI.AssayLib" on the desktop, and click Import Highlighted.



4. This assay is now available for use. See section 2.3.4 to select and use assay.