PRODUCT INSERT

Instrument Compatibility: Cellometer™ Spectrum, K2, and Ascend
Cellaca™ MX and PLX

ViaStain™ Calcein-AM/PI Cell Viability Kit

Part number: CSK-0118

Test number: 100 Tests

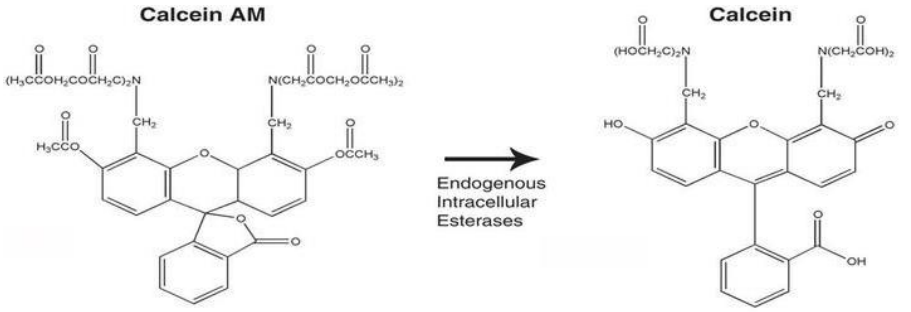
Storage: 2 to 8 °C (Propidium Iodide)
-16 to -24 °C (Calcein AM)

Table of Contents

1 Introduction

1.1 Assay Description

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 bright green fluorescing calcein. The newly created calcein is more hydrophilic and becomes trapped inside the cells with intact membranes (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 percentage viability for nucleated cells. When Calcein AM is used in conjunction with PI, it is possible to determine the percentage of 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 (2). 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 (3). 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. (1,4,5).

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

1.2 Materials Included

Reagents	Volume	Catalog number	Number of Tests
Propidium Iodide Staining Solution (Component A) **Calcein AM Staining Solution** (Component B)	500 µL 200 µL	CSK-0118	100 Tests

1.3 Materials Required

- Microcentrifuge tube
- Pipette and pipette tips
- Distilled H_2O (dH_20)
- Phosphate Buffered Saline (1X PBS) ***OR*** cell culture media
- Trypsin EDTA (if working with adherent cells)
- Disposable counting slide or plate, depending on cell counter/image cytometer:
    - Cellometer counting slide (Cat. # SD100 or PD100) for Cellometer K2 and Spectrum
    - Ascend counting slide (Cat. # ASD-CHM3 or ASD-CHM8) for Cellometer Ascend
    - Counting plate (Cat. # CHM24-A100 or CHM24-B100) for Cellaca systems

1.4 Compatible Instruments and Software Requirements

- Cellometer K2 with Cellometer K2 or Cellometer Matrix™ software
- Cellometer Spectrum with Fluorescence Optics Modules S1-534-470 and S1-655-527 with Cellometer Spectrum Software
- Cellaca MX High-throughput Cell Counter with Matrix™ software
- Cellaca PLX High-throughput Image Cytometer with Matrix™ software

2 Assay Protocol

2.1 Cell Preparation for Staining

2.1.1 Suspension Cells

1. Prepare cell sample at 2-3 x 10^6 cells/mL.
    a. Dilute or concentrate as needed.

2.1.2 Adherent Cells

1. Using 1X Trypsin EDTA, trypsinize cells until they have lifted off the plate (~15 min).
2. Prepare cell sample at 2-3 x 10^6 cells/mL.
    a. If available, use the Cellometer Sample Adjustment Calculator to determine the sample volume required to obtain desired concentration.
3. Centrifuge cells at 300 x g for 5 min.
4. Remove the supernatant and re-suspend cells in 1 mL of 1X PBS or cell culture media.

2.1.3 Biological Samples

1. If using whole blood, cord blood, or bone marrow no additional preparation is required.

2.2 Preparation of Calcein AM Working Solution

1. Pipette 2 µL Calcein AM (**Component A**) into 18 µL of dH_2O to prepare a Calcein AM Working Solution.
2. Mix by pipetting up and down at least 15 times or vortexing.
    ***NOTE**: Solution is stable up to 3 h.*

2.3 (*optional)* Generation of a Calcein AM Negative, PI Positive Control

1. Heat-kill cells by placing in a boiling water bath for 10 min.
2. Allow them to cool to room temperature.
3. Mix live and heat killed dead cells at a desired ratio or use the control as is.

    ***NOTE**: If using the control as is on the Cellometer Ascend or Cellaca MX/PLX, verify that it is set to focus in brightfield or FL2 (PI channel), since the green calcein signal (FL1 channel) will be absent, and the instrument will not have anything to focus on.*

2.4 Staining Procedure

When staining cultured cells (suspension or adherent), prepare samples according to Section 2.4.1 below. For biological samples, such as whole blood, cord blood, or bone marrow, prepare according to Section 2.4.2.

2.4.1 Staining Procedure for Cultured Cells

1. Add 40 µL of cell sample to a microcentrifuge tube.
2. Add 5 µL of Calcein-AM Working Solution and 5 µL of PI (**Component B**) to the tube containing cells.
3. Gently pipette the sample up and down 10 times.
4. Incubate for 20 min at 37 °C in the dark.
5. Proceed to imaging and data acquisition according to cell counter being used:
    a. Cellometer K2: Section 3
    b. Cellometer Ascend: Section 4
    c. Cellaca MX or PLX: Section 5
    d. Cellometer Spectrum: Section 6

2.4.2 Staining Procedure for Biological Samples

1. Pipette 10 µL of fresh blood sample into 70 µL of 1X PBS.
2. Pipette 40 µL of the diluted blood sample into a new microcentrifuge tube.
3. Add 5 µL of Calcein AM Working Solution and 5 µL of PI (**Component B**) to the cell sample.
4. Gently pipette the sample up and down 10 times.
5. Incubate for 20 min at 37 °C in the dark.
6. Proceed to imaging and data acquisition according to cell counter being used:
    a. Cellometer K2: Section 3
    b. Cellometer Ascend: Section 4
    c. Cellaca MX or PLX: Section 5
    d. Cellometer Spectrum: Section 6

3 Cellometer K2 Imaging and Data Acquisition

1. Gently mix the sample by pipetting up and down at least 10 times.
2. Pipette 20 µL of each sample into a counting chamber.
   ***NOTE**: If using SD100 slides, peel plastic film off both sides before loading.*
3. Select the appropriate assay for Calcein AM/PI vitality measurement.
   ***NOTE**: If assay is unavailable, please contact support at USCAN.service@revvity.com.*
4. Verify that the dilution factor is set to 1.25.
5. Insert slide into the Cellometer K2 instrument.
6. Preview brightfield and fluorescent images. Focus, if necessary.
7. Click **Count**.

4 Cellometer Ascend Imaging and Data Acquisition

1. Gently mix the sample by pipetting up and down at least 10 times.
2. Pipette 20 µL of each sample into a counting chamber.
   ***NOTE 1**: Peel plastic film off both sides before loading.*
   ***NOTE 2**: Loading volume for Cellometer Ascend 8-chamber slides is 10 µL.*
3. Select the appropriate assay for Calcein AM/PI vitality measurement in the Matrix software.
4. Verify that the dilution factor is set to 1.25.
5. Insert slide into the Cellometer Ascend to **Preview** or **Count**.
   a. If **Skip Preview** is set to **No**, software will go into preview mode where fluorescent exposures can be adjusted.
   b. If **Skip Preview** is set to **Yes**, software will immediately proceed to counting sample.

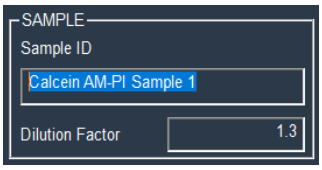
5 Cellaca Imaging and Data Acquisition

1. Gently mix the sample by pipetting up and down at least 10 times.
2. Pipette 50 µL of each sample into a loading well of the counting plate.
3. Load plate into the Cellaca instrument with A1 in the top left corner.
4. Select the appropriate assay for Calcein AM/PI vitality measurement in the Matrix software.
5. Verify that the dilution factor is set to 1.25.
6. Click **Preview.**
7. Click **Auto Focus**, if cells are not in focus.
8. Preview brightfield and fluorescent images and adjust fluorescent exposures, as needed.
9. Click **Count**.

6 Cellometer Spectrum Imaging and Data Acquisition

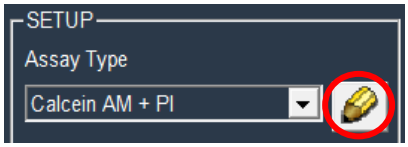
Prior to data acquisition it is always advised to review the auto save set-up to make sure the acquired data is properly saved (see Section 7.2).

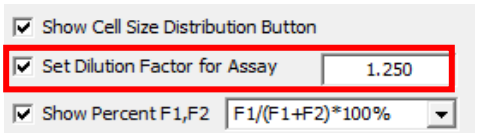
1. Gently mix the cell sample by pipetting up and down at least 10 times.
2. Load 20 µL of stained sample into a Cellometer counting chamber.
3. Insert the slide into the Cellometer instrument.
4. Wait 60 sec for the cells to settle in the chamber.
5. Type a name for your sample into the Sample ID text box.
6. Verify the default dilution factor:
    a. For cultured cells, the dilution is 1.25.
        ***NOTE**: The dilution gets rounded up on the main software screen and is displayed as 1.3.*
    b. For Biological Samples (Calcein AM + PI **CS**) the dilution is 10.



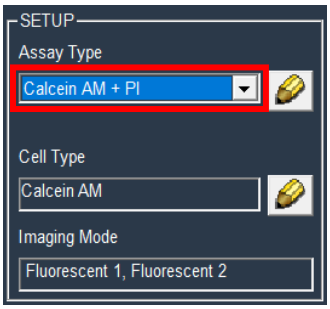
***NOTE 1**: If additional dilutions were performed during sample preparation, adjust the dilution factor accordingly.*

***NOTE 2**: To adjust the dilution factor, click on the pencil icon, locate the "Set Dilution Factor for Assay" in the dialog pop-up screen, and type in the value.*

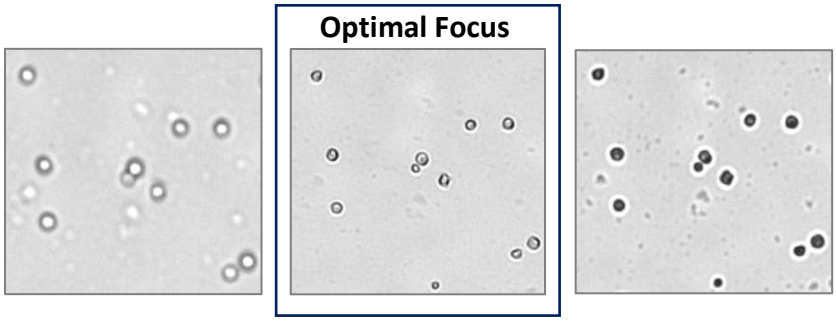




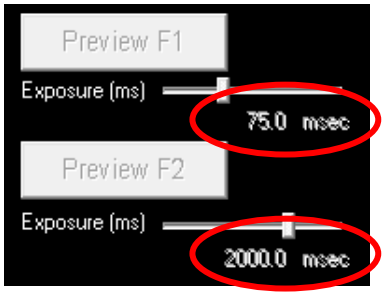
7. Select **Calcein AM + PI** or **Calcein AM + PI_CS** from the Assay drop-down menu.
    a. If assay is not present in the drop-down menu, import it (see Section 8.2).
    b. Assay should be run using default settings (see Section 7.3).
    c. Section 7.4 shows user how to create Calcein AM + PI for Biological Samples (Calcein AM + PI_CS) using the Calcein AM + PI assay.

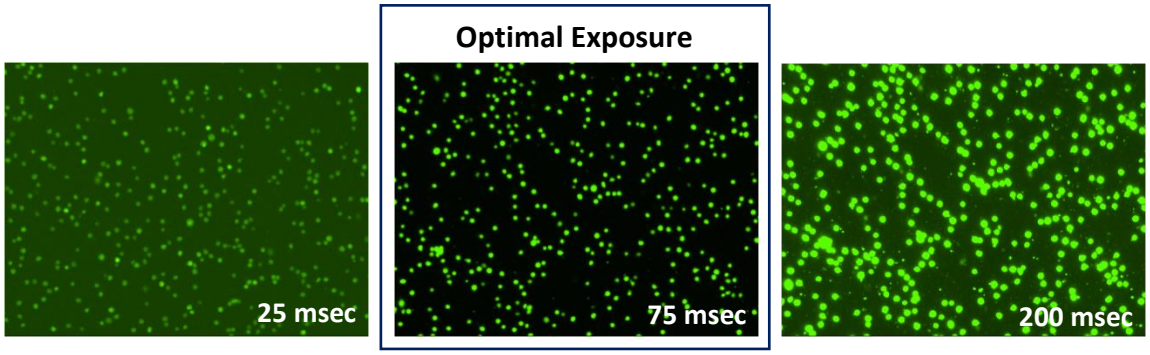


8. Click Preview B1 at the bottom left of the main software screen.
9. Adjust focus for the bright field image using the focus knob.
    a. Cells in focus will have a bright center and dark outline with a crisp contrast between background and the cell membrane. See Focus Guide below.



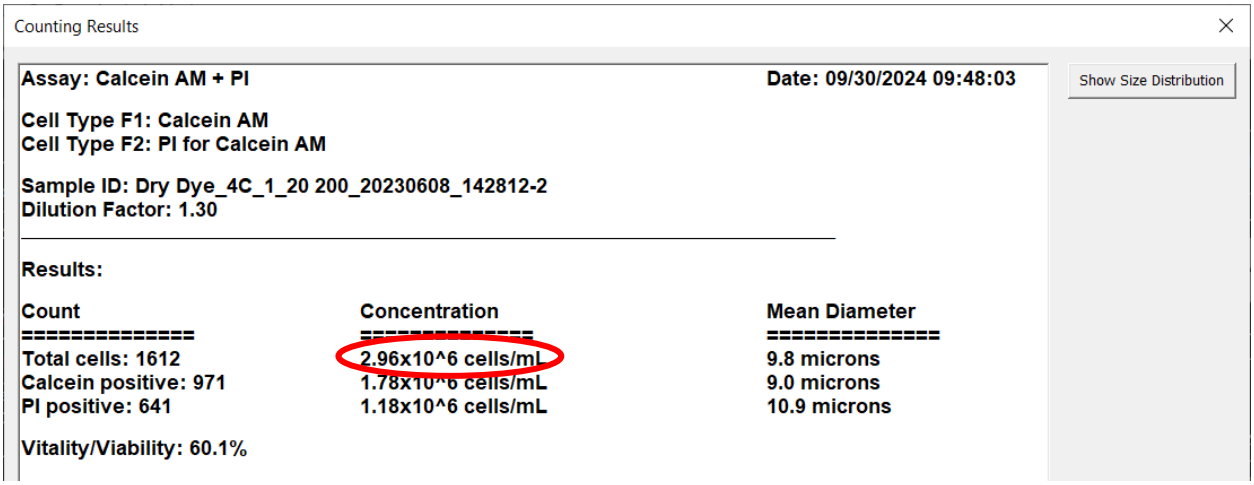
10. Click Stop Preview.
11. Click Preview F1 button and verify that the fluorescence signal displays as 100% of range.
    a. Default exposure time for F1 (Calcein AM) is 75 msec for cultured samples and 200 msec for biological samples.
        i. Optimal exposure time will generate a bright image with well-defined fluorescent spots (75 msec; see focus guide below).
        ii. Under-exposure will yield dark images with weak spots demonstrating insufficient fluorescent signal (25 msec).
        iii. Over-exposure will yield images that are too bright with fluorescent spots that are large and sometimes overlapping (200 msec). Spots are also less distinct from background.





12. Click Preview F2.
    a. Default exposure time for F2 (PI) is 2,000 msec for cultured samples and 4,000 msec for biological samples.
13. Click the Count button at the bottom of the screen.
14. When counting is complete, the Results Table will appear on the screen.
    a. The total cell concentration should be between 3 x 10^5 and 1 x 10^7 cells/mL (see example in the report below). Recount a concentrated or diluted cell sample as needed.

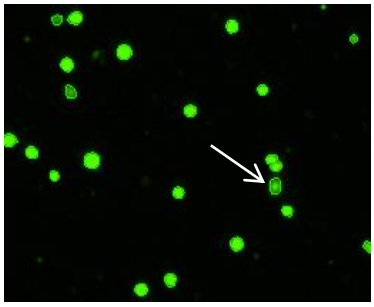
The default report displays:



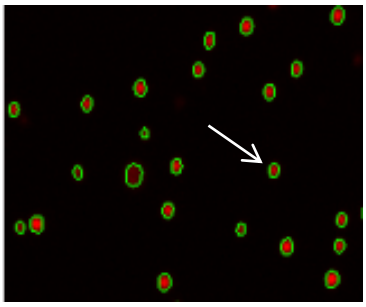
- **Calcein positive** (metabolically-active) cell counts, concentration, and mean cell diameter.

- **PI-positive** (non-viable) cell counts, concentration, and mean cell diameter.
- **Total cell** counts, concentration, and mean cell diameter.
- **Vitality/Viability %** of the cell sample: $\frac{Calcein\ positive\ cells}{Calcein\ positive\ cells + PI\ positive\ cells}$

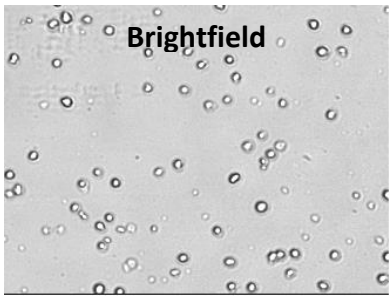
15. Click the **Close** button at the bottom right corner of the Counting Results table.
16. Select the F1 Image and check Counted at the right-hand side of the screen.
17. Review the counted image to confirm that the Calcein-positive cells are counted correctly.
    a. Individual cells within clumps should be circled in green, indicating that they are being counted individually.

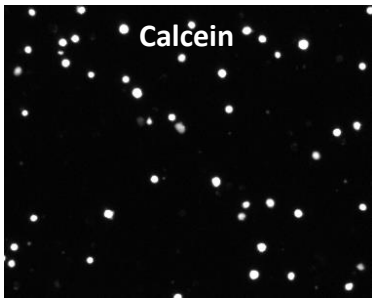


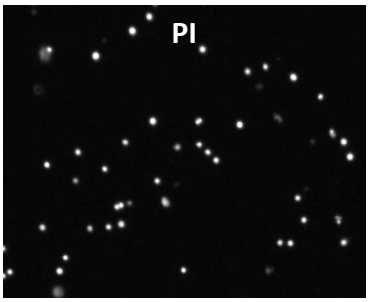
18. Click F2 Image to confirm that the PI-stained cells are being counted correctly as well.
    ***NOTE**: If cells are not being counted correctly, please contact Revvity Technical Support for assistance with optimization of counting parameters (see Section 11).*



19. To confirm that data has been saved appropriately, open the file location to check for brightfield and fluorescent cell images.
    a. Saved **Raw** images may be opened in the Cellometer software for re-analysis.





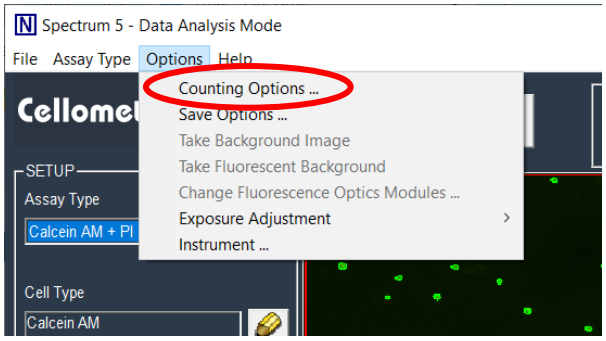


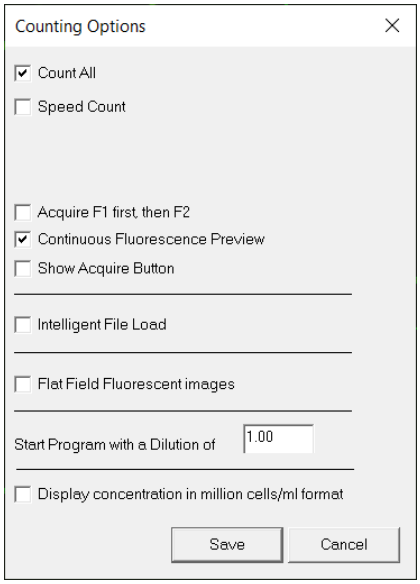
***NOTE**: A screen-capture software may be used to save both uncounted and counted colorized fluorescent images for presentation and publication.*

7 Cellometer Spectrum Software Settings

7.1 Counting Options Screen

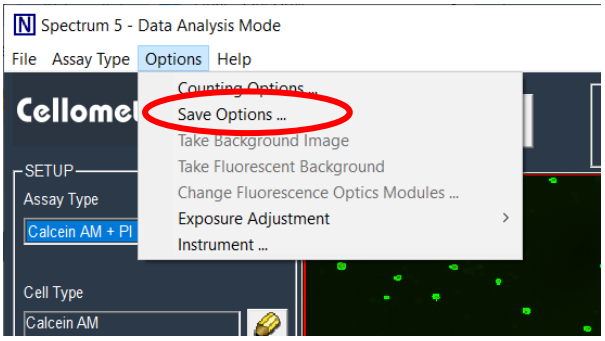
1. Click on the **Options** Tab and select **Counting Options**….
    a. Default settings for the Calcein AM/PI assay are shown.



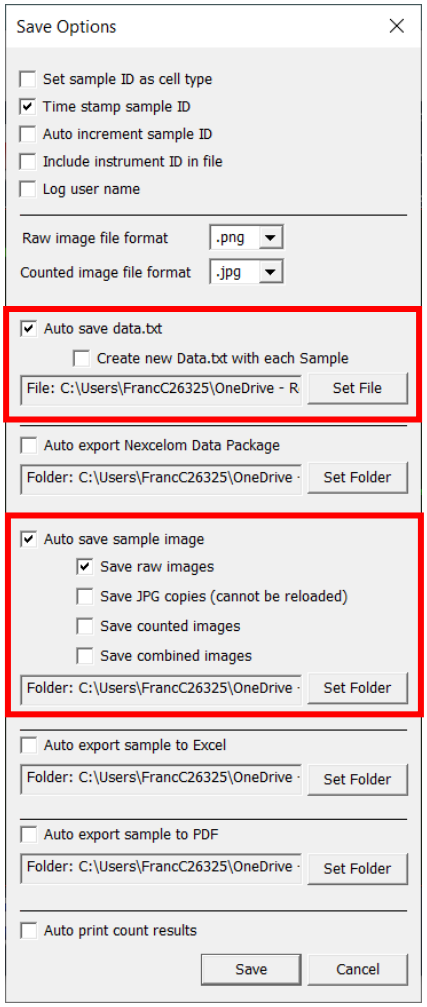


7.2 Save Options Screen

2. Click on the **Options** Tab and select **Save Options**….
    a. Default settings for the Calcein AM/PI assay are shown.



    b. Images are only saved if “Auto save data.txt” is selected in the save options menu.
    c. Saved **Raw** images may be opened in the Cellometer software for re-analysis.

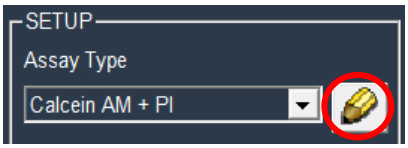


7.3 Default Calcein AM/PI Software Settings

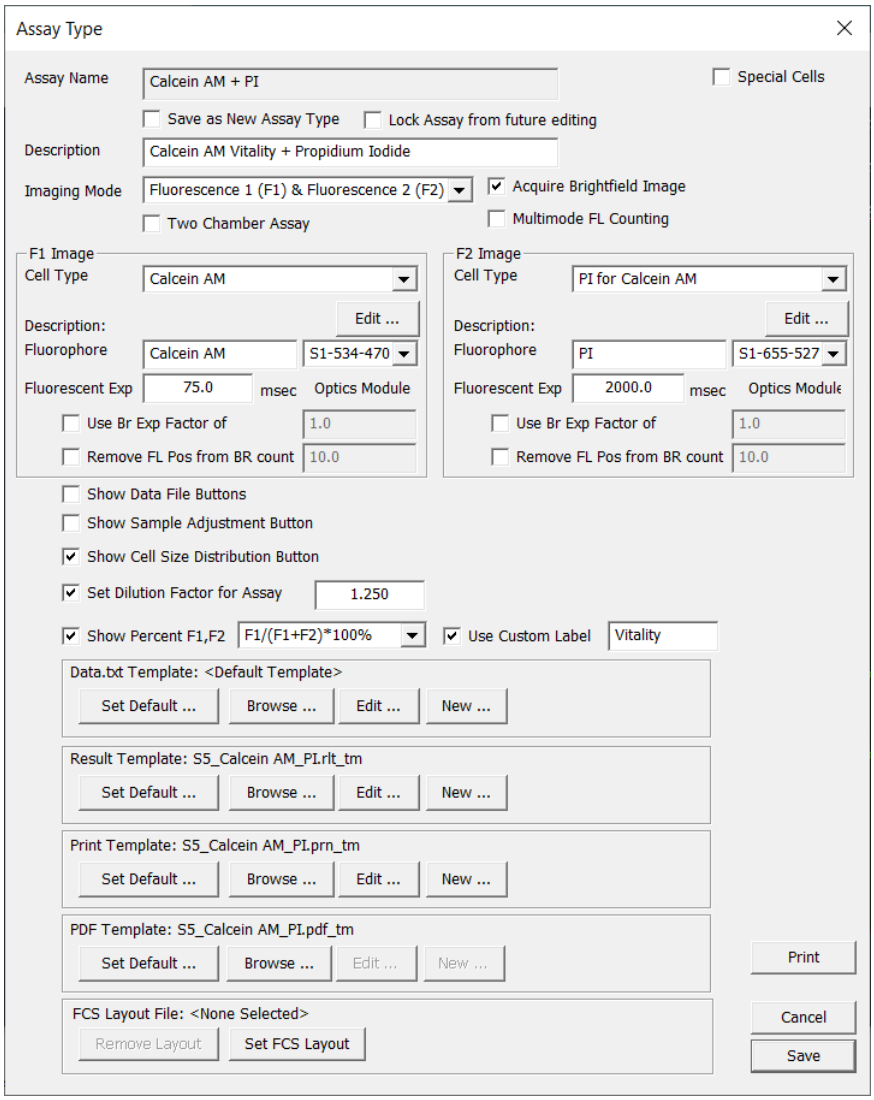
7.3.1 Dialog Screen Settings

These default software settings should be used to review or create the Calcein AM + PI assay. Detailed instructions on how to create a novel assay are located in the user manual.

1. Click on the pencil icon under Assay on the main software screen.

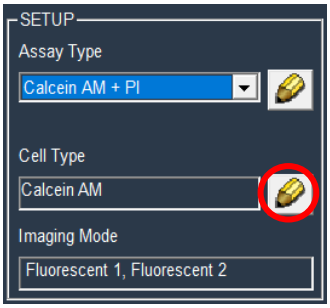


2. Default Dialog Settings are shown.

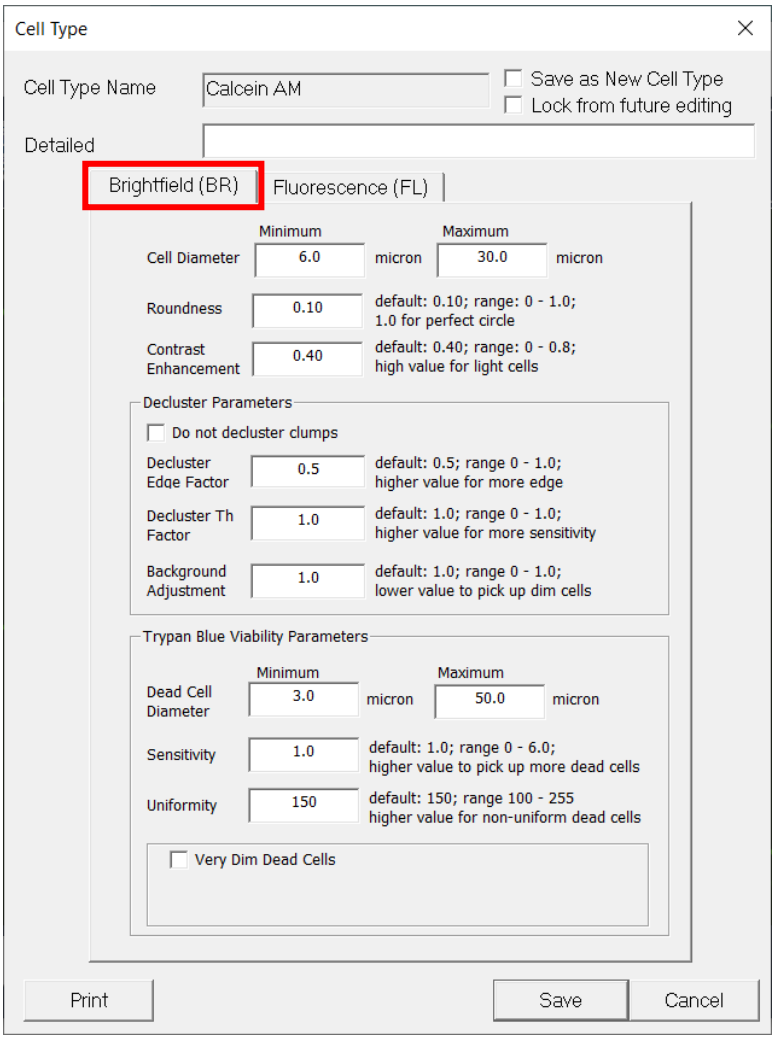


7.3.2 Cell Type Settings

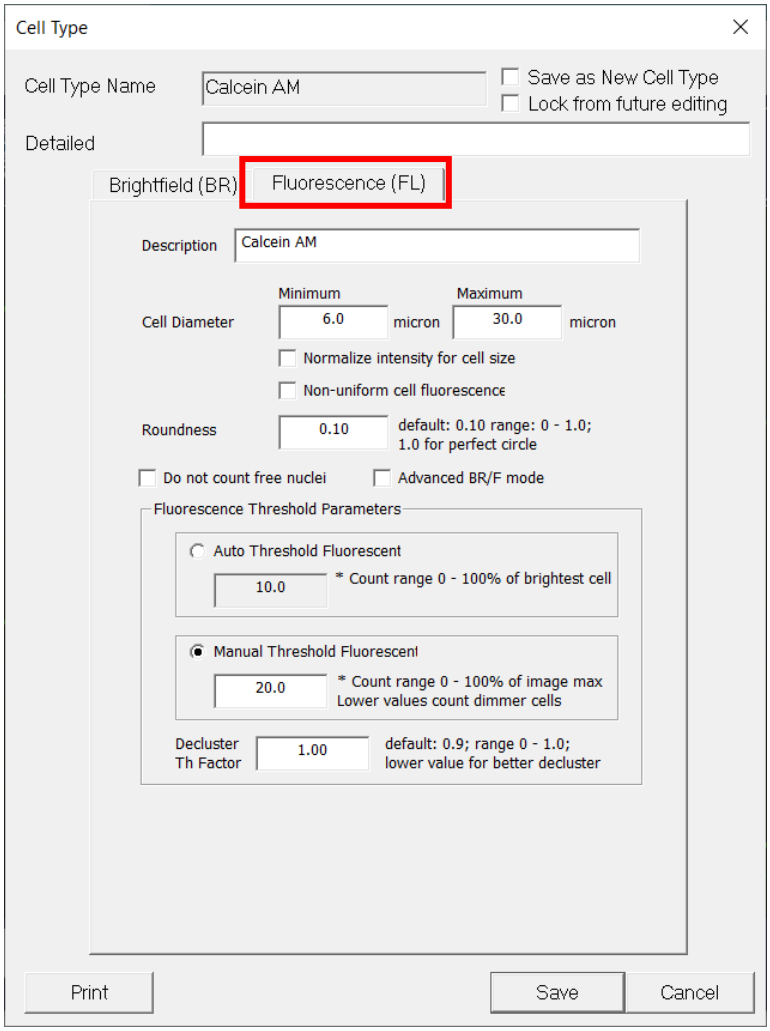
1. Click on the pencil icon under Cell Type on the main software screen.



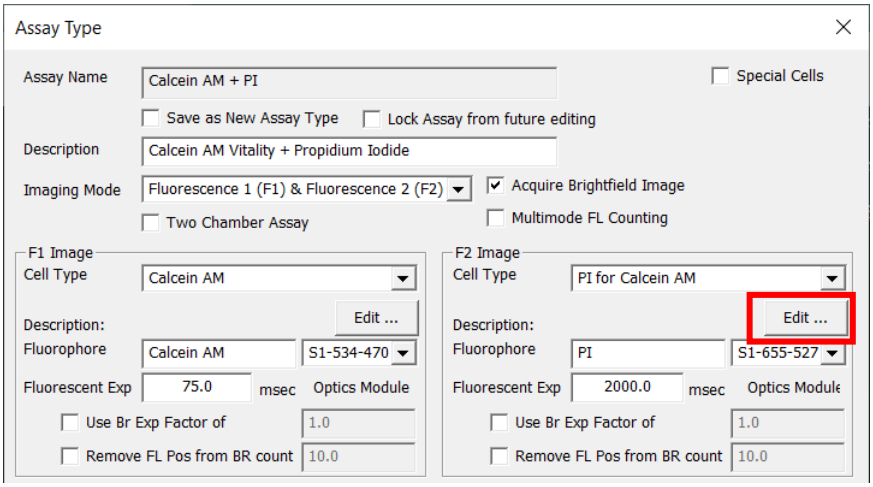
2. Default Brightfield (BR) tab Settings for the F1 image (Calcein) are shown.



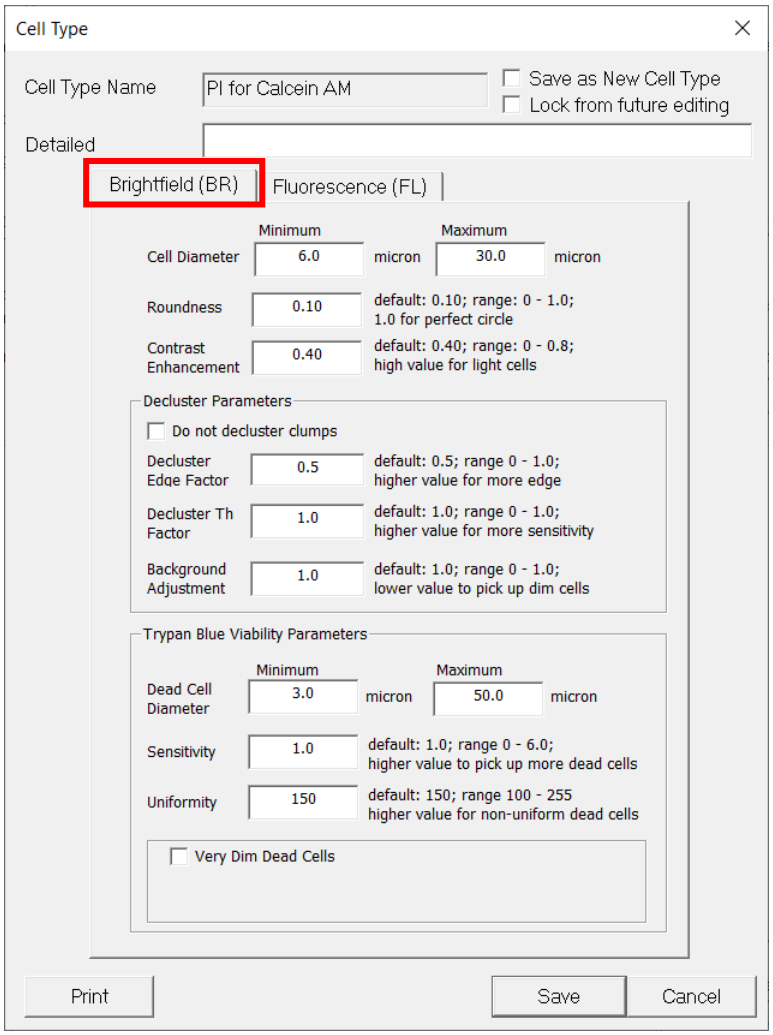
3. Click on the Fluorescence (FL) tab.
4. Default Fluorescence (FL) tab Settings for the F1 image (Calcein AM) are shown.



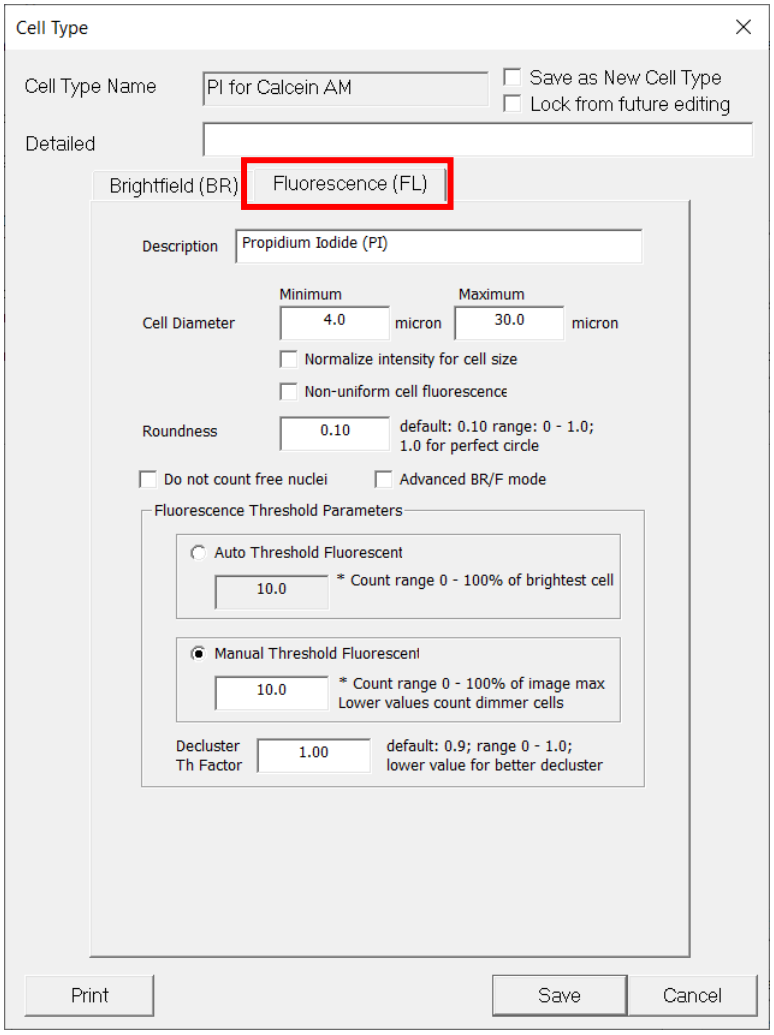
5. Click on the pencil icon under Assay on the main screen.
6. Click the Edit button on the right-hand side of the dialog box to edit F2 Image Cell Type.



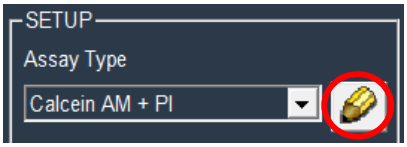
7. Default Brightfield (BR) tab Settings for the F2 image (PI) are shown.



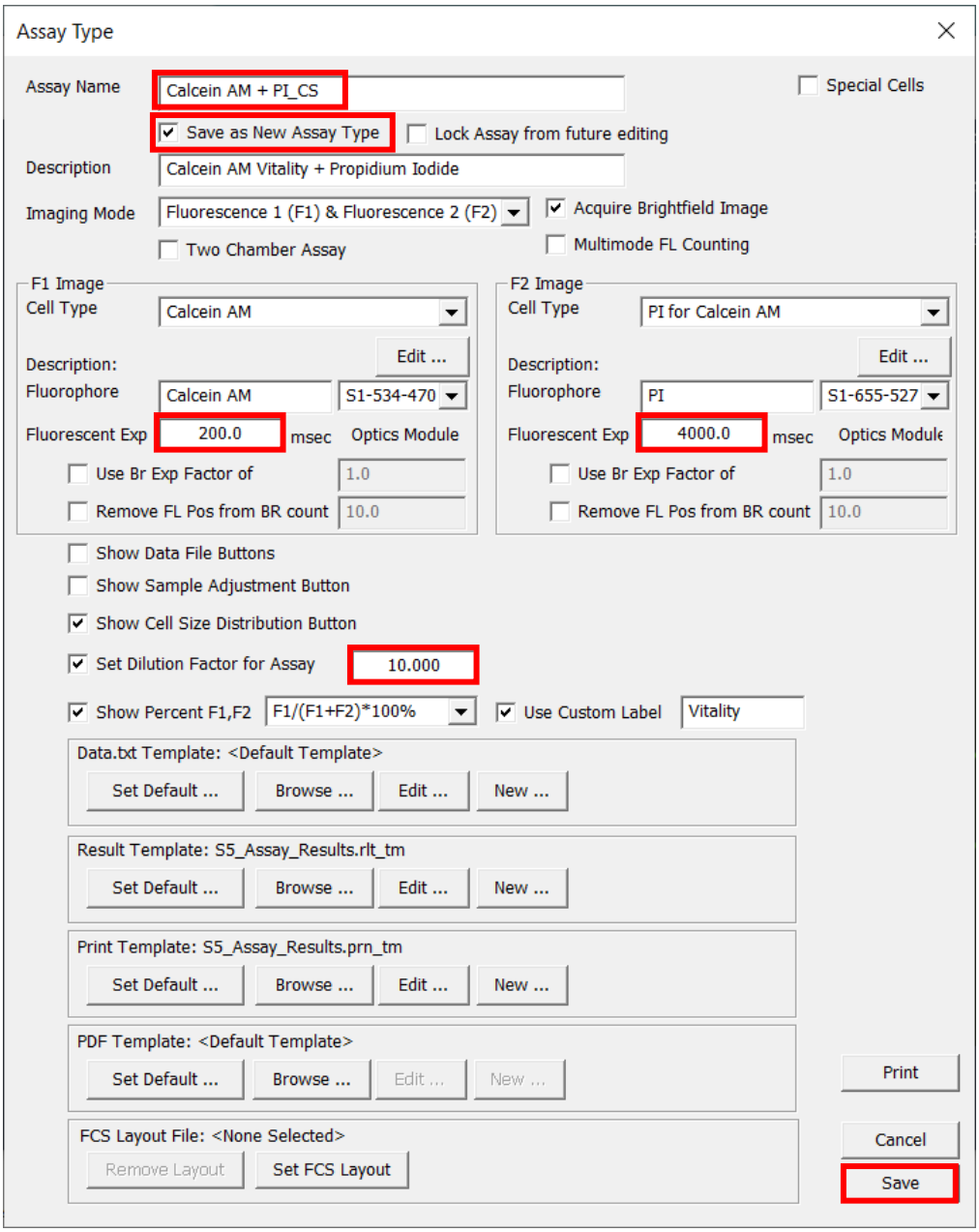
8. Click on the Fluorescence (FL) tab.
9. Default Fluorescence (FL) tab Settings for the F2 image (PI) are shown.



7.4 Software Settings for Biological Samples



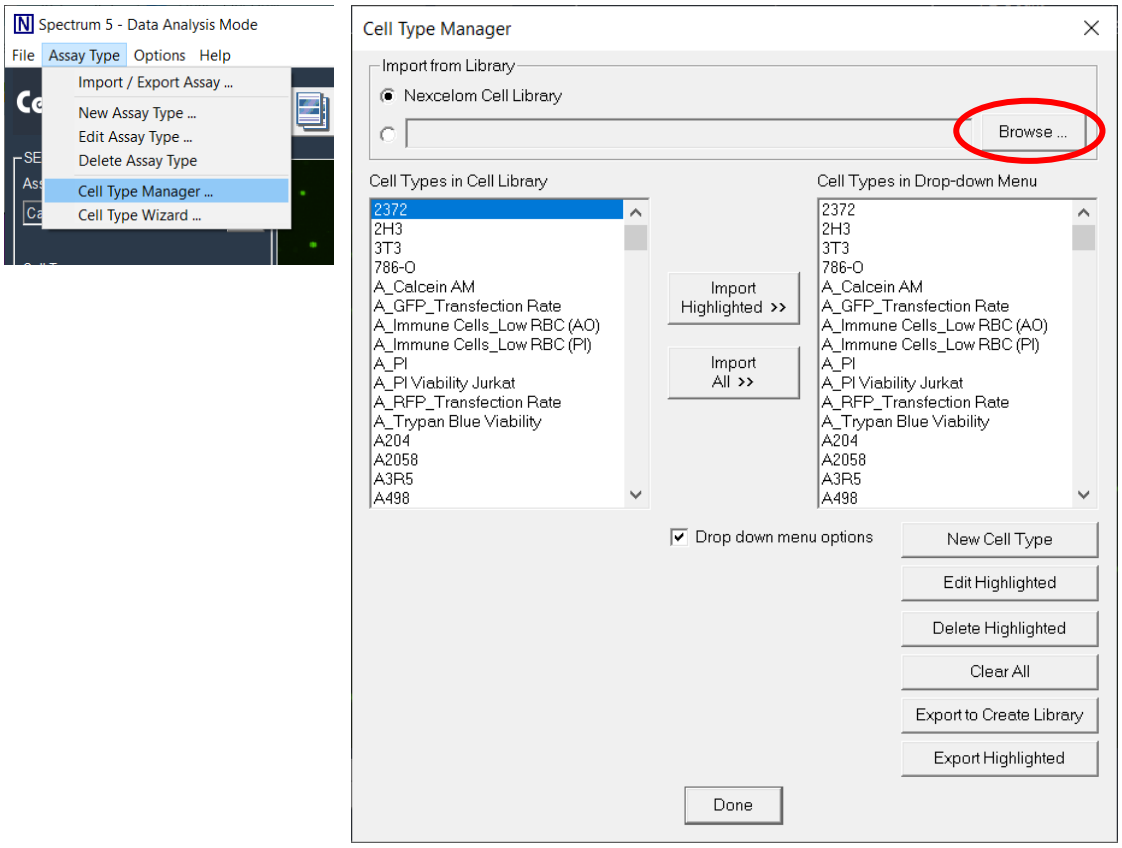
1. Click on the pencil icon under Assay on the main screen.
2. Adjust the current settings.
    a. Check the **Save as New Assay Type** box.
    b. Rename Assay to **Calcein AM + PI_CS**.
    c. Change the **Fluorescent Exp** for Calcein AM to 200.
    d. Change the **Fluorescent Exp** for PI to 4000.
    e. Change the **Dilution Factor for Assay** to 10.
    f. Click Save.



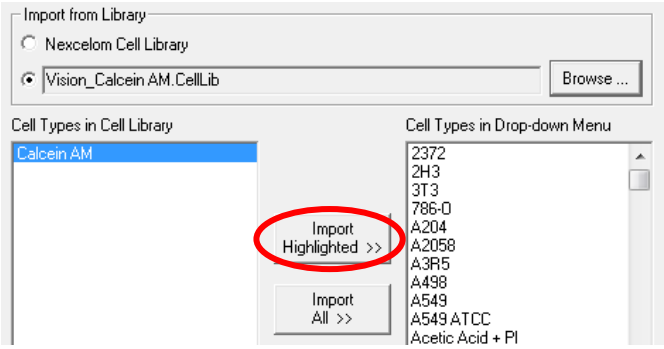
3. The Calcein AM + PI_CS assay will now be available from the dropdown menu. Proceed with data aquisition of biological samples as outlined in Section 6.

8.1 Importing a 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 and navigate to the location of the cell type file (will end with “.CellLib”). For the Calcein AM/PI assay, there is a Vision_Calcein AM.CellLib that can be requested through Revvity Customer Support.



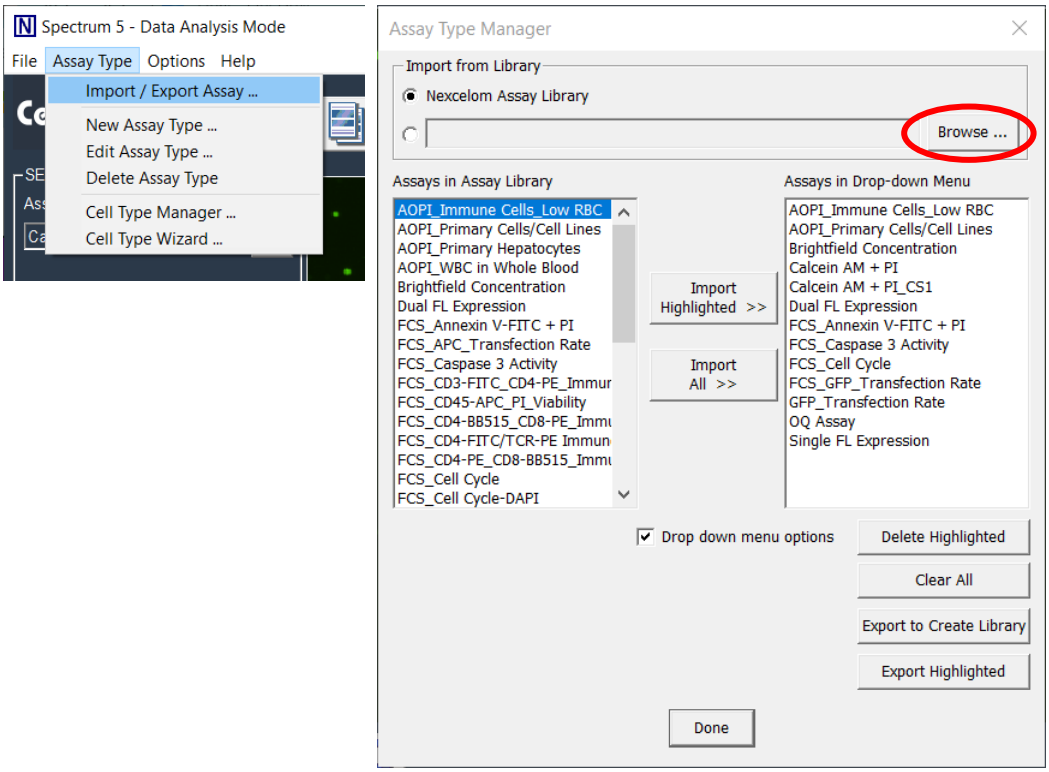
3. Highlight the cell type in the **Cell Types in Cell Library** and click **Import Highlighted**. For the Calcein AM/PI assay, the Calcein AM cell type should be imported.



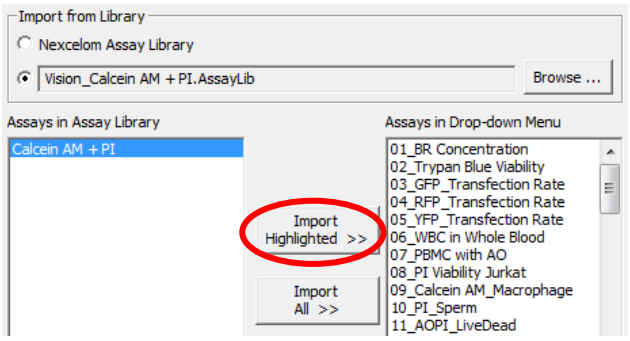
4. Repeat steps to import the PI cell type.

8.2 Importing an Assay

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.



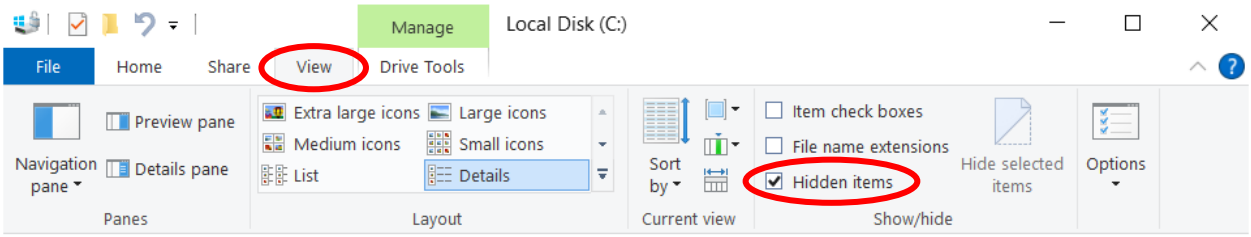
3. Highlight the assay in the **Assays in Assay Library** and click **Import Highlighted**. For the Calcein AM/PI assay, the Calcein AM +PI assay should be imported.
4. This assay will now be available from the dropdown menu.



5. The Calcein AM + PI_CS assay will now be available from the drop down menu.

8.3 Importing a Result or Print Template

1. From the File Explorer navigator open the **C: Drive** and locate the **ProgramData** folder.
   1.1. If the ProgramData folder is not listed, it may be hidden. To unhide a folder, click on the **View** Tab and ensure the **Hidden items** check box is selected.



2. Open the **ProgramData** folder.
3. Locate and open the **Nexcelom_Spectrum_v321** (or software equivalent) folder.
4. Open the **Template** folder.
5. Add the result template (Calcein AM and PI.rlt_tm) and/or print template (Calcein AM and PI .prn_tm) files into the Template folder. They will now be available to choose from when editing the assay.

9 Storage and Handling

Store the Propidium Iodide (Component A) between 2 and 8 °C and the Calcein AM (Component B) between -16 and -24 °C. Please consult the Safety Data Sheet for more safety information, found on **www.revvity.com/cellcountingreagents**.

10 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.

11 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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