Celígo®

ViaStain™ Calcein AM/Hoechst/PI Viability Kit

Product Number: CSK-V0006-1

Sample Kit: CSK-V0006-S
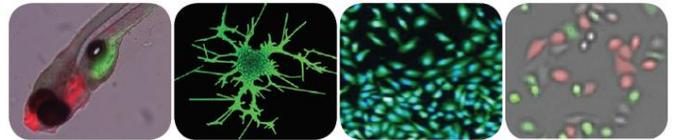
Nexcelom Bioscience

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

Table of Contents

Nexcelom Bioscience LLC. | 360 Merrimack Street, Building 9 | Lawrence, MA 01843
Telephone: 978.327.5340 | Fax: 978.327.5341 | Email: info@nexcelom.com | www.nexcelom.com
8002291 Rev. E

P a g e | **1**

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.
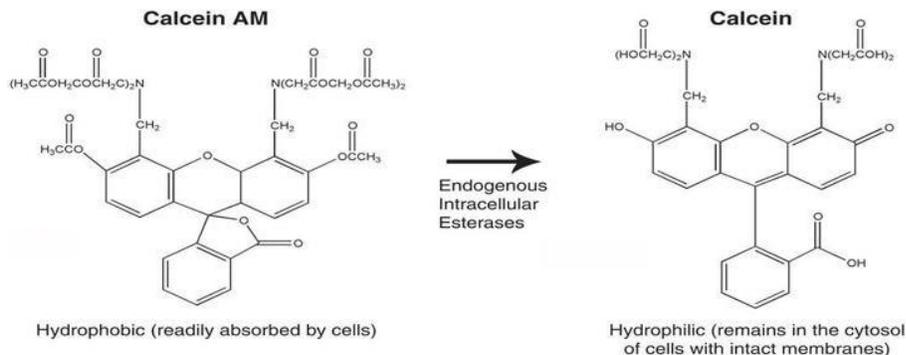
Stains such as propidium iodide (PI), 7-AAD, and ethidium bromide (EB), are membrane exclusion dyes that are frequently used to stain non-viable nucleated cells with compromised membranes. When calcein AM is used in conjunction with PI, it is possible to determine % vitality / viability based on the number of metabolically-active (green fluorescent) and non-viable (red fluorescent) cells in a sample (2). To enumerate the total number of nucleated cells, the cells were counterstained with Hoechst 33342.

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).

1.2 Materials and Reagents

- ViaStain™ Calcein AM/Hoechst/PI Viability Kit
  Cat. # CSK-V0006-1 (Stains 5, 96-well plates)
  - (Component A) Propidium iodide, 200 μL
  - (Component B) Hoechst 33342, 40 μL
  - (Component C) Calcein AM, 50 μL

- ViaStain™ Calcein AM/Hoechst/PI Viability Kit – Sample kit (not available for individual re-sale)
  Cat. # CSK-V0006-S (Stains 1, 96 well plates)
  - (component A) Propidium iodide, 40 μL
  - (component B) Hoechst 33342, 8 μL
  - (Component C) Calcein AM, 10 μL

- ViaStain™ Calcein-AM/Hoechst/PI Viability Kit Instructions

1.3 Instrument and Software Requirements

- Celigo® Imaging Cell Cytometer

Nexcelom Bioscience LLC. | 360 Merrimack Street, Building 9 | Lawrence, MA 01843
Telephone: 978.327.5340 | Fax: 978.327.5341 | Email: info@nexcelom.com | www.nexcelom.com

8002291 Rev. E

P a g e | **2**

2.0 Assay Protocol

2.1 Preparation of Control Samples

1. A positive control may be generated by exposing cells to pharmacological agents such as α-TOS, Etoposide, or Staurosporine. For Celigo experiments A549 cells were treated with 50 µM α-TOS or 100 nM Staurosporine.

2. A negative control (untreated cells) should be tested to determine baseline cell concentration viability of the cells.

2.2 Preparation of Adherent Cells for Staining

1. Adherent cells do not have to be trypsinized or pre-treated for this assay. Cell growth, staining and imaging may be done in the same plate.

2.3 Staining Procedure: Add and read

For a 96-well plate prepare a 4x master mix using the following reagents: 1x PBS, calcein AM, Propidium iodide (PI) and Hoechst 33342.

1. Prepare a 4x Master Mix staining solution:

    a. Pipette 4942 µl of 1x PBS into a 15 mL centrifuge tube.

    b. Add 40 µl of the Propidium iodide solution (Component A) to 1 x PBS in step a.

    c. Add 8 µl of the Hoechst 33342(Component B) to 1x PBS in step a.

    d. Add 10 µl of the calcein AM (Component C) to 1x PBS in step a.

    e. Vortex tube for 10 seconds.

2. Pipette 50 µl of 4x staining solution per well containing 150 µl of volume. Final volume per well will now be 200 µl.

3. Incubate for 30 minutes at 37° in the dark.

4. This an add and read assay, no washing required.

5. Plate is ready for imaging on Celigo.

3.0 Software Settings

3.1 Celigo Assay Setup

Viability Assay can be analyzed in two different ways:

1. **Cell Viability: Live + Dead + Total (see section 3.2)**
    - ➢ 3 –channel application Green, Red, Blue
    - ➢ pre-labelled channels
    - ➢ Simple analysis – no gating required
    - ➢ Bright-field images are not acquired

2. **Expression Analysis: Target 1+2+3+Mask (see section 3.3)**
    - ➢ 4-channel application Green, Red, Blue, and Bright-field
    - ➢ Customizable channel names
    - ➢ Data analysis via population gating is required

### 3.2 Celigo Setup for Cell Viability: Live + Dead + Total

Start Tab:

1. Create a new scan and name file appropriately.
2. If experimental settings had been previously optimized then select these to be used.

Scan Tab:

Setup image acquisition settings for each channel as described below. Ensure signal of objects is well separated from background. Ideal object signal 100-150 pixel intensity to ideal background 1-25 pixel intensity. Additional washes maybe necessary to reduce background.

1. Select Celigo Application: Cell Viability (Live + Dead + Total).
2. Setup Live Channel
   a. Select Green illumination for calcein AM stain.
   b. Exposure time should be around 10,000 µs (Gain 0).
3. Setup Dead Channel
   a. Select Red illumination for Propidium Iodide stain.
   b. Exposure time should be around 40,000 µs (Gain 0).
4. Setup Total Channel
   a. Select Blue illumination for Hoechst stain.
   b. Exposure time should be around 80,000 µs (Gain 0).
   c. Register Hardware autofocus using the Blue / Hoechst channel.
5. Switch to Green / Calcein AM channel, "Find focus" and "Set Offset".
6. Switch to Red / Propidium Iodide channel, "Find focus" and "Set Offset".
7. Select wells to acquire and "Start Scan".

Nexcelom Bioscience LLC. | 360 Merrimack Street, Building 9 | Lawrence, MA 01843
Telephone: 978.327.5340 | Fax: 978.327.5341 | Email: info@nexcelom.com | www.nexcelom.com

8002291 Rev. E

P a g e | **4**

	Live		**Dead**		**Total**

Channel — Live | **Channel** — Dead | **Channel** — Total

Image Acquisition Settings
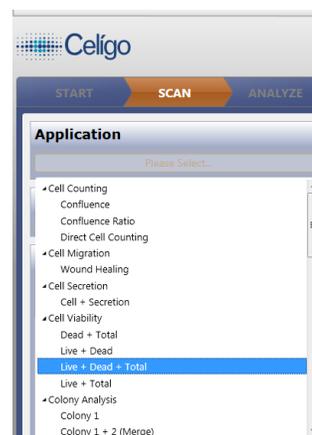
	Live	Dead	Total
Type:	Custom Channel	Custom Channel	Custom Channel
Focus Offset:	0	0	0

Configuration

	Live	Dead	Total
Illumination:	Green 483/536	Red 531/629	Blue 377/447
Acquisition Resolution:	1 µm/pixel	1 µm/pixel	1 µm/pixel
Exposure Time (µs):	10000	40000	100000
	Auto Calc	Auto Calc	Auto Calc
Gain:	0	0	0
	Auto Calc	Auto Calc	Auto Calc

Analyze Tab:

The Celigo Cell Viability application segments all three fluorescent channels. Fluorescent objects will be identified in each channel. Green and Red objects (for live and dead cells) will be counted only if they are super-imposed with a blue fluorescent object (for nuclear stain). Therefore, segmented green and red objects (such as fluorescent debris) not associated with a blue nucleus will be rejected from the analysis.

1. Select "Well Mask" and "Automatic" to exclude the outer part of the well.
2. Select "Fluorescence" algorithm for all 3 channels.
3. Adjust the "Intensity Threshold" to ensure proper identification of the nucleus. Default value of "4" with "High Precision" works well for most fluorescent stains. Values of "2" to "3" can be used for dimmer samples.
4. Keep the "Cell Diameter" between "8" and "10".
5. Select "Separate Touching Objects" in the Total channel to ensure proper separation of nuclei in close proximity.

Live | **Dead** | **Total**

General

	Live	Dead	Total
Well Mask:	✓	✓	✓
Well Mask Usage Mode:	Automatic	Automatic	Automatic
% Well Mask:	100.000	100.000	100.000

Identification

	Live	Dead	Total
Channel:	Live	Dead	Total
Algorithm:	Fluorescence	Fluorescence	Fluorescence
Intensity Threshold:	4	3	3
Precision:	High	High	High
Cell Diameter (pixel):	10	10	8
Background Correction:	☐	☐	☐
Separate Touching Objects:	☐	☐	✓

6. Expected Segmentation:
    a. Use the "Image Display" and "Graphic Overlay" controls to visualize segmentation.

    b. Typical images and fluorescent objects identification should look as follow:
7. Select wells to analyze and "Start Analyze".
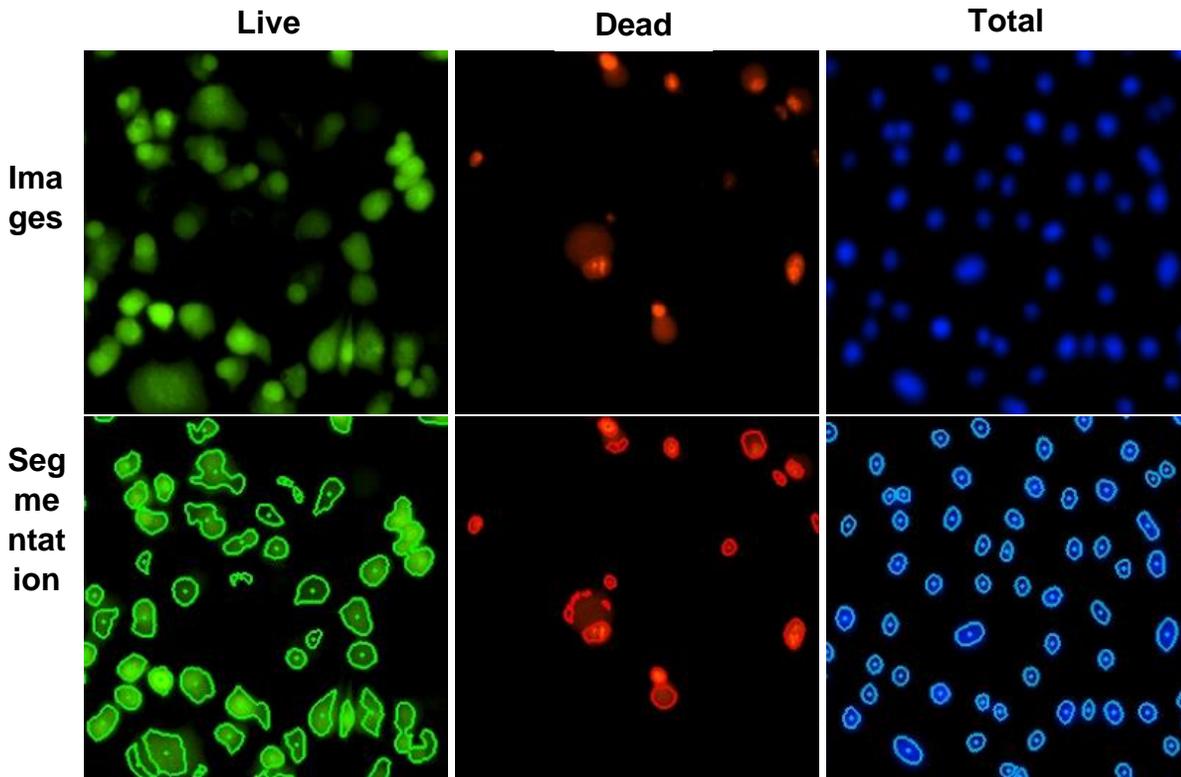
Results Tab:

Image analysis uses a method that first identifies the nuclei in the image, then evaluates whether objects from the Live and Dead channels overlap. If they do overlap they are reported as Live +, Dead, Live+ and Dead. Live + objects and Dead objects not associated or overlapping a nuclear area (i.e. debris or detached cell membrane) are not included in analysis.

Nexcelom Bioscience LLC. | 360 Merrimack Street, Building 9 | Lawrence, MA 01843
Telephone: 978.327.5340 | Fax: 978.327.5341 | Email: info@nexcelom.com | www.nexcelom.com
8002291 Rev. E
P a g e | **6**

Table of Data reported:

Name	Live (+) corrected	Live (+)	Dead	Total
Percent (relative to Total)	●	●	●	
Counts	●	●	●	●
Mean Intensity (AVE, SD)	●	●	●	●
Integrated Intensity (AVE, SD)	●	●	●	●

Live + Dead + Total   **Segmentation**

Live cell with green overlay super-imposing a blue nucleus overlay

Dead cell with red overlay super-imposing a blue nucleus overlay

Green overlay dismissed from analysis because not super-imposing a blue nucleus overlay

Red overlay dismissed from analysis because not super-imposing a blue nucleus overlay
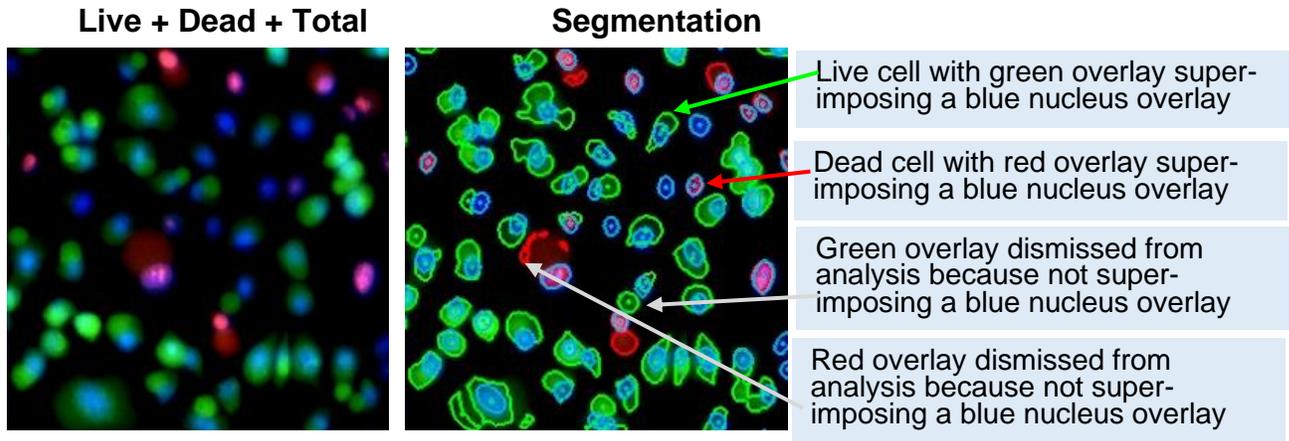
### 3.3 Celigo Setup for Expression Analysis Target 1 + 2 + 3 + Mask

Start Tab:
1. Create a new scan and name file appropriately.
2. If experimental settings had been previously optimized then select these to be used.

Scan Tab:
1. Select Celigo Application: Expression Analysis Target 1+2+3+Mask.
2. Rename Channel, Graphic Overlay, & Class label.
3. Setup Blue (Mask) Channel.
    a. Select Blue Illumination.
    b. Exposure time should be around 80,000 µs.

Customize Application

Edit customizable names:

Channel (1)	Green
Channel (2)	Red
Channel (3)	BF
Channel (Mask)	Blue
Feature (1)	Green
Feature (2)	Red
Feature (3)	BF
Feature (Mask)	Blue
Class (1)	CAM+ PI-
Class (2)	CAM+PI+
Class (3)	CAM-PI+
Class (4)	CAM-PI-

Analysis Settings

Nexcelom Bioscience LLC. | 360 Merrimack Street, Building 9 | Lawrence, MA 01843
Telephone: 978.327.5340 | Fax: 978.327.5341 | Email: info@nexcelom.com | www.nexcelom.com
8002291 Rev. E
P a g e | **7**

c. Click Focus Setup, then click Auto Register to register hardware focus position.

d. Adjust Illumination exposure.

> ➢ Ideal object pixel intensity should be between 100-150 with background signal no higher than 25 pixel intensity.

4. Setup Green Channel

   a. Select Green Illumination.
   b. Exposure time should be around 10,000 µs.
   c. Click Find Focus, or adjust manually.
   d. Click Set Offset.
   e. Adjust Illumination exposure.
   > ➢ Ideal object pixel intensity should be between 100-150 with background signal no higher than 25 pixel intensity.

5. Setup Red Channel

   a. Select Red Illumination.
   b. Exposure time should be around 40,000 µs.
   c. Click Find Focus, or adjust manually.
   d. Click Set Offset.
   e. Adjust Illumination exposure.
   > ➢ Ideal object pixel intensity should be between 100-150 with background signal no higher than 25 pixel intensity.

6. Setup BF Channel

   a. Select Brightfield Illumination.
   b. Click Auto Calc.
   > ➢ Auto Calc will return an illumination with background pixel intensity between 120-150.
   > ➢ Exposure time should be around 4000 µs, depending on media color.

   c. Click Find Focus, or adjust manually.
   d. Click Set Offset.

7. Click Selection button on plate map, then select wells to be imaged.

8. Click Start Scan to begin image acquisition.

Nexcelom Bioscience LLC. | 360 Merrimack Street, Building 9 | Lawrence, MA 01843
Telephone: 978.327.5340 | Fax: 978.327.5341 | Email: info@nexcelom.com | www.nexcelom.com

8002291 Rev. E

P a g e | **8**

Analyze Tab:

1. Check the Well Mask box, select Automatic, and adjust percent if necessary.
2. Set analysis parameters for the Blue (mask) Channel.
   a. Turn ON Blue image display and graphic overlay.
   b. Turn OFF the other image displays and graphic overlay.
   c. Select a green well to view images.
   d. Adjust Intensity Threshold to ensure proper identification of the nucleus. Default value of "4" with "high Precision" works well for nuclear stains.
   e. Keep the "Cell Diameter" at "10".
   f. Increase "Dilation Radius" to "2".
   g. Select "Separate Touching Objects" to ensure proper separation of nuclei in close proximity.
3. Click Gate Tab.

Analysis Settings

Current: Untitled Analysis Settings 1

General

Well Mask:	✓
Well Mask Usage Mode:	Automatic
% Well Mask:	100.000

Identification

Algorithm:	Fluorescence
Intensity Threshold:	4
Precision:	High
Cell Diameter (pixel):	10
Dilation Radius (pixel):	0
Background Correction:	☐
Separate Touching Objects:	✓

Pre-Filtering

Feature Type: Green

Cell Area (pixel^2):
10 — 10000

Cell Intensity Range:
0 — 255

Min Cell Aspect Ratio: 0.000

Example images and graphic overlay of nuclei Mask

Nexcelom Bioscience LLC. | 360 Merrimack Street, Building 9 | Lawrence, MA 01843
Telephone: 978.327.5340 | Fax: 978.327.5341 | Email: info@nexcelom.com | www.nexcelom.com
8002291 Rev. E
P a g e | **9**

Gate Tab:

Similar to flow cytometry, we use the Celigo gating interface to identify sub-populations of cells in various phases of viability. Plotting the size of the identified objects and the intensities of the calcien AM and Propidium Iodide is required.

1. In the gating area, select "+" to create a plot.
2. Select "Histogram" plot type and choose "Area".
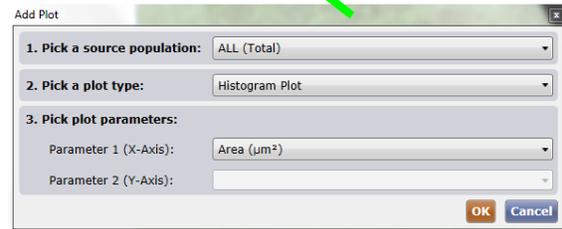
3. Select "Range" gate that excludes small and large objects in order to create "Pop 1" of objects
4. Assign the gated cells to the "total" class.
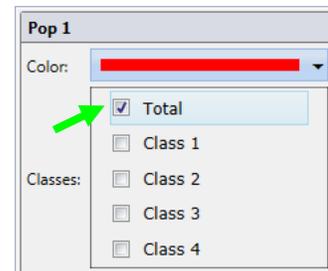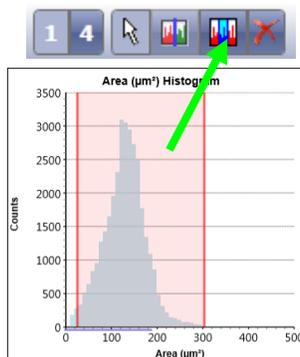
5. Select "+" to create a plot
6. Select Scatter plot and select Green: Mean Intensity for x-axis and Red: Mean Intensity for y-Axis.
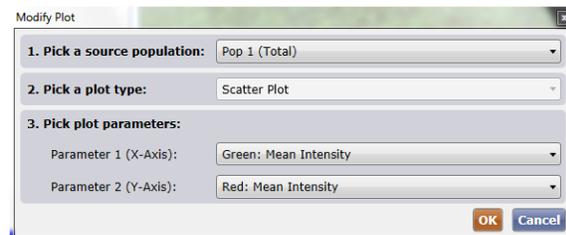
7. Select quadrant gate and click on scatter plot just above and left of blue dots.
8. Adjust xy-axis ranges to focus in on low intensity area. Adjust location of quadrant to best sit left of live population.

Nexcelom Bioscience LLC. | 360 Merrimack Street, Building 9 | Lawrence, MA 01843
Telephone: 978.327.5340 | Fax: 978.327.5341 | Email: info@nexcelom.com | www.nexcelom.com

8002291 Rev. E

P a g e | **10**
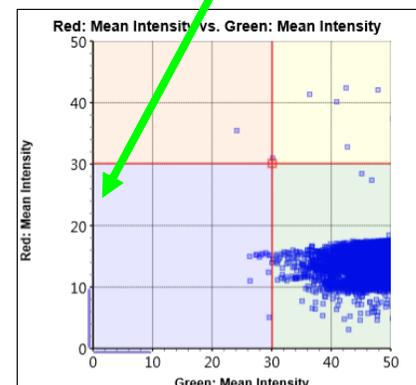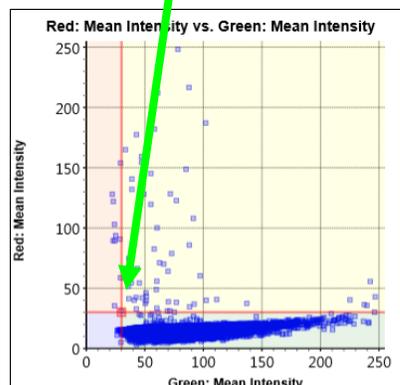
9. Check appropriate boxes to label classes.
10. Check control wells to verify quadrant is in correct location.
11. Select Start Analysis.
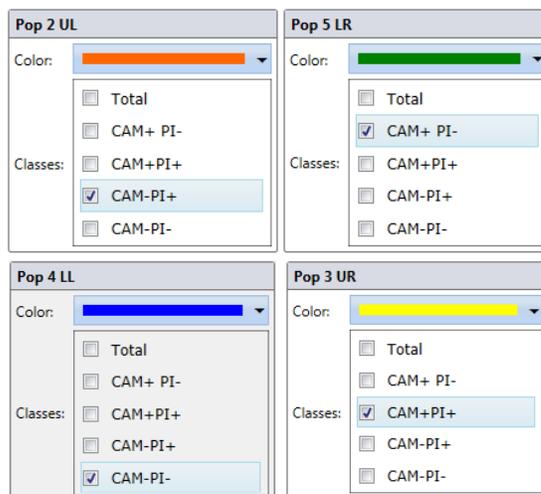
Results:

Image analysis and gating uses a method that first identifies the nuclei in the Mask image channel, then measures FL signature in the additional channels. Objects are then plotted according to their size and FL signature. Gates are used to create subpopulations. Check boxes identify what populations will be reported.

Table of Data reported:

Name	Class 1	Class 2	Class 3	Class 4	Total
Percent (relative to Total)	●	●	●	●	
Counts	●	●	●	●	●
Mean Intensity (AVE, SD)	●	●	●	●	●
Integrated Intensity (AVE, SD)	●	●	●	●	●

Nexcelom Bioscience LLC. | 360 Merrimack Street, Building 9 | Lawrence, MA 01843
Telephone: 978.327.5340 | Fax: 978.327.5341 | Email: info@nexcelom.com | www.nexcelom.com
8002291 Rev. E
P a g e | **11**

4.0 Reference

4.1 Quick Reference

Image plate at the suggested following exposures to obtain object pixel intensities between 150-175: These values are not absolute and may require further optimization.
- Hoechst – Total Channel @ 80,000 µs, Focus Setup with HWAF or IBAF.
- PI – Dead Channel @ 40,000 µs, Focus offset value of -20 µm.
- Calcein AM – Live Channel @ 10,000 µs, Focus offset value of -20 µm.

4.2 Technical Support

- Celigo Learning Center (online) at www.nexcelom.com/celigo-learning   .
- Nexcelom Technical Support is available from 9am to 5:30pm EST.
  E-mail: support@nexcelom.com.
  Phone: 978-327-5340.

4.3 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.

4.4 Storage and Handling
- For long term storage, store ViaStain™ Propiduim iodide (Component A) between 2°C to 8°C.
- For long term storage, store ViaStain™ Hoechst 33342 (Component B) between -16°C to -24°C. Thaw to room temperature before use.
- For long term storage, store ViaStain™ Calcein AM (Component C) between -16°C to -24°C. Thaw to room temperature before use.

Nexcelom Bioscience LLC. | 360 Merrimack Street, Building 9 | Lawrence, MA 01843
Telephone: 978.327.5340 | Fax: 978.327.5341 | Email: info@nexcelom.com | www.nexcelom.com
8002291 Rev. E
P a g e | 12

5.0 Ordering Information

5.1 How to Reorder

For orders shipping to destinations in the United States:
- When ordering with a Purchase order:
  - Fax a copy of your order to 978-327-5341.
  - 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/

For orders shipping to destinations outside the United States:
Contact your local distributor or Nexcelom Representative to place your order/

Nexcelom Bioscience LLC. | 360 Merrimack Street, Building 9 | Lawrence, MA 01843
Telephone: 978.327.5340 | Fax: 978.327.5341 | Email: info@nexcelom.com | www.nexcelom.com
8002291 Rev. E
P a g e | **13**