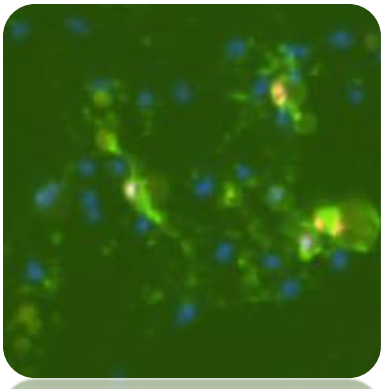
# ViaStain™ No Wash Annexin V – FITC Kit For Celigo

**Product Number**: CSK-V0007-1, CSK-V0007-2

Sample Kit: CSK-V0007-S (Not available for purchase)



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

Page left blank intentionally

## Table of Contents

# 1.0 Introduction

## 1.1 Assay Description

Apoptosis, or programmed cell death, is a natural process of cellular self-destruction. Apoptosis is a part of routine cell turnover and tissue homeostasis, prevalent in epithelial cells, erythrocytes, and other cell types genetically programmed to have a limited life span. It is also important in embryogenesis, maintenance of immune tolerance, and development of the nervous system. Apoptosis can be induced either by a stimulus, such as irradiation or toxic drugs, or by removal of a repressor agent. The cells disintegrate into membrane-bound particles that are then eliminated by phagocytosis.

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.

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 J*. 8:155-181

## 1.2 Materials Included

- ViaStain™ No Wash Annexin V – FITC Kit For Celigo
  **Cat. # CSK-V0007-1** (Stains 1, 96-well plate), **CSK-V0007-2** (Stains 5, 96-well plate)
  - (Component A) Propidium iodide
  - (Component B) Hoechst 33342
  - (Component C) Annexin V-FITC
  - (Component D) Annexin V Binding Buffer
- ViaStain™ No Wash Annexin V – FITC Kit For Celigo Instruction Booklet

## 1.3 Additional Materials required, but not included

- 96-well flat bottom, clear bottom, black walled
- Cells with appropriate media

## 1.4 Instrument and Software Requirement

- Celigo® Imaging Cytometer Instrument

# 2.0 Assay Protocol for No-wash Annexin V Assay

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

## 2.2 Preparation of Adherent Cells

1. Adherent cells do not have to be trypsinized for this assay. Cell growth, treatment, staining and imaging may be done in the same plate.
2. Plate cells and let adhere overnight in incubator.
3. Treat cells with appropriate compounds that induce Apoptosis.

## 2.3 Staining Procedure for 96-well Plate Type

### 2.3.1 Create Stock Solution

For a 96-well plate prepare a 1.5x master mix staining solution using the following reagents:

1. Pipette 9864 mL of Annexin V Binding Buffer into a 15 mL centrifuge tube
2. Add 100 µl of the Annexin V-FITC
3. Add 30 µl of the Propidium Iodide
4. Add 6 µl of the Hoechst 33342
5. Cap and vortex tube for 10 seconds to mix reagents

### 2.3.2 Staining Protocol for 96 well plate

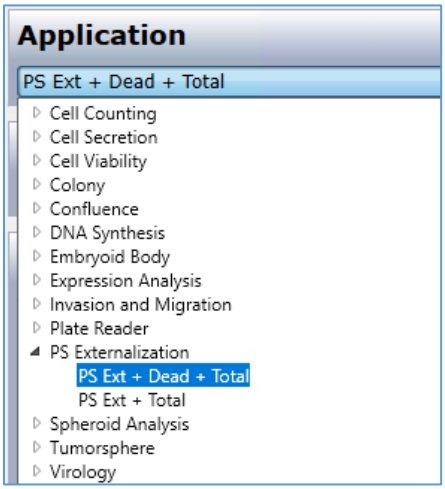
1. Remove liquid in wells so only 50 µl is remaining in wells
2. Pipette 100 µl of 1.5x staining solution per well containing 50 µl of volume. Final volume per well will now be 150 µl
3. Incubate for 30-45 minutes at 37°C 5% $CO_2$ in the dark

## 3.0 Celigo Assay Setup Options

Apoptosis assay can be analyzed in **two different ways (3.1 and 3.2)**:

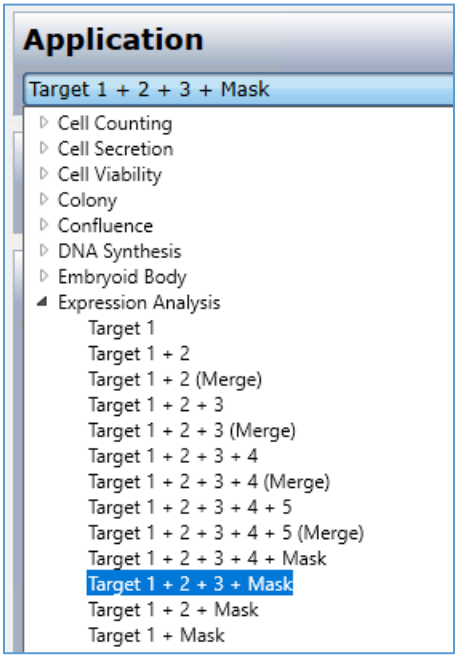
### 3.1 Cell Apoptosis: PSE + Dead + Total (see section 4.0)

- 3-channel application Green, Red, Blue
- pre-labelled channels
- Simple analysis – no gating required
- Bright-field images are not acquired
- Phosphatidylserine Externalization (PS Ext)




### 3.2 Expression Analysis: Target 1+2+3+Mask (see section 5.0)

- 4-channel application Green, Red, Brightfield, and Blue
- Customizable channel names
- Data analysis via population gating is required
- Obtain Brightfield image for observation only



# 4.0 Celigo Software Settings for Cell Apoptosis: PSE + Dead + Total

## 4.1 HOME and SETUP Tabs:

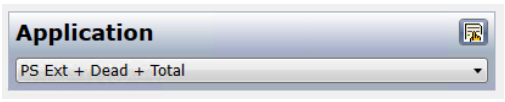
1. Create a new scan and name file appropriately.
2. If experimental settings had been previously optimized then select these to be used.

## 4.2 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.

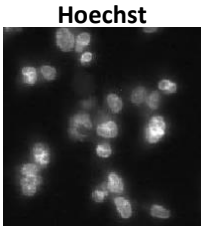
### 4.2.1 Select an Application

1. Select Celigo Application: PS Externalization (PS Ext + Dead + Total).



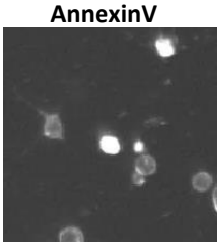

### 4.2.2 Setup Total Channel

1. Select Blue illumination for Hoechst stain.
2. Set Exposure time should be around 80,000-100,000 µs (Gain 0).
3. In Focus Setup, Register Auto for Hardware Auto Focus for Total Channel.



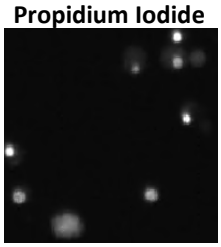

### 4.2.3 PS Ext Channel Setup

1. Select Green illumination for Annexin V-FITC stain.
2. Exposure time should be around 60,000-80,000 µs (Gain 0).
3. Click “Find focus” and “Set Offset”.




### 4.2.4 Dead Channel Setup

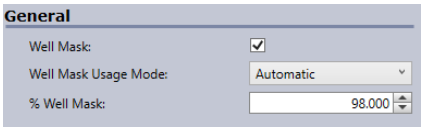
1. Select Red illumination for Propidium Iodide stain.
2. Exposure time should be around 20,000-30,000 µs (Gain 0).
3. Click “Find focus” and “Set Offset”.
4. Select wells to acquire and “Start Scan”.




## 4.3 ANALYZE Tab:

The Celigo Cell Apoptosis application segments all three fluorescent channels. Fluorescent objects will be identified in each channel. Green and Red objects (for apoptotic 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.

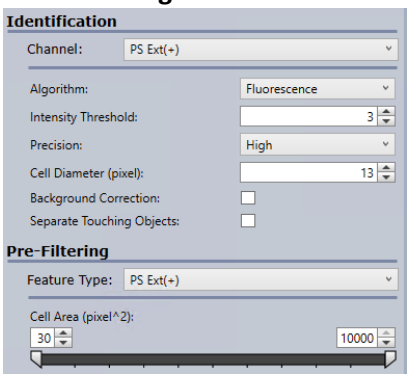
### 4.3.1 General Section Analysis Setup




1. Select “Well Mask” and “Automatic” to exclude the outer part of the well.
2. Shrink to 98%

**PS Ext Setting**

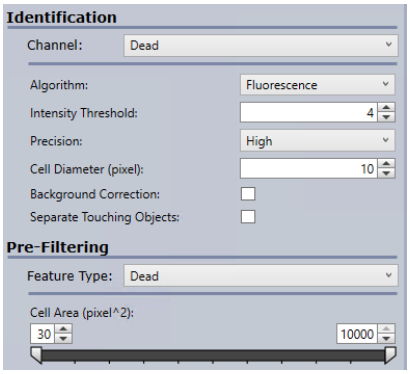
### 4.3.2 PS Ext (+) Analysis Setup




1. Intensity Threshold: select 2-3
2. Cell Diameter: select 13
3. Minimum Area: type 30

**Dead Settings**

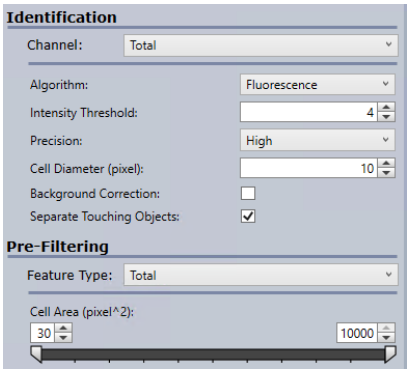
### 4.3.3 “Dead” Analysis Setup




1. Intensity Threshold: select 3-4
2. Cell Diameter: select 10
3. Minimum Area: type 30

**Total Settings**

### 4.3.4 “Total” Analysis Setup




1. Intensity Threshold: select 4
2. Cell Diameter: select 10
3. Separating Touching Objects: Checked
4. Minimum Area: type 30

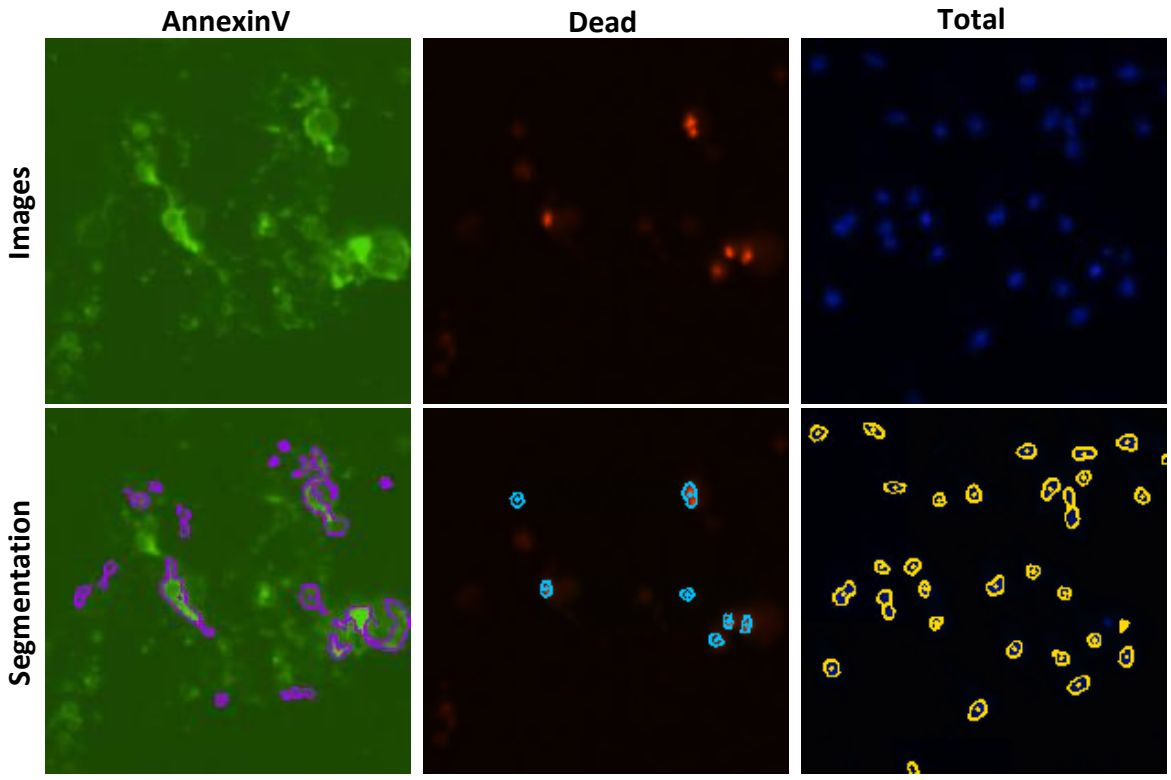
### 4.3.5 Well Selection and Start Analysis

1. On Plate Map, click “Selection” and select wells to analyze
2. Click “Start Analyze”

**Expected Segmentation:**

Use the “Image Display” and “Graphic Overlay” controls to visualize segmentation.


Typical images and fluorescent objects identification should look as follow:



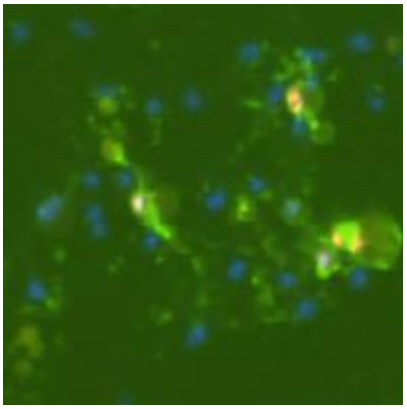
### 4.4 RESULTS Tab:

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.

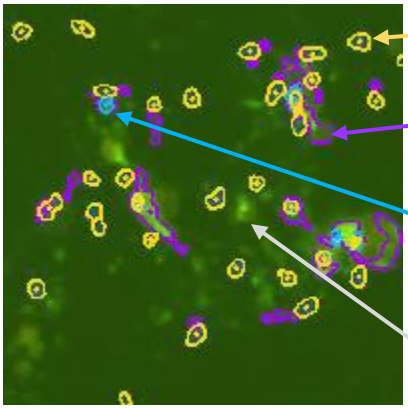
**Table of Data Reported**

| Name | PS Ext (+) corrected | PS Ext (+) | Dead | Total |
|---|---|---|---|---|
| **Percent (relative to Total)** | ● | ● | ● | |
| **Counts** | ● | ● | ● | ● |
| **Mean Intensity (AVE, SD)** | ● | ● | ● | ● |
| **Integrated Intensity (AVE, SD)** | ● | ● | ● | ● |

**PS Ext + Dead + Total**



**Segmentation Overlays**



Blue Nuclei with Yellow Graphic Overlay identifies all cells

Annexin V cell with green overlay super-imposing a blue nucleus

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

Annexin PS Ext overlay dismissed from analysis because not super-imposing a blue nucleus overlay

# 5.0 Celigo Software Settings for Expression Analysis: Target 1+2+3+Mask

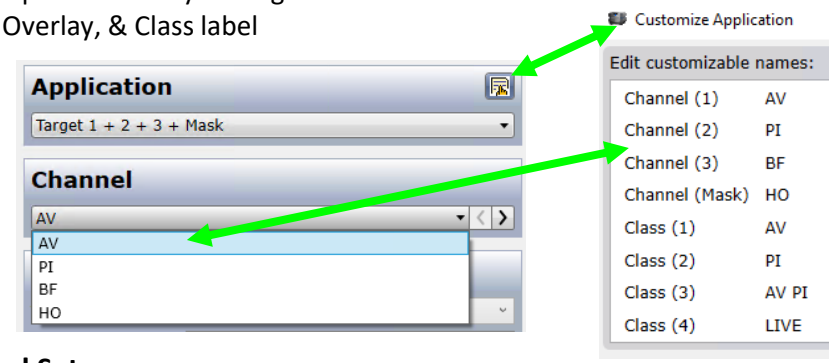
## 5.1 HOME and SETUP Tabs:

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

## 5.2 SCAN Tab:

### 5.2.1 Select an Application

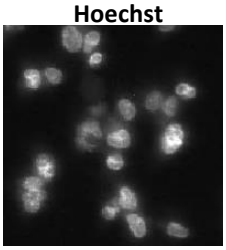
1. Select Celigo Application: Expression Analysis Target 1+2+3+Mask
2. Rename Channel, Graphic Overlay, & Class label




### 5.2.2 Mask "Hoechst" Channel Setup

1. Select Mask (HO) Channel
2. Select Blue Illumination
3. Exposure time should be around 80,000-100,000 µs
4. Click Focus Setup, then click Auto Register to register hardware focus position.
5. Adjust Illumination exposure. Ideal object pixel intensity should be between 100-150 with background signal no higher than 25 pixel intensity

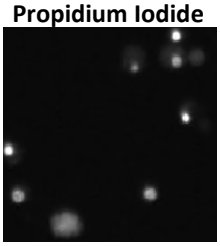
**Hoechst**



### 5.2.3 "Annexin V" Channel Setup

1. Select Annexin V (AV) Channel
2. Select Green Illumination
3. Exposure time should be around 50,000-90,000 µs
4. Click Find Focus, or adjust manually.
5. Click Set Offset
6. Adjust Illumination exposure. Ideal object pixel intensity should be between 100-150 with background signal no higher than 25 pixel intensity

**Propidium Iodide**

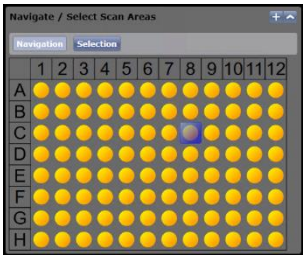


### 5.2.4 "Dead" Channel Setup

1. Select Red Illumination
2. Exposure time should be around 30,000-60,000 µs
3. Click Find Focus, or adjust manually.
4. Click Set Offset
5. Adjust Illumination exposure. Ideal object pixel intensity should be between 100-150 with background signal no higher than 25 pixel intensity

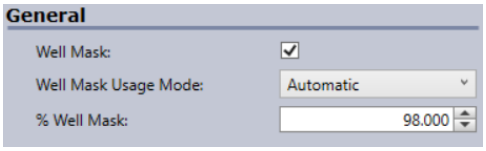
### 5.2.5 "Brightfield" Channel Setup

1. Select Brightfield (BF) Channel
2. Select Brightfield Illumination
3. Set Exposure time to Auto-Exposure Auto-Gain
4. Ideal background pixel intensity should be between 125-130
5. Click Find Focus, or adjust manually.
6. Click Set Offset
7. Click Selection button on plate map
8. Select wells to be imaged
9. Click Start Scan to begin image acquisition



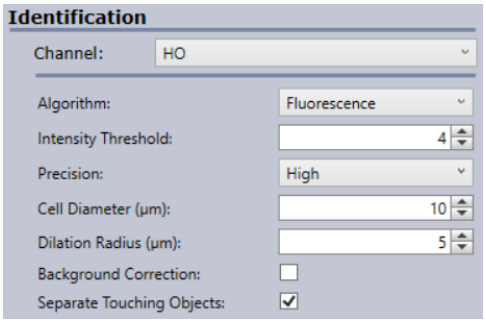

## 5.3 ANALYZE Tab:

### 5.3.1 General Section Analysis Setup



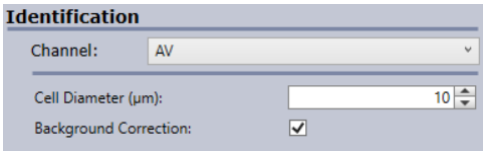

1. Select "Well Mask" and "Automatic" to exclude the outer part of the well
2. Shrink to 98%

### 5.3.2 Mask (HO) Analysis Setup




1. Turn ON Blue image display and graphic overlay
2. Turn OFF the other image displays and graphic overlay
3. Select a green well to view images
4. Adjust Intensity Threshold to ensure proper identification of the nucleus. Default value of "4" with "high Precision" works well for nuclear stains.
5. Set the "Cell Diameter" to "10"
6. Increase "Dilation Radius" to "5"
7. Select "Separate Touching Objects" to ensure proper separation of nuclei in close proximity.

### 5.3.3 Annexin V (AV) Analysis Setup



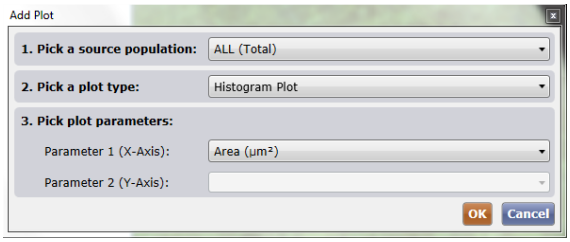

1. Switch to Annexin V (Green) Channel
2. Check Background Correction ON
3. Click Gate Tab

## 5.4 GATE Tab:

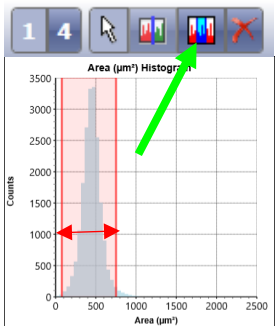
Similar to flow cytometry, we use the Celigo gating interface to identify sub-populations of cells in various phases of apoptosis. Plotting the size of the identified objects and the intensities of the Annexin V and Propidium Iodide is required.

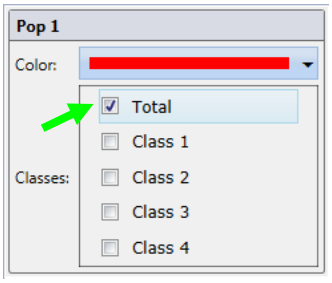
### 5.5.1 Histogram of Area Setup

1. In the gating area, select "+" to create a plot
2. Select "Histogram" plot type and choose "Area"



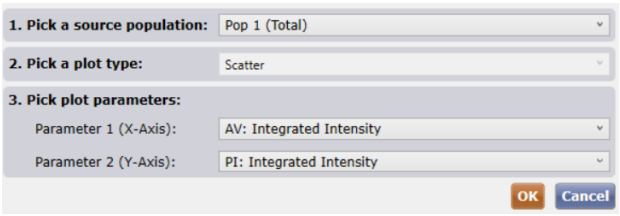

3. Select "Range" gate
4. Then draw (↔) over histogram area where majority of population is located in order to create "Pop 1" of objects. This excludes small and large objects.
5. Assign the gated cells to the "Total" class.



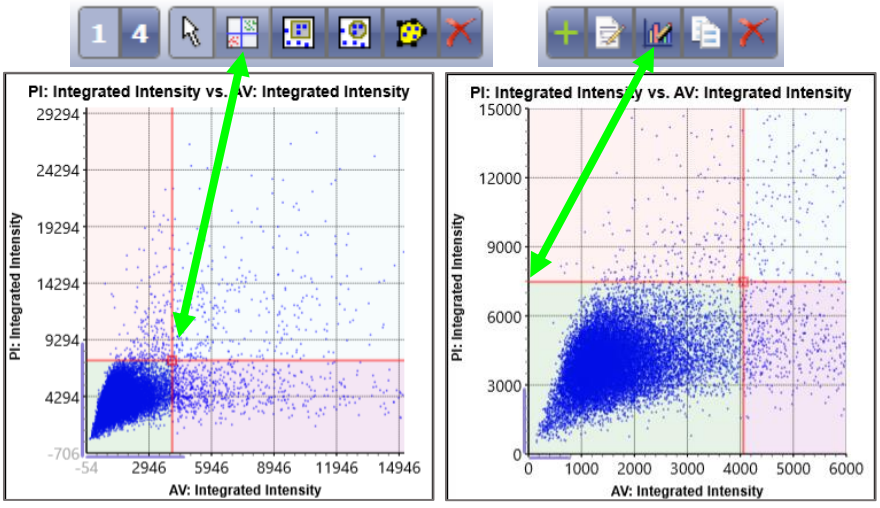

### 5.5.2 Scatter Plot of Green vs Red Mean Intensity Setup

1. Select "+" to create a plot
2. Select Scatter plot and select
3. Green: Mean Intensity for X-axis, and
4. Red: Mean Intensity for Y-Axis.



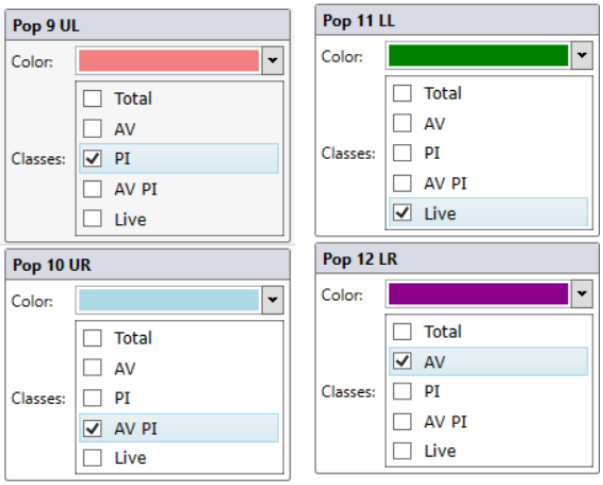

5. Select quadrant gate and click on scatter plot just above and left of blue dots.

6. Adjust xy-axis ranges to focus in on low intensity area. Adjust location of quadrant to best sit left of live population

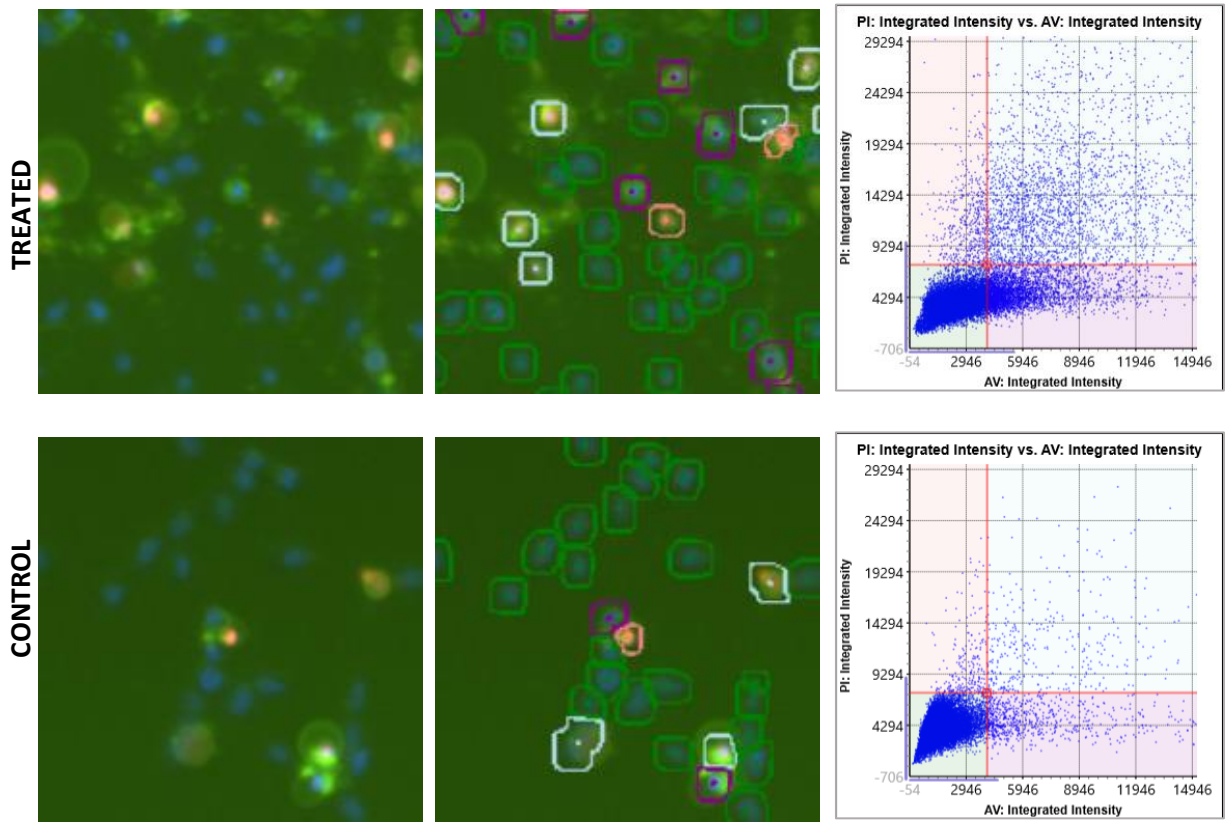


### 5.5.3 Check Populations for Reporting Subclasses

1. Check appropriate boxes to label classes for AV, PI, AV PI, and Live
2. Check control wells to verify quadrant is in correct location.
3. Select Start Analysis




**Example Segmentation, Plots and Gates:**



### 5.6 RESULTS Tab:

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) | • | • | • | • | • |

# 6.0 Additional Resources

## 6.1 Technical Support

- Celigo Learning Center (online) at www.nexcelom.com/celigo-learning
- Nexcelom Technical Support is available from 9am to 5:00pm EST.
  E-mail: support@nexcelom.com
  Phone: 978-327-5340

## 6.2 Storage and Handling

- For storage conditions please see the product label on each individual component.

## 6.3 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 6 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.

# 7.0 Ordering Information

## 7.1 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