PRODUCT INSERT

Instrument Compatibility: Celigo™

ViaStain™ Live Caspase 3/7 Detection for 2D/3D Culture

Part number: CS1-V0002-1

Test number: 2.5 x 96-well plates

Storage: 2 to 8 °C

Table of Contents

1 Introduction

1.1 Assay Description

The ViaStain™ Live Caspase 3/7 Detection for 2D/3D Culture kit is designed to detect caspase 3/7 activity in live cells. The ability to perform kinetic apoptosis assays allows researchers to continuously measure the caspase 3/7 activity within the cell population. This no wash assay has been shown to effectively work in both 2D and 3D cultures.

The caspase-3 substrate reagent consists of a nucleic acid-binding dye with a fluorescent probe that is attached to a four-amino acid peptide sequence DEVD (Asp-Glu-Val-Asp) forming a cell membrane-permeable DEVD-DNA complex. While the nucleic-acid dye is linked to the DEVD peptide sequence, the dye is unable to bind to DNA and remains non-fluorescent. During apoptosis, caspase 3/7 proteins cleave the DEVD-DNA dye complex and thereby release the high-affinity DNA dye, which translocates to the nucleus and binds to the DNA, producing a bright green, fluorescent signal.

1.2 Materials Supplied

Reagent	Catalog number	Volume	Number of Tests
Caspase 3/7	CS1-V0002-1	100 µL	2.5 x 96-well plates

1.3 Materials Required

- Conical tube
- Pipette and pipette tips
- (*optional*) Multichannel pipette
- Serological pipettes and pipette aide
- Phosphate Buffered Saline (1X PBS)
- 96-well flat, clear bottom, black walled plate *(Recommended:* Revvity ViewPlate #6005225*)*
- (*optional*) 96-well U-bottom ULA (ultra-low attachment) for spheroid formation *(Recommended:* Revvity CellCarrier ULA #6055330*)*
- Cells with appropriate media
    - For 2D cultures, suspension or adherent cells can be used. Go to section 2 for additional details and procedures.
    - For 3D/spheroid format, go to section 3 for additional details and procedures.

1.4 Instrument and Software Requirements

- Celigo™ Image Cytometer (4- or 5-channel)

2 Protocol for Caspase 3/7 Detection of 2D samples – adherent or suspension

2.1 Preparation of Control Samples

It is highly recommended to generate positive and negative controls for this assay, whether using adherent or suspension cells. Positive and negative controls should be processed at the same time using the staining and data acquisition procedures below.

- Positive control:
  - Expose cells to an apoptotic inducing pharmacological agent. For example, MDA-MB-231 cells were treated with 3 µM Staurosporine for a period of 5 to 7 h.
- Negative control:
  - Cells that are untreated (no drug) should be included in experimental design to determine the baseline caspase 3/7 activity.

2.2 Adherent Cell Plating and Staining Procedure

The Caspase 3/7 reagent can be used for continuous monitoring or as an endpoint measurement. See corresponding procedures below. The ViaStain™ Live Caspase 3/7 Detection for 2D/3D Culture should be used at a final concentration between 2 – 5 µM. For Celigo, we recommend a starting concentration of 2 µM. Volumes below are for a 96-well plate; adjust as needed for other formats.

2.2.1 Adherent Cell Plating

1. In a 96-well plate, seed approximately 5,000 – 10,000 adherent cells per well and allow the adherent cells to attach overnight.
   a. Prepare a cell suspension of 5-10 x 10^4 adherent cells/mL in 10 mL media.
   b. Pipet 100 µL of cells per well in 96-well plate.
      ***NOTE**: Outer wells of a 96-well plate can have significant variability, so we recommend avoiding them, if possible.*
   c. Incubate overnight at 37 °C with the appropriate CO_2.

2.2.2 Adherent Cell Caspase 3/7 Staining Procedure for Continuous Monitoring

1. Prepare a 2X solution (4 µM) of caspase 3/7 substrate in media.
   a. Add 40 µL of 1 mM caspase 3/7 substrate to 10 mL media.
2. Prepare drug treatments at a 2X concentration (100 µL drug per well).
3. Remove all media from adherent cells in wells.
4. Add 100 µL of the 2X caspase 3/7 to all wells.
5. Add 100 µL of 2X drug treatment to wells.
   ***NOTE**: Combining the 2X solutions from steps 4 and 5 in each well will result in final 1X caspase 3/7 and 1X drug treatment concentration.*
6. Incubate at 37 °C with the appropriate CO_2 for continuous monitoring throughout the experiment.
7. Image at multiple time points during the drug treatment on Celigo. See section 4 for Celigo parameters.

2.2.3 Adherent Cell Caspase 3/7 Staining Procedure as Endpoint Measurement

1. Treat cells with drugs according to experimental design.
2. Prepare a 2 µM caspase 3/7 master mix in PBS (use within 3 h of preparation):
    a. Add 40 µL of stock caspase 3/7 to 20 mL of PBS.
3. Once drug treatment is completed, aspirate all media from wells.
4. Pipet 200 µL of the master mix into each well.
5. Incubate for 30 min at 37 °C.
6. Image on Celigo. See section 4 for Celigo parameters.

2.3 Suspension Cell Plating and Staining Procedure

The Caspase 3/7 reagent can be used for continuous monitoring or as an endpoint measurement. See corresponding procedures below. The ViaStain™ Live Caspase 3/7 Detection for 2D/3D Culture should be used at a final concentration between 2 – 5 µM. For Celigo, we recommend a starting concentration of 2 µM. Volumes below are for a 96-well plate; adjust as needed for other formats.

2.3.1 Suspension Cell Plating and Caspase 3/7 Staining Procedure for Continuous Monitoring

1. In a 96-well plate, seed approximately 10,000 – 20,000 suspension cells per well with caspase 3/7 stain.
    a. Prepare a cell suspension of 1-2 x 10^5 cells/mL in 10 mL media.
    b. Add 40 µL of caspase 3/7 stock to cell suspension for a 4 µM (2X) concentration.
    c. Pipet 100 µL of cells per well in 96-well plate.
        ***NOTE**: Outer wells of a 96-well plate can have significant variability, so we recommend avoiding them, if possible.*
2. Prepare drug treatments at a 2X concentration (100 µL drug per well).
3. Pipet 100 µL of 2X drug treatment to wells.
    ***NOTE**: Combining the 2X solutions from steps 1 and 3 in each well will result in final 1X caspase 3/7 and 1X drug treatment concentration.*
4. Centrifuge plate (quick 200-400 x g) to settle cells.
5. Incubate at 37 °C with the appropriate CO_2 for continuous monitoring throughout the experiment.
6. Image at multiple time points during the drug treatment on Celigo. See section 4 for Celigo parameters.

2.3.2 Suspension Cell Plating and Caspase 3/7 Staining Procedure as Endpoint Measurement

1. In a 96-well plate, seed approximately 10,000 – 20,000 suspension cells per well.
    a. Prepare a cell suspension of 1-2 x 10^5 cells/mL in 10 mL media.
    b. Pipet 100 µL of cells per well in 96-well plate.
        ***NOTE**: Outer wells of a 96-well plate can have significant variability, so we recommend avoiding them, if possible.*
2. Prepare drug treatments at a 2X concentration (100 µL drug per well).
3. Pipet 100 µL of 2X drug treatment to wells.
    ***NOTE**: Combining the 2X solutions from steps 1 and 3 in each well will result in final 1X drug treatment.*
4. Incubate at 37 °C with the appropriate CO_2 for drug treatment according to experimental design.

5. Prepare a 4 µM (2X) caspase 3/7 substrate solution by adding 40 µL caspase 3/7 substrate to 10 mL of PBS.
6. Centrifuge plate (quick 200-400 x g) to settle cells.
7. Remove 100 µL from each well, being careful not to disturb cells at well bottom.
8. Pipet 100 µL of 2X caspase 3/7 substrate solution to each well.
9. Incubate for 30 min at 37 °C.
10. Image on Celigo. See section 4 for Celigo parameters.

    ***NOTE**: If cells have clumped together or rolled to well edge, pipet up/down to resuspend cells and quick centrifuge at 200-400 x g to settle cells evenly on bottom.*

3 Protocol for Caspase 3/7 Detection of 3D samples - spheroid models

3.1 Preparation of Control Samples

- Positive control:
  - Expose 3D model (spheroids) to an apoptotic inducing pharmacological agent.
- Negative control:
  - 3D cell models (spheroids) that are untreated (no drug) should be included in experimental design to determine the baseline caspase 3/7 activity.

3.2 Preparation of Multicellular Tumor Spheroids (MCTSs)

Prepare MCTSs in either flat bottom or U-bottom plates 96-well plates.

> ***NOTE**: Outer wells of a 96-well plate can have significant variability, so we recommend avoiding them, if possible.*

3.3 Spheroid Plating and Staining Procedure

The Caspase 3/7 reagent can be used for continuous monitoring or as an endpoint measurement. See corresponding procedures below. The ViaStain™ Live Caspase 3/7 Detection for 2D/3D Culture should be used at a final concentration between 2 – 5 µM. For Celigo, we recommend a starting concentration of 2 µM. Volumes below are for a 96-well plate; adjust as needed for other formats.

3.3.1 Spheroid Caspase 3/7 Staining Procedure for Continuous Monitoring

1. Prepare a 4X concentration of caspase 3/7 substrate (8 µM) in media.
   a. Add 40 µL caspase 3/7 substrate to 5 mL of PBS.
2. Prepare drug treatments at a 4X concentration (100 µL drug per well).
3. Adjust well volume so that each wells contains 100 µL media.
4. Add 50 µL of the 4X caspase 3/7 to all wells.
5. Add 50 µL of 4X drug treatment to wells.

   ***NOTE**: Combining the 4X solutions from steps 4 and 5 into each well already containing 100 µL of media will result in final 1X caspase 3/7 (2 µM) and 1X drug treatments.*
6. Incubate for 60 min at 37°C.
7. Image at multiple time points during the drug treatment on Celigo. See section 5 for Celigo parameters.

3.3.2 Spheroid Caspase 3/7 Staining Procedure as Endpoint Measurement

1. Prepare a 2X master mix of 4 µM caspase 3/7 substrate.
   a. Add 40 µL caspase 3/7 stock to 10 mL of PBS.
2. Add 100 µL of the 2X caspase solution to each well of a 96-well plate containing 100 µL of media with spheroid/cells.
3. Incubate for 60 min at 37 °C.
4. Image on Celigo. See section 5 for Celigo parameters.

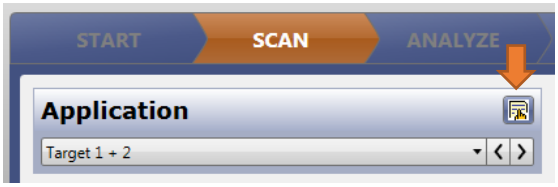
4 Celigo Parameters for Caspase 3/7 Detection of 2D samples

4.1 START Tab

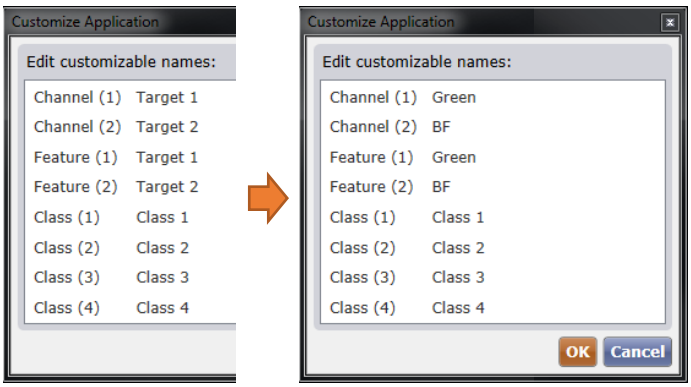
1. Select Create a New Scan.
2. Select appropriate Plate Category and Vendor Type.
3. Type in a Plate ID or select one already created.
4. Click Load Plate.

4.2 SCAN Tab

4.2.1 Select an application



1. Select Application Expression Analysis Target 1+2.
2. Customize the channels by clicking on the "Customize Analysis Application" button.
    a. Change channel and feature names to the following: Target 1 (Green), Target 2 (Brightfield).
    b. Click OK.



4.2.2 Brightfield Channel Setup

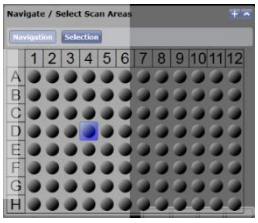
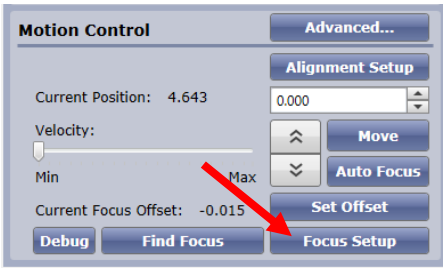
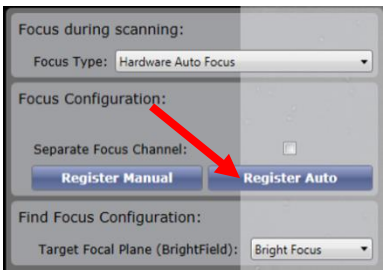


Plate Map Navigation Area

1. Select a well with enough caspase 3/7+ cells (such as a treated well) by clicking on plate map navigation area.
2. Change Channel to BF. Ensure that the Type is set to Auto Exposure/Gain Channel.
3. Change the illumination to Brightfield.
4. Click Apply for Auto Exposure in Brightfield illumination.

5. Click Focus Setup.
6. Register Auto (with Hardware Auto Focus).
7. Close dialog by clicking Focus Setup.

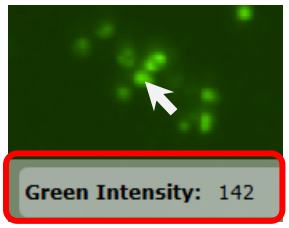


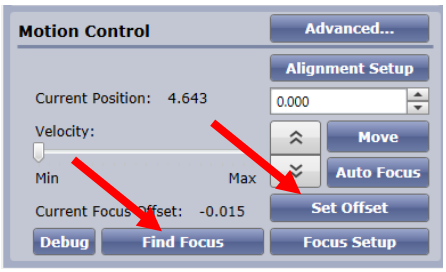


4.2.3 Green Channel Setup

1. Change Channel to Green.
2. Change illumination to Green fluorescence.
3. Set illumination exposure time for green channel to 50,000 µs.
    - This exposure time should correspond to objects (apoptotic cells) average pixel intensity values between 100-150 RFUs. We recommend an exposure of 50,000 – 80,000 µs.
4. Click Find Focus or manually adjust z-focus with arrows to find the correct focus.
5. Click Set Offset once correct focal plane is found.
6. Click Selection on plate map navigation area and highlight wells for imaging.
7. Click Start Scan.

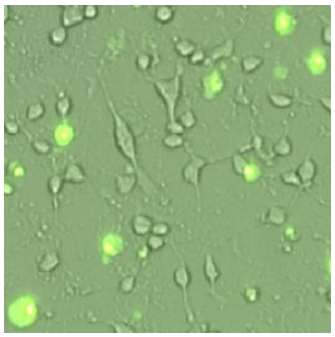
Hover mouse over object to observe pixel intensity display



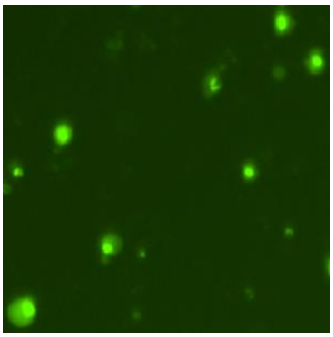


Images of different channel combinations:

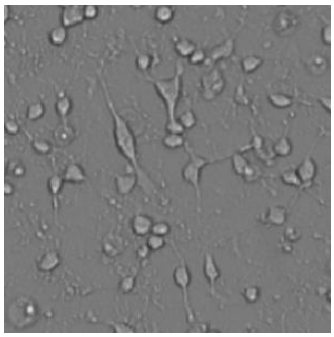
Caspase 3/7 + BF



Caspase 3/7 only



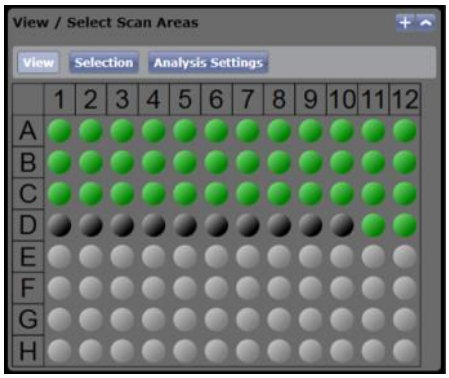
BF only



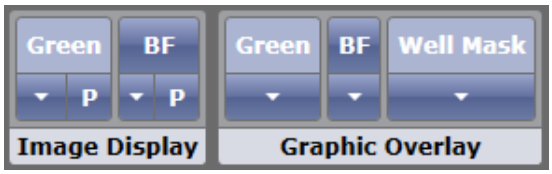
4.3 ANALYZE Tab

4.3.1 Green Channel Analysis Setup

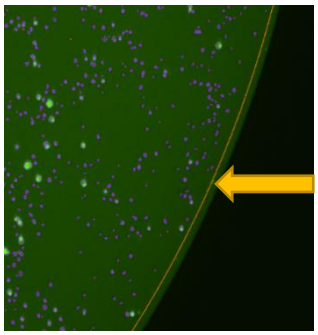
As images are acquired for each well, the plate navigation map will show green wells for images saved to database.

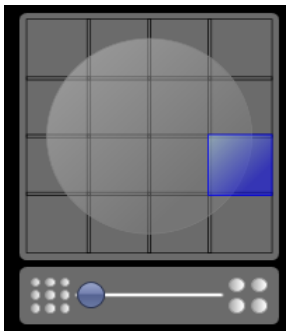


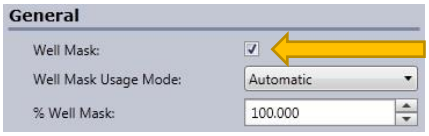
1. Select a green well in plate map navigation area to view image in display area.
2. Turn On Green Graphic Overlay, turn OFF BF Graphic Overlay so that you are only viewing green graphic overlay.
    - Light blue indicates ON
    - Dark blue indicates OFF



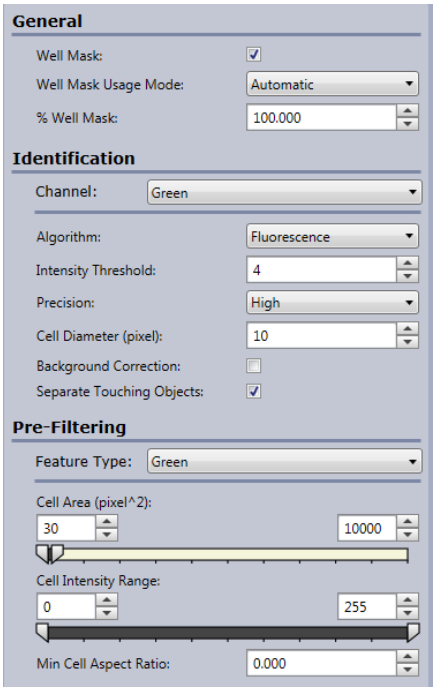
3. Click on an edge image in Well Map to view edge of well.
4. Check box ON for Well Mask, to exclude object detection outside well area.







5. Change Identification Channel to Green.
6. Select Algorithm of Fluorescence (Default).
7. Adjust Intensity Threshold value to properly detect (outline) nuclei in image.
    - Higher values detect only brighter objects
    - Lower values will include more dim objects
8. Adjust Cell Diameter (µm) to be the average expected diameter of object.
    - Higher values will expand current objects area
    - Lower values will decrease current objects area
9. Check box Separate Touching Objects to separate objects that are close together and identified as one object.
10. In Pre-filtering, increase lower limit of Cell Area to eliminate objects smaller than object of interest. Suggested minimum values of 30-50 Cell Area (pixel^2).



4.3.2 Brightfield Analysis Setup

1. Change the identification channel to BF.
2. Turn ON BF Graphic Overlay, turn OFF Green Graphic Overlay so that you are only viewing BF graphic overlay.
3. Select Algorithm Bright field.
4. Adjust Intensity Threshold and Cell Diameter to properly detect BF Cells.
5. Adjust Pre-filtering values as needed.
6. Once the analysis parameters for Green and BF are optimized, click Start Analysis.

4.4 RESULTS Tab

1. Double click on a well in the results page to view the whole-well image.
2. Scroll in to see high resolution images and graphic overlay.
3. Once data analysis is finished, click Export Well-Level Data into CSV file format.
4. Data reported are counts, standard deviation, average mean intensities, integrated intensity measurements of all channels, and analysis parameters for current analysis.

5 Celigo Parameters for Caspase 3/7 Detection of 3D Samples (Spheroids)

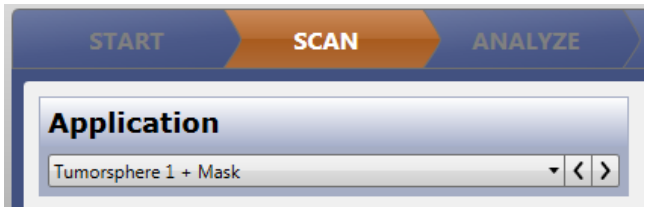
5.1 START Tab

1. Select Create a New Scan.
2. Select appropriate Plate Category and Vendor Type.
3. Type in a Plate ID or select one already created.
4. Click Load Plate.

5.2 SCAN Tab

5.2.1 Select an application

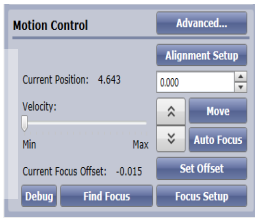
1. Select Application Tumorsphere 1 + Mask.

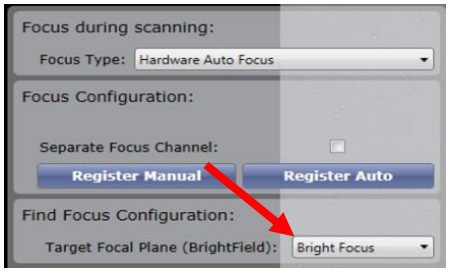


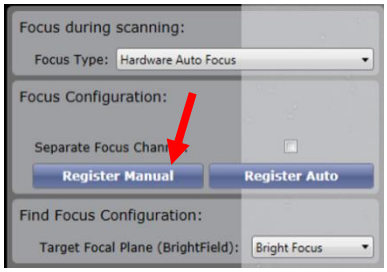
5.2.2 Mask (Brightfield) Channel Setup

1. Select a well with sufficient amount of caspase 3/7+ cells (such as a treated well) by clicking on plate map navigation area.
2. Change Channel to Mask.
3. Change the illumination to Brightfield. Ensure that the Type is set to Auto Exposure/Gain Channel.
4. Click Apply for Auto Exposure in Brightfield illumination.
5. Click Focus Setup.
6. Turn on Live camera.
7. Manually adjust focus to get crisp outer edge of spheres with up/down arrows (see images below).
8. Register Manual (with Hardware Auto Focus).
9. Close dialog by clicking Focus Setup.

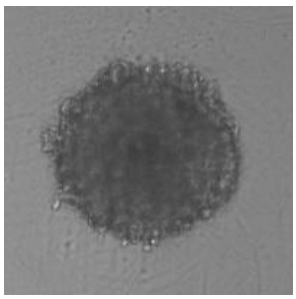
Plate Map Navigation Area



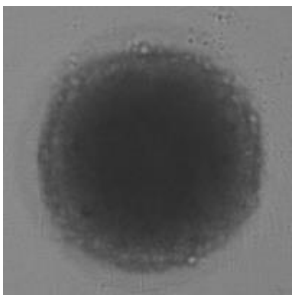




Crisp Sphere Edge (GOOD)



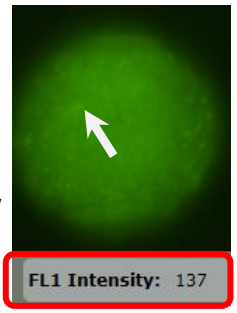
Blurry Sphere Edge (BAD)



5.2.3 Green Channel Setup

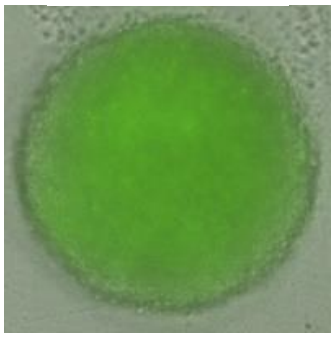
1. Change Channel to FL1.
2. Change illumination to Green fluorescence.
3. Set illumination exposure time for green channel to 30,000 µs.
    - This exposure time should correspond to objects (caspase3/7+ cells or spheres) average pixel intensity values between 100-150 RFUs
4. Click Selection on plate map navigation area and highlight wells for imaging.
5. Click Start Scan.

Hover Mouse over object to observe Pixel Intensity Display

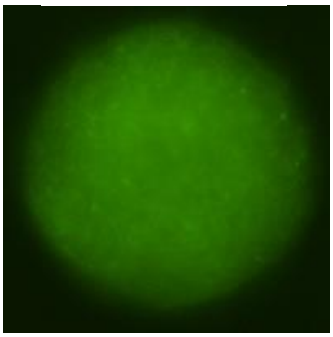


Images of different channel combinations for a drug treated Caspase 3/7 positive sphere:

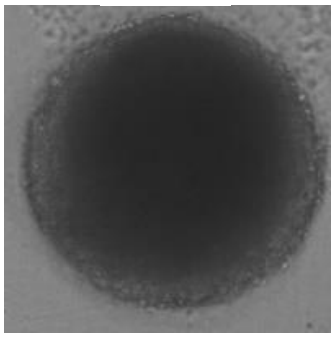
Caspase 3/7 + BF



Caspase 3/7 Only

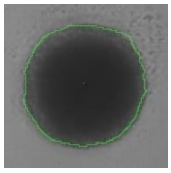


BF Only



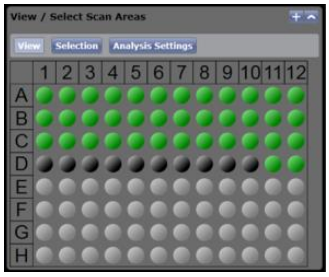
5.3 ANALYZE Tab

5.3.1 Brightfield (Mask) Analysis Setup

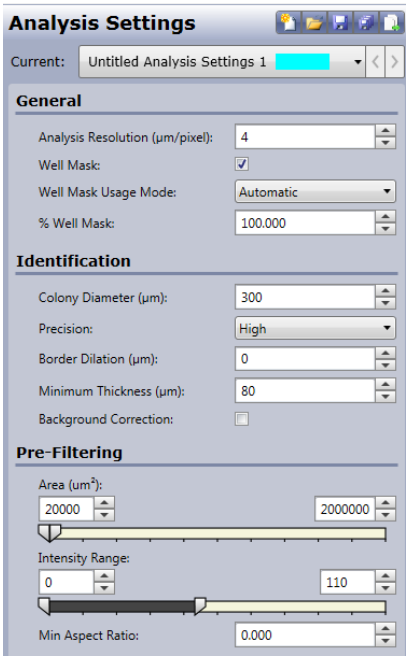


The “Mask” channel is a single graphic overlay mask used to identify objects in one channel, typically the last channel. The Mask channel is used as a Total object count. Additional channels will use the mask channel graphic overlay to measure fluorescent and brightfield intensity for each object. Therefore, analysis parameters for the mask channel are the only ones needing to be optimized in Analyze Tab.

As images are acquired for each well, plate navigation map with show green wells for images saved to database.



1. Select a green well in plate map navigation area to view image in display area.
2. Turn On Mask Graphic Overlay, turn OFF FL1 Graphic Overlay so that you are only viewing Mask graphic overlay (green outline around sphere).
    - Light blue indicates ON
    - Dark blue indicates OFF
3. Check box ON Well Mask, to exclude object detection outside well area.
4. Select an average Colony Diameter value.
5. Adjust Precision for amount of detail needed at edge of sphere.
6. Adjust Minimum Thickness for desired roundness.
    - Increasing values will smooth out long protrusions on spheres
    - Decreasing values will create more jagged edges on sphere
7. Turn ON Background correction if needed.
8. Click Start Analysis.



5.4 RESULTS Tab

1. Double click on a well in the results page to view the whole-well image.
2. Scroll in to see high resolution images and graphic overlay.
3. Once data analysis is finished, click Export Well-Level Data into CSV file format.
4. Data reported are well counts, average mean intensities of all channels, integrated intensity measurements, and analysis parameters for current analysis.

6 Additional Resources

6.1 Citations

1. Cohen GM. (1997) Caspases: the executioners of apoptosis. *Biochem J*. 326: 1–16.
2. Cen H, et al. (2008) Devd-Nucview488: A Novel Class of Enzyme Substrates for Real-Time Detection of Caspase-3 Activity in Live Cells. *FASEB J.* 22(7):2243-2252.
3. Elmore S. (2007). Apoptosis: A Review of Programmed Cell Death. *Toxicol. Pathol*. 35(4): 495-516.
4. Rastogi RP, *et al.* (2009). Apoptosis: Molecular Mechanisms and Pathogenicity. *EXCLI Journal*. 8:155-181

6.2 Quick Reference Recommended Exposures and Settings

Image plate at the suggested following exposures to obtain object pixel intensities between 100-150. These values are not absolute and may require further optimization.

- Caspase 3/7 – Total Channel @ 30,000 µs, Focus Setup with HWAF (hardware-based autofocus) or IBAF (image-based autofocus)

7 Storage and Handling

Store ViaStain™ Live Caspase 3/7 Detection for 2D/3D Culture between 2 to 8 °C for long term storage. Please consult the Safety Data Sheet for more safety information, found on **www.revvity.com/cellcountingreagents**.

8 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.

9 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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