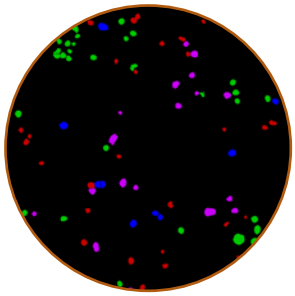
Molecular Devices

High-content analysis of the various stages of apoptosis with the MetaXpress Cell Health Application Module

TOTAL IMAGING SOLUTION APPLICATION NOTE #1



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INTRODUCTION

The study of cell health has become critical for drug discovery and development. In some diseases, such as cancer, mutations may cause apoptotic pathways to malfunction, allowing uncontrolled proliferation of tumorigenic cells. In contrast, premature cell death is a problem in many neuronal diseases including Parkinson's and Alzheimer's, as well as in many immune and autoimmune diseases.[1, 2, 3]

Cell-based assays provide an efficient method of modeling the toxicological effects of drugs and for discriminating anti-proliferative effects from induction of apoptosis or necrosis. Identification of the mechanism of cell death may allow for modification of the potential drug and prevention of unintentional toxicity. A variety of commercially available fluorescent probes have been developed to distinguish the various stages of cell health.

The Cell Health Application Module for MetaXpress™ software from Molecular Devices is designed for the analysis of cell-based assays of apoptosis and necrosis using three different dyes. The software module was designed with the flexibility to operate with multiple dye combinations and allows the obtainment of multiple customizable cell-by-cell outputs. A simple interface minimizes set-up efforts and provides the flexibility to customize settings to suit a specific experiment. The module includes Adaptive Background Correction™ (ABC) to provide more robust segmentation and assay repeatability and correct uneven backgrounds throughout the image by adapting to local content.

This study illustrates the flexibility and simplicity of the MetaXpress Cell Health module using the Vybrant® #7 assay kit, a homogenous assay using various nuclear dyes as indicators of Cell Health. For more information on the Cell Health Application Module, download the data sheet at www.moleculardevices.com/pdfs/MX_CellHealth_Datasheet_Rev_B.pdf. For more information on other assays tested with the Cell Health Application Module, download the poster "Quantitation of Apoptosis, Necrosis, and Cell Death Using High Content Screening" at www.moleculardevices.com/pdfs/SBSSBS04_LiveDead_CellHealth.pdf.

MATERIALS

- Cells: DU 145 cells (ATCC Cat. #HTB-81)
- Cell culture media: HyQ/DME media with 10% dFCS (Fisher Scientific Cat. #SH3024301 and #SH3008803HI)
- PBS (Fisher Scientific Cat. #BW17512F)
- Staurosporine (Sigma Cat. #S6942)
- DMSO (Sigma Cat. #D2438)
- Vybrant #7 Kit (Molecular Probes Cat. #V23201)
- Plates: 96-well Black/Clear Assay Plates with lids, tissue-culture treated polystyrene, flat-bottom (Corning/Costar Cat. #3603)

METHODS

Cell culture

Grow adherent DU 145 cells in HyQ/DME media with 10% dFCS at 37°C, 5% CO_2. Split cells 1–2 times a week at a ratio of 1:2 to 1:5.

Cell health assay

Step 1: Before each assay, plate DU 145 cells in 96-well plates at ~7,000 cells per well. Incubate overnight at 37°C in 5% CO_2.

Step 2: Add to cells varying concentrations (serial dilutions from 0.05–2 µM) of staurosporine or vehicle control (DMSO) diluted in cell culture medium. Incubate at 37°C, 5% CO_2 for up to 16 hours.

Step 3: Dilute Vybrant #7 probes (Hoechst 33342, YO-PRO-1, and PI) in PBS according to the kit protocol.

Step 4: Add diluted probes to each well and incubate for 30 minutes.

Step 5: Acquire images on the ImageXpress$^{MICRO_{TM}}$ system using MetaXpress image acquisition software (all Molecular Devices imaging platforms are compatible with the Cell Health Application Module). Filters compatible with the Vybrant #7 dyes are indicated in Table 1. Use the laser autofocus to increase acquisition speed if available.

Table 1 shows the fluorescent filter combinations used for acquisition.

Table 1. Filter Sets Used For Imaging Vybrant #7 Fluorophores

Fluorescent Probe	Filter Set
Hoechst 33342 (H33342)	BrightLine® Dapi-5060B (Semrock)
YO-PRO-1	BrightLine FITC-3540B (Semrock)
Propidium Iodide (PI)	Texas Red TXRED-4040B (Semrock)

Automated image analysis

For more information on the MetaXpress Cell Health Application Module, download the "Cell Health Application Module for MetaXpress" data sheet on our web site at www.moleculardevices.com/pdfs/MX_CellHealth_Datasheet_Rev_B.pdf

Select three wavelengths within the Cell Health Application Module dialog. (See Figure 2.) W1 (Wavelength 1) corresponds to a wavelength that could be used to identify all nuclei. W2 corresponds to a dye used to identify cells in early or late apoptosis and W3 for cells in late apoptosis or necrosis. For W2 and W3, dyes staining the cytoplasm, the nucleus or both compartments can be chosen and these locations indicated to the software for better image analysis. For the Vybrant #7 kit, H33342 is used for W1, YO-PRO-1 or H33342 is used for W2 and PI is used for W3. For each of the wavelengths, choose appropriate size constraints and contrast levels for identifying positive nuclear staining.

Tables 2 and 3 show all site-by-site and cell-by-cell parameters that can be determined by the Cell Health Application Module including the measurements used in this application note.

Table 2. Site-by-Site Measurements Available With the MetaXpress Cell Health Application Module†

Measurement	Description
Total Cells	Total number of cells detected in the All nuclei image (W1)
Viable Cells, **% Viable Cells**	Total number and percent of cells not apoptotic or necrotic
Early Apoptotic Cells, **% Early Apoptotic Cells**	Total number and percent of cells positive for Apoptotic staining (W2) but not Dead staining (W3)
Late Apoptotic Cells, **% Late Apoptotic Cells**	Total number and percent of cells positive for Apoptotic staining (W2) and Dead staining (W3)
Necrotic Cells, **% Necrotic Cells**	Total number and percent of cells negative for Apoptotic staining (W2) but positive for Dead staining (W3)
All Cells Total Area, All Cells Mean Area	Total and average area of all nuclei detected in the All nuclei image (W1)
All Cells W1 Integrated Intensity, All Cells W1 Average Intensity	Total and average intensity measured in the W1 source image using the nuclear areas
All Cells W2 Integrated Intensity, All Cells W2 Average Intensity	Total and average intensity measured in the W2 source image using the nuclear areas
All Cells W3 Integrated Intensity, All Cells W3 Average Intensity	Total and average intensity measured in the W3 source image using the nuclear areas

† Note: Specific measurements used for this assay are shown in **color**.

Table 3. Cell-by-Cell Measurements Available With the Cell Health Application Module

Measurement	Description
Cell: Nuclear Area	The nuclear area measured in μm^2
Cell: W1 Integrated Intensity, Cell W1 Average Intensity	Total and average intensity measured in the W1 source image using this nuclear area
Cell: Health Classification	Classification of the nucleus as either Viable, Early Apoptotic, Late Apoptotic or Necrotic
Cell: W2 Integrated Intensity, Cell W2 Average Intensity	Total and average intensity measured in the W2 source image using this nuclear area
Cell: W3 Integrated Intensity, Cell W3 Average Intensity	Total and average intensity measured in the W3 source image using this nuclear area

Log the measurements to the MDCStore™ database or Microsoft Excel® for further evaluation. For IC_{50} determination, use AcuityXpress™ cellular informatics software, which is integrated with the MDCStore database.

RESULTS

DNA stains for the detection of apoptotic cells

High-content screening allows differentiation of subpopulations of cells, distinguishing live, necrotic, and early and late apoptotic cells within the same well. There is a variety of commercially available fluorescent

probes suitable for detecting and quantifying cells undergoing either apoptotic or necrotic cell death. These probes vary in selectivity, branch of the pathway being monitored, signal-to-noise ratio (S/N) and ease-of-use.

During apoptosis, both the nuclear envelope and chromatin are degraded. The Vybrant #7 apoptosis assay kit uses three distinct fluorescent probes specific to DNA to detect this degradation.

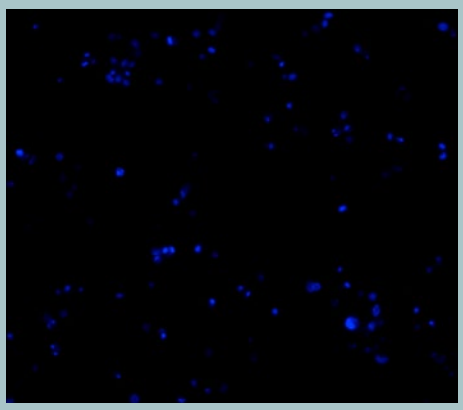
Hoechst 33342 enters all cells regardless of cell health. The average intensity of DNA labeling substantially increases in apoptotic cells as the nuclei condense early in apoptosis. Therefore, Hoechst 33342 can be used as both a marker for all nuclei as well as for apoptotic nuclei. Hoechst 33342 is excited by UV light and emits blue light.[4]

YO-PRO-1 is selectively taken up by apoptotic cells. YO-PRO-1 is excited by blue light and emits green light.[5]

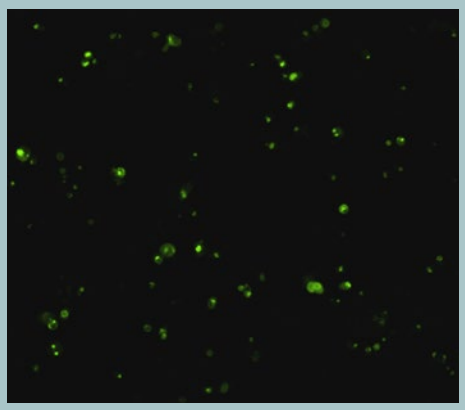
Propidium Iodide (PI) is membrane-impermeant and generally excluded from viable cells. During late stage apoptosis and necrosis, the membrane increases its permeability allowing PI to enter the cell and label DNA. PI is excited by green light and emits red light.[6]

DU145 cells were grown, treated with either DMSO or staurosporine for 12 hours, and labeled with the fluorescent markers as indicated in the methods. The ImageXpress^{MICRO} system was used with automated multiwavelength acquisition to acquire three fluorescent images for each site using the fluorescent filters described in Table 1.

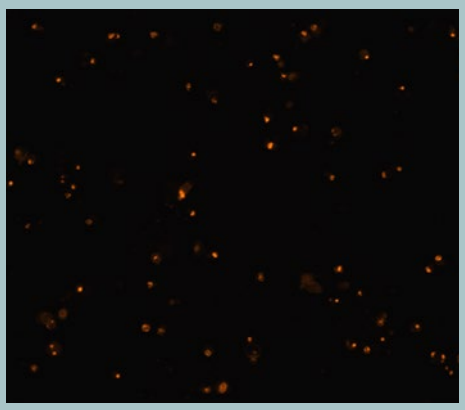
fluorescent images produced by the Vybrant #7 kit (figure 1)



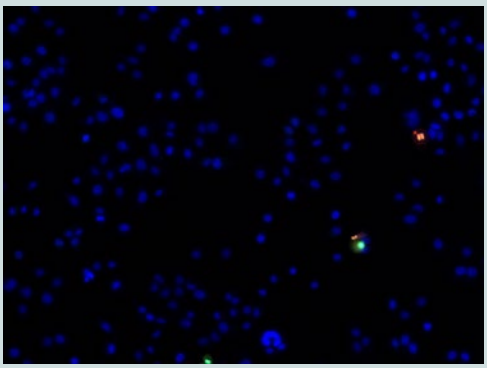
Hoechst 33342



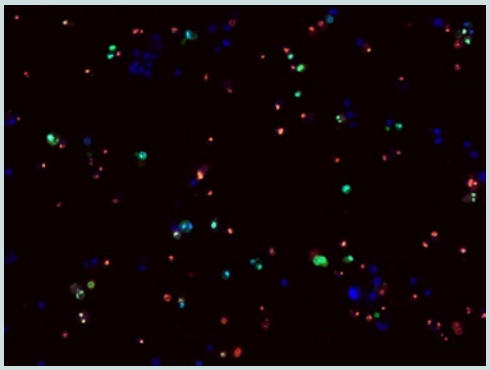
YO-PRO 1



Propidium Iodide



DMSO (overlay of 3 dyes)

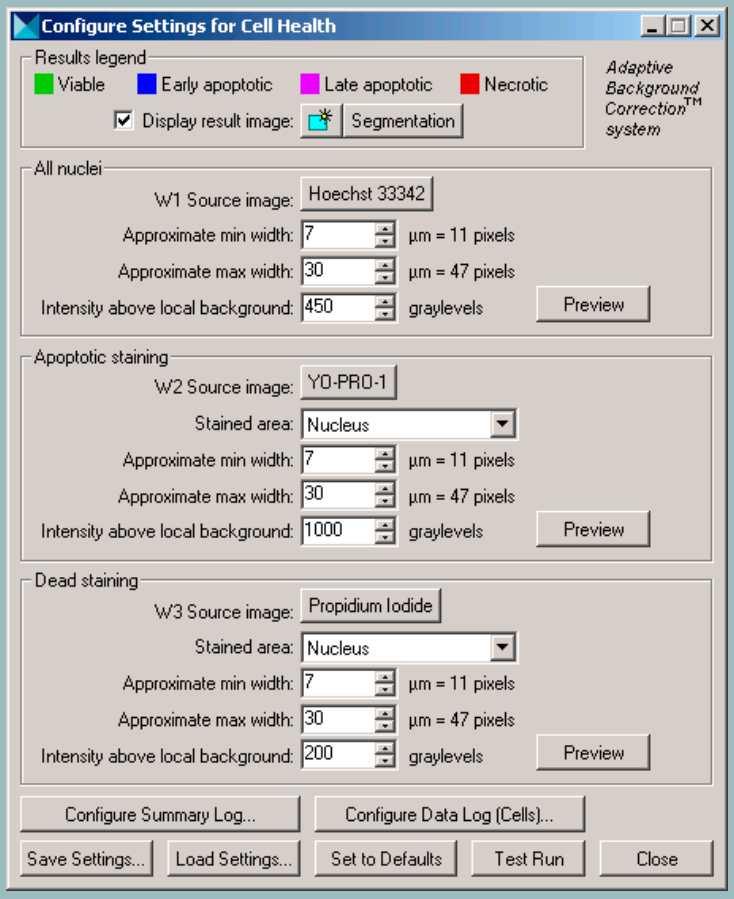


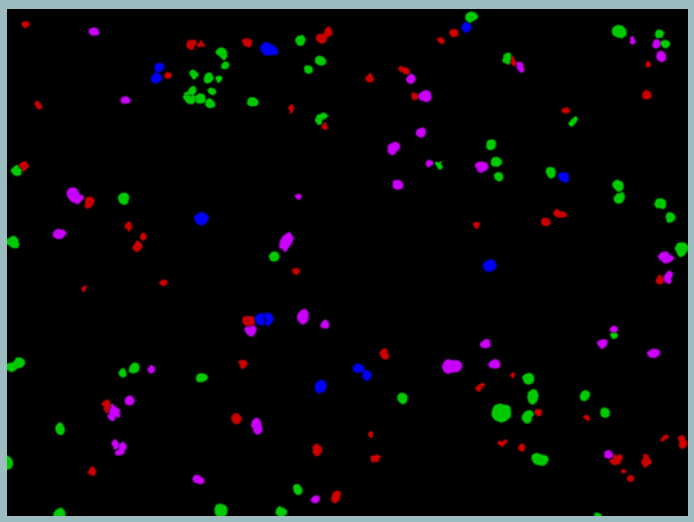
1 μM staurosporine (overlay of 3 dyes)

As shown in Figure 1, very distinct staining patterns were obtained with the three dyes in the presence of the maximum concentration of staurosporine (single channel images on the top of the Figure 1 or their overlay on the bottom) whereas very little YO-PRO1 and PI staining were present in untreated cells (overlay of DMSO vehicle-treated cells on the bottom) indicating the specificity of the dyes for various phases of the apoptotic process and the ability of the imaging system to separate signals from the dyes.

In order to automatically analyze the images, the Cell Health Application Module was chosen for its flexibility and ease of use. (See Figure 2.) Indeed, application modules allow for flexible, simple customization of fluorescent markers used and cell compartments labeled. The Cell Health Application Module uses Adaptive Background Correction to correctly segment positive staining cells in each of the wavelengths chosen. Three user-configurable parameters are used for each wavelength, as well as the specific compartments labeled. "Approximate min width" and "Approximate max width" specify the size range expected for each stained compartment. "Intensity above local background" is adjusted as appropriate for optimal detection of positively stained cells. Specific site-by-site and cell-by-cell measurements are user-selectable for logging to the MDCStore database, Microsoft Excel, or a text file. Tables 2 and 3 show the measurements available in the Cell Health Application Module. Analysis results stored in the MDCStore database may be used with AcuityXpress informatics software for data mining and visualization.

Cell Health Application Module for MetaXpress (figure 2)





Left: A simple dialog is used to configure the Cell Health Application Module. The module easily differentiates the early stages of apoptosis and can be run with any combination of dye corresponding to the 4 phenotypes. Source image names refer to filter sets used.

Top: Cells were treated with 1 µM staurosporine for 12 hours and stained with Hoechst 33342, YO-PRO-1 and PI. Image analysis results are shown as a colored overlay on the source images. Viable nuclei are shown in green. Early apoptotic nuclei are shown in blue. Late apoptotic nuclei are shown in purple. Necrotic nuclei are shown in red.

Nuclear condensation is one of the earliest steps of apoptosis, allowing this assay to differentiate between early and very early apoptosis. Nuclear condensation is seen as an increased intensity of the Hoechst 33342 signal. Uptake of YO-PRO-1 happens at a later point in apoptosis but prior to membrane permeabilization. A comparison of image analysis using brightly stained Hoechst 33342 versus YO-PRO-1 identified a significant number of cells with condensed nuclei but no detectable staining with YO-PRO-1. (See Figure 2.)

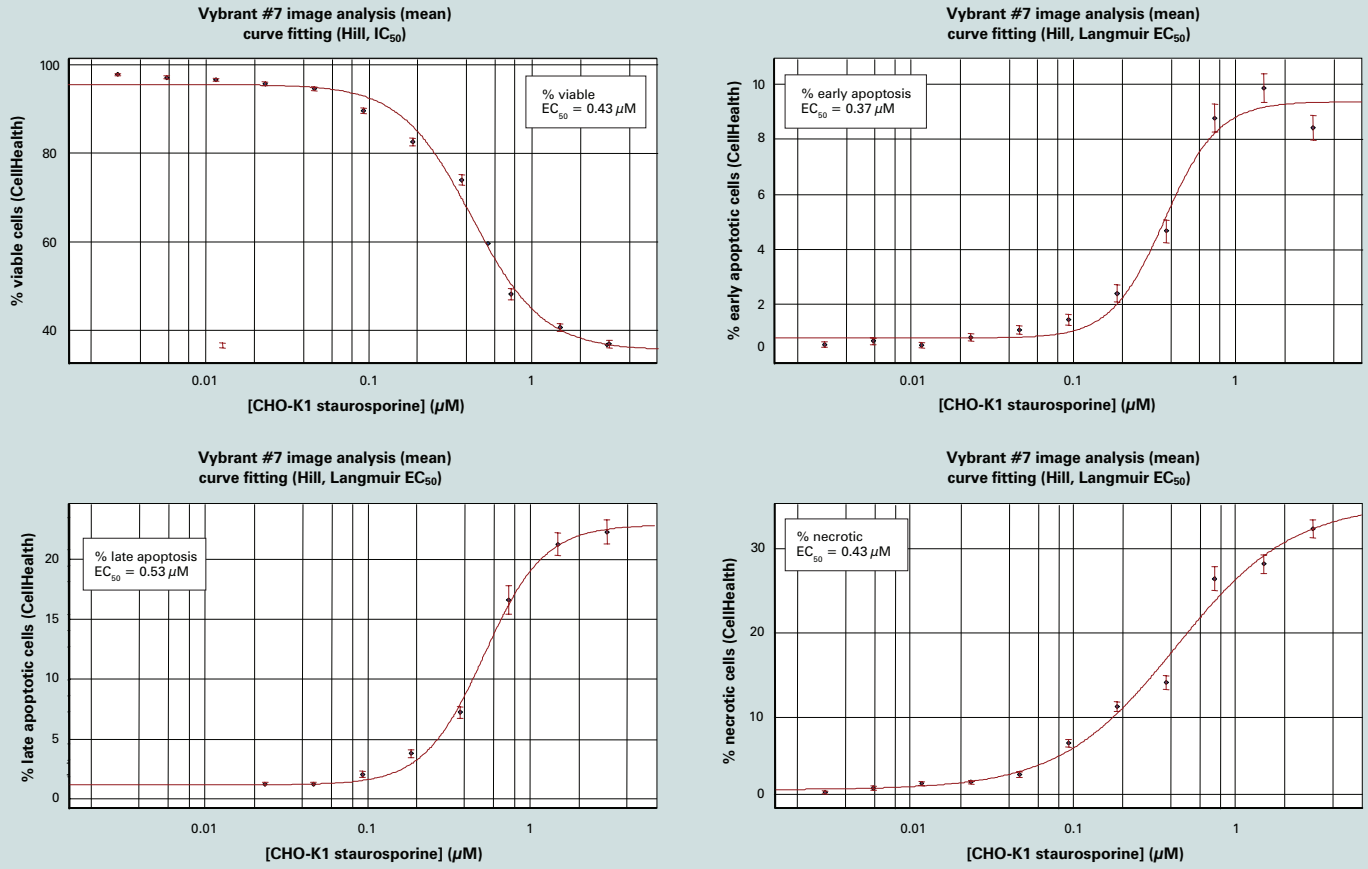
Dose-Response Analysis

AcuityXpress was used for curve-fitting of the various dose responses obtained from the analysis. The Cell Health Application Module is used to generate robust dose-response data with the Vybrant #7 kit. After 12 hours treatment with varying concentrations of the kinase inhibitor staurosporine, cell viability dramatically decrease at concentrations between 0.1 and 2 µM. (See Figure 3.) The results also demonstrated a concentration-dependent shift of cellular phenotypes, with more cells in early apoptosis at the lowest concentrations, and more cells in late apoptosis at 0.3 µM and higher concentrations of staurosporine.[7]

CONCLUSIONS

This experiment shows the ease-of-use of the Cell Health Application Module applicable for the rapid screening of a large number of agents for their effects on cell health using the Vybrant #7 apoptosis assay kit from Molecular Probes. The flexibility of this module makes it compatible with a wide range of other dyes

AcuityXpress analysis of staurosporine response (figure 3)



Response of cells to increasing dosages of staurosporine (12-hour treatment). Image analysis and cell classification are performed using the Cell Health Application Module. Curve fitting is performed with AcuityXpress.

to indicate cell health. It is also a powerful tool for screening potential toxic or non-toxic therapeutic agents and excluding toxic agents, as well as analyzing specific pathways of toxicity. For more information, see the poster "Quantitation of Apoptosis, Necrosis and Cell Death Using High Content Screening", www.moleculardevices.com/pdfs/SBSSBS04_LiveDead_CellHealth.pdf

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