Customised Multi-Parametric Image Analysis For Accelerated Toxicity Screening

[¥]Ben Haworth, [¥]Andrea Gough, Jayne Hesley, Oksana Sirenko, Grischa Chandy and Steven Luke.
Molecular Devices, LLC, Sunnyvale, United States & [¥]Molecular Device (UK) Ltd, Wokingham, United Kingdom

Introduction

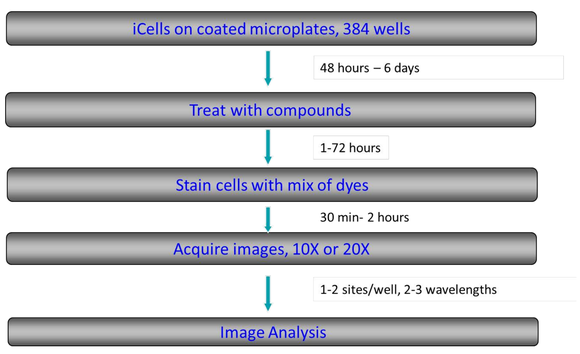
Predictive *in vitro* assays suitable for safety testing are extremely important for reducing the incidence of late-stage drug attrition. Cell-based cytotoxicity methods utilizing high content and high-throughput imaging are well-established. However, standard assays and analyses are sometimes insufficient and customized measurements may be required to fully capture some toxic effects. These experiments utilize a software tool that allows easy generation of customized analysis modules that enable more multiplexing and specific outputs than has been available in the past. We have used both standard cell lines and human iPSC-derived hepatocytes and neurons with doses of different classes of compounds in order to demonstrate several models for assessing general and mechanistic toxicity using the power of high content imaging. Unique multi-parametric image analysis was used to monitor cell viability, apoptosis, proliferation, and mitochondria membrane potential.

Additionally, intuitive custom software modules can be used to quantitate many more cellular responses to drug treatment in live or fixed cells such as:
• Nuclear count • Nuclear morphology • Live Cell count • Whole cell morphology • Mitochondria count • Loss of mitochondria membrane potential • Apoptosis • Necrosis • Autophagy • Glycogen levels • Phospholipidosis • Steatosis • Vacuole formation • Neurite outgrowth • Puncta on neurites

These assays were conducted in 96 or 384 well microplates and multiple assays can be run in each well. Each assay will yield multiparametric results which can be evaluated using MetaXpress® 5 Image Analysis Software and AcuityXpress™ High Content Data Analysis Software to determine the most significant responses to study. Some examples of various analyses with easy-to-create Custom Modules are shown below.

Materials & Methods

- **iCell® Hepatocytes and Neurons** – Cellular Dynamics Intl.
- **Assay Reagents**
  CellEvent™ Caspase 3/7 Apoptosis Detection Reagent – Life Technologies (Invitrogen) P/N C10423
  Calcein, AM Cell Viability reagent – Life Technologies (Invitrogen) P/N C3100MP
  MAP2B-AlexaFluor 647 Antibody – BD Pharmingen P/N 560382
  MitoTracker® Orange CMTMRos - Life Technologies (Invitrogen) P/N M7510
  CellMeter™ JC-10 Mitochondrial Membrane Potential Kit – AAT Bioquest P/N 22801
- **High content Imaging** – Molecular Devices, LLC
  ImageXpress® Micro XL High Throughput Imaging System with transmitted light option and standard fluorescence DAPI, FITC, TRITC, Cy5 filter cubes and 4X Plan Fluor, 10X Plan Apo, or 20X S Plan Fluor ELWD objectives



- **Automated Analysis**
  MetaXpress 5 Image Analysis Software with Custom Module Editor processes images using either pre-configured software modules or easily customizable modules to characterize phenotypic changes and allow specific outputs

  AcuityXpress™ High Content Data Analysis Software provides tools for statistics, curve fitting, hierarchical clustering, and principal component analysis to provide additional insights into assay results

Measuring Cell Proliferation using Transmitted Light

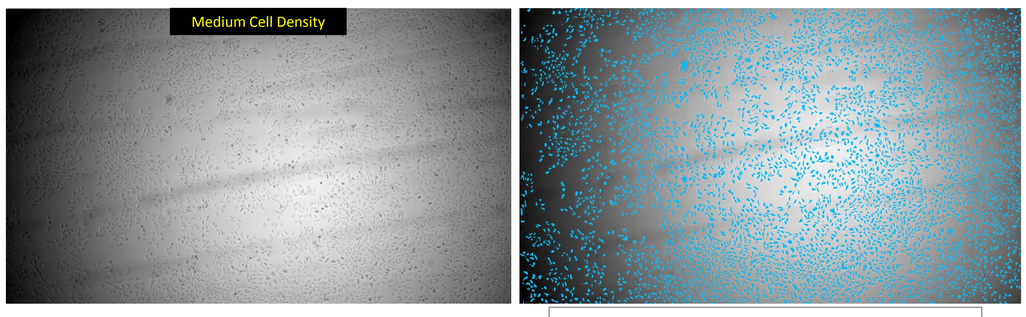
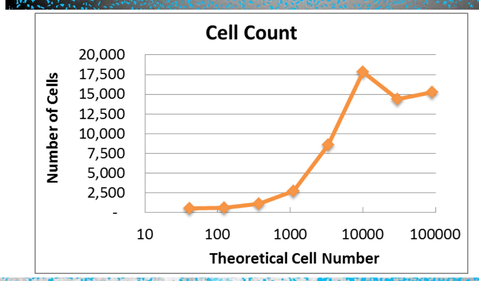
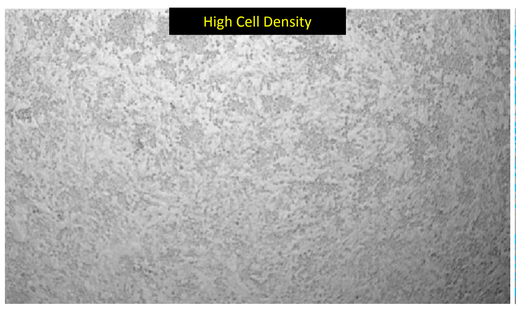


Figure 1A. Images of unlabeled CHO cells taken with a 4X objective were successfully analyzed using a simple Custom Module even if they displayed artifacts from scratches on the microplate or highly variable background. Top: Raw image is shown on the left and segmented cells are shown in light blue on the upper right. Right: The results from counting individual cells became unreliable at the highest concentrations when cells were nearly confluent. X axis is an estimate of cells in each field-of-view based on well plating calculations. The correlation coefficient (R^2) from 41-10,000 cells was 0.98.





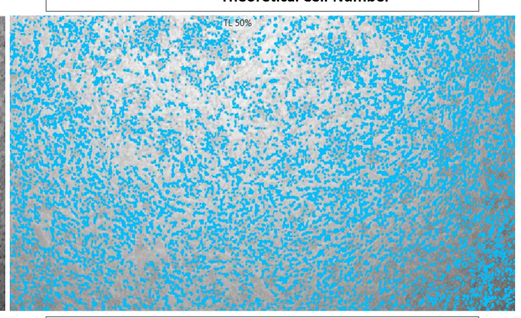
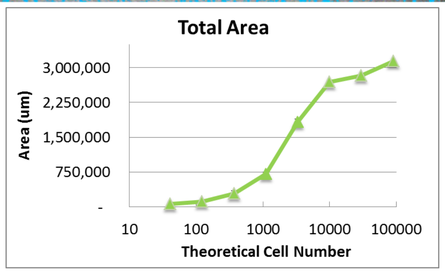
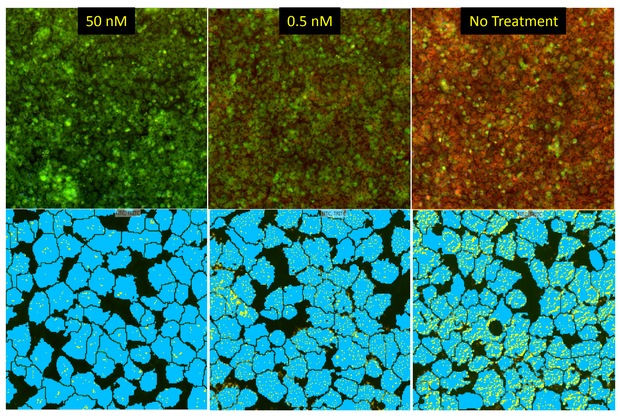


Figure 1B. Top: Raw image of a well with high cell density is shown on the left and the light blue overlay on the right shows the segmented image. The analysis identified both individual cells and cell clumps so that a total area covered by cells was reported. Right: This type of analysis yielded meaningful measurements from wells with both low and high cell density .



Single-dye Mitochondria Membrane Potential Assay

Mitochondrial depolarization is an early signal of hypoxic damage or oxidative stress. Cells were dosed with compounds and the mitochondria membrane potential was monitored with the dye, JC-10. Images were analyzed using the a custom module that would measure both the orange fluorescence intensity in the healthy mitochondria and the green intensity of the cytoplasm, which increases when membrane integrity is failing. This assay can be used either as an end-point or real time live-cell assay with or without a nuclear counter-stain.



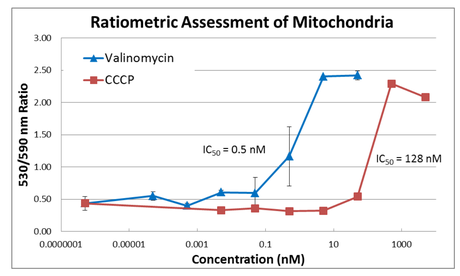
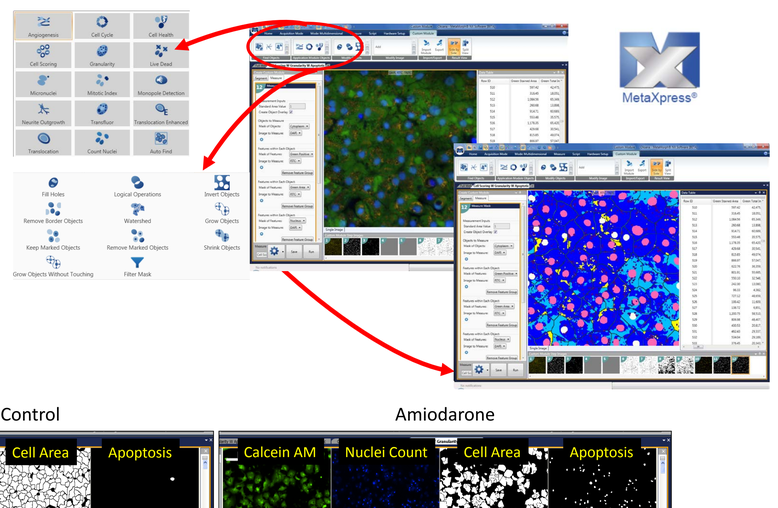


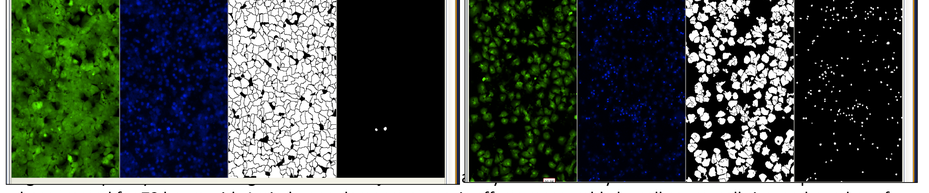
Figure 2. iCell Hepatocytes treated with Valinomycin for 60 minutes. Live cells were imaged with a 10X objective. Top left: Overlay of green cytoplasm and orange mitochondria. Bottom left: Resulting mask after analysis with a custom module (zoomed) shows mitochondria identified (yellow) in a dose response to the compound. Cell-by-cell analysis of average intensity is possible even without a nuclear stain.

Multi-Parametric Hepatotoxicity

Toxic effects vary depending on mechanism of the compound's activity. Several parameters can be measured simultaneously in each well or each cell using a Custom Module. Pre-configured MetaXpress software modules for commonly used analyses (such as Cell Scoring, Granularity, Live/Dead identification) can be combined with a multitude of image analysis tools (such as Logical AND/OR, watershed, dilate objects, omit border objects) to create a custom module for any assay (Figure 3). These modules can be applied to images and run in MetaXpress PowerCore™ High Content Image Processing Software in large batches for high throughput applications. Custom modules have been demonstrated with hepatocytes which quantitate cell size or shape, total number of viable cells, autophagy, and cells positive for phospholipidosis or steatosis (some data not shown here).

Figure 3. The Custom Module Editor in MetaXpress 5 software allows linking preconfigured analysis modules with many other image analysis tools in a step-wise manner. The results of each step are displayed in a filmstrip at the bottom of the set-up screen. The example, showing Hepatocytes (right) stained with Hoechst (blue nuclei), MitoTracker (red mitochondria), and Calcein AM viability stain (green cytoplasm) was analyzed for total nuclei (pink), apoptotic nuclei (white), number and area of cells containing live-cell stain (dark blue) and healthy mitochondria (light blue).

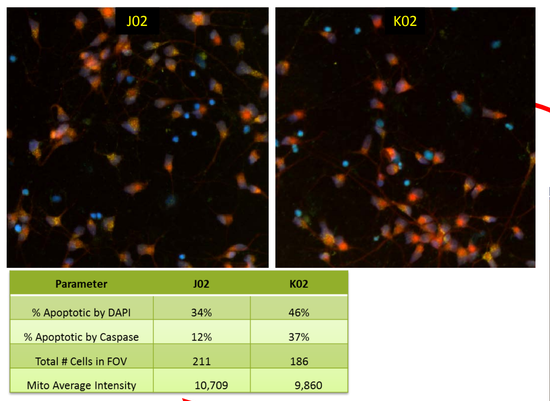




those treated for 72 hours with Amiodarone demonstrate toxic effects measurable by cell count, cell size, and number of apoptotic nuclei.

Four Color Multi-Parametric Neuronal Toxicity

Custom modules can be designed to quantitate neurite outgrowth length, branching, or number per cell or to quantitate total number of viable cells, autophagy, apoptosis, or presence of synaptic puncta on cell bodies or outgrowths (some data not shown here). Assays like these have been done in 24 well tissue culture plates and 96 or 384 well microplates.



Parameter	J02	K02
% Apoptotic by DAPI	34%	46%
% Apoptotic by Caspase	12%	37%
Total # Cells in FOV	211	186
Mito Average Intensity	10,709	9,860

Figure 5A. The Custom Module Editor in MetaXpress 5 Image Analysis software allows measurement of apoptosis in human iPSC-derived neurons after 24 hour Staurosporine treatment . Neurons (top left) stained with Hoechst (blue nuclei), Caspase 3/7 apoptosis stain (green nuclei), MitoTracker (yellow mitochondria), and MAP2-AlexaFluor 647 (red) were imaged with a 20X objective.

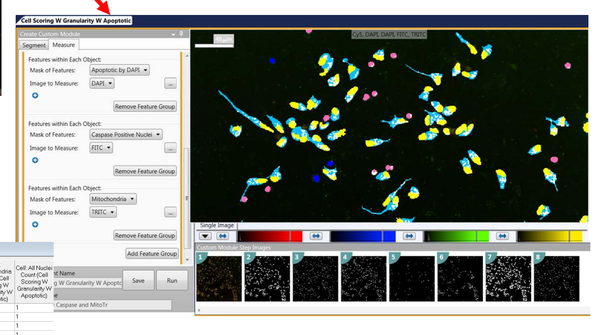


Figure 5B. The segmentation mask of a 4 color neuron toxicity image. The mask shows segmentation results for total nuclei (yellow), apoptotic nuclei by Caspase 3/7 staining (pink), apoptotic by nuclear condensation, small and bright in Hoechst channel, (royal blue) number of MAP2 positive (aqua blue) and healthy mitochondria (white). The user chooses the output-of-interest to be generated on a cell-by-cell basis as well as average or sum per field-of-view.

Summary

- Combining high-throughput imaging and customizable high-content analysis allows multiple useful applications for toxicity assessment in assays using either cell lines or stem-cell derived human cells.
- MetaXpress 5 image analysis software allows users to rapidly analyze multi-parametric toxicity assays and report only the data output that is relevant.
- With the freedom to design your own image analysis modules, cell-based toxicity testing can go far beyond simple live/dead assays.

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