Phenotypic Cell-Based Assays with a New Automated Imaging System and Cell Analysis Software

Oksana Sirenko, Felix Spira, Matthew Hammer, Jayne Hesley, Kayla Hill, Grischa Chandy

Molecular Devices LLC, San Jose, California, USA;

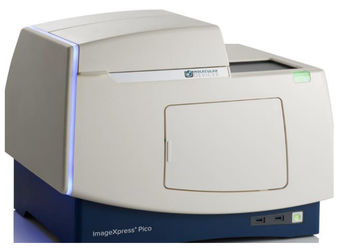
INTRODUCTION

There is a great need to automate complex cell-based assays with multi-parametric readouts while maintaining high data quality and precision. A compact, automated imaging system was used to develop several multi-parametric assays utilizing iPSC-derived cardiomyocytes, neurons, and hepatocytes. Multiple readouts include cell viability, apoptosis, mitochondria membrane potential and autophagy for defining mechanisms of toxicity in live or fixed cells. Also, we provide examples of label–free cell analysis for evaluation of cytotoxicity and cell proliferation. We demonstrate the utility of a new automated imaging system for biological research and present several assay models that will be useful for both academic and biopharma environments.

INSTRUMENT

Cell-based assays were performed using the ImageXpress® Pico Automated Cell Imaging System in combination with the CellReporterXpress Automated Imaging and Analysis Software. The imager provides 4 fluorescence channels plus transmitted light and colorimetric assays, with a variety of magnifications, environmental control (EC), injectors, and time-lapse capacity to enable automatic monitoring of cell proliferation, differentiation, compound toxicity, and a variety of other cell-based assays. The analysis software uses novel algorithms for object recognition that simplify workflow and analysis and provide multi-parametric readouts.

- **ImageXpress Pico** Automated Cell Imaging System
  - Equipped with 4 fluorescence channels plus transmitted light; 4x-63x objectives; EC;
- **CellReporterXpress** Automated Imaging and Analysis Software
  - Includes 19 pre-configured assay protocols



METHODS

Cell Culture: Human iPSC-derived neurons, cardiomyocytes, or hepatocytes and the appropriate media were purchased from Cellular Dynamics International, Fujifilm Co (CDI). Cells were plated into 384-well black clear bottom plates (Greiner) at a density of 10,000 cells per well and cultured as recommended by protocols from CDI. HeLa or PC12 cell lines (ATCC) were cultured according to manufacturer's recommendations. Treatment with compounds was performed 24 hours post plating; cultures were exposed to treatment for 3 days, or as indicated in the figures.

Cell Staining: To assess phenotypic changes, cells were stained live using a mixture of three dyes: the viability dye Calcein AM (1 µM), the mitochondria potential dye MitoTracker Orange (0.2 µM), and the nuclear dye Hoechst (2 µM) (all from Life Technologies). For visualizing the actin cytoskeleton, cells were fixed with 4% formaldehyde (Sigma) and stained with AlexaFluor® 488 (AF488) labeled phalloidin stain. Neurons were stained with anti-TuJ-1 antibodies (BD Biosciences).

Assessment of Cell Morphology and Viability

Imaging and analysis methods provide efficient tools for characterization of multiple readouts including cell viability, characterization of cell shape, cell adhesion and spreading, cytoskeleton integrity (morphology), and mitochondria potential.

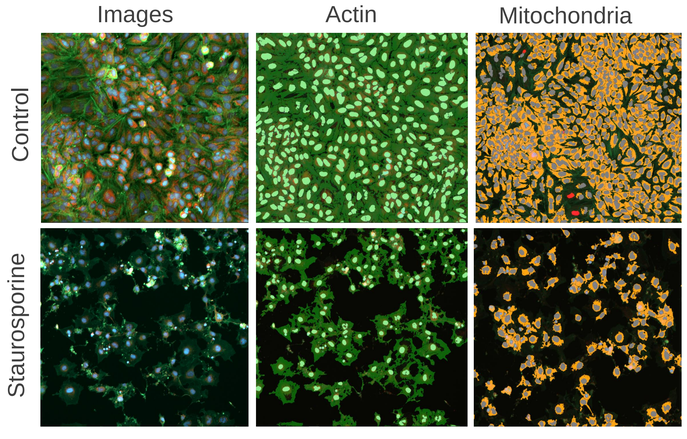


Figure 1. Images and the analysis masks for multi-parametric analysis. HeLa cells were treated for 72 hours, and then stained with a nuclear stain (Hoechst 33342), actin cytoskeleton stain (AlexaFluor® 488 (AF488) labeled phalloidin), and MitoTracker Orange CMTMRos. Images and analysis masks compared for control cells and cells treated with 0.1 µM staurosporine. Cells were imaged with the DAPI, FITC, and TRITC using a 10x Plan Fluor objective. The images (left) show nuclei (blue), actin cytoskeleton (1:100, green), mitochondria (orange). Images were analyzed using the Cell Scoring analysis modules optimized for the quantitation of phalloidin positive cells (middle) and MitoTracker Orange positive cells (right). The analysis masks: light green - positive nuclei, red - negative nuclei, green - actin cytoskeleton, orange - intact mitochondria.

	EC_{50} (µM) ± Standard Deviation				
Analysis Readouts	**Staurosporine**	**Mitomycin C**	**Paclitaxel**	**Etoposide**	**Doxorubicin**
Number of Actin Positive Cells	0.012 ± 0.001	0.178 ± 0.140	$5.46 \times 10^{-4} \pm 3.48 \times 10^{-5}$	0.820 ± 0.340	0.020 ± 0.012
Actin Positive Cell Total Area	0.067 ± 0.009	4.952 ± 0.354	$8.56 \times 10^{-4} \pm 5.69 \times 10^{-5}$	13.54 ± 1.229	0.649 ± 0.054
Actin Positive Cell Total Integrated Intensity	0.038 ± 0.005	3.614 ± 0.378	$6.33 \times 10^{-4} \pm 4.21 \times 10^{-4}$	10.10 ± 1.301	0.501 ± 0.078
Number of Cells with Intact Mitochondria	0.013 ± 0.001	0.110 ± 0.045	$4.50 \times 10^{-4} \pm 2.64 \times 10^{-5}$	0.543 ± 0.190	0.014 ± 0.006
Mitochondria Positive Cell Total Integrated Intensity	0.017 ± 0.002	1.691 ± 0.222	$4.18 \times 10^{-4} \pm 2.98 \times 10^{-5}$	6.336 ± 1.086	0.197 ± 0.053

RESULTS:

Compound-Specific Effects on Neurite Outgrowth

Phenotypic readouts for neurite outgrowth (iCell Gaba Neurons, CDI) included quantitative characterization of the extent and complexity of neural networks by multiplexed measurements. We have characterized multiple measurements and tested several known neurotoxic compounds. Neurite outgrowth was characterized by the extent of the outgrowth (length of total outgrowth or mean outgrowth per cell), the number of neurite processes (total number of processes), and the extent of branching (total number of branches and mean number of branches per cell).

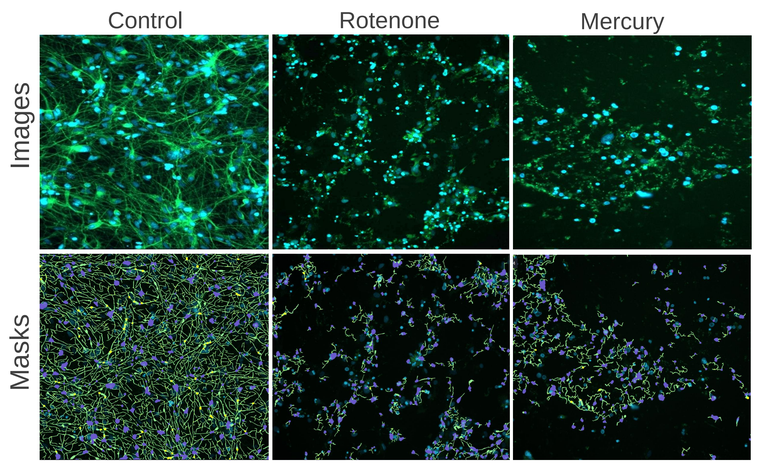


Figure 2. Images of b-tubulin (green) stain and the software analysis traces shown for the control and compound treated cells. iCell Neurons were treated with compounds for 3 days, and were then fixed and stained with AF488-conjugated anti-β-tubulin (TUJ-1) antibodies (1:100, BD Biosciences). Images were taken by the ImageXpress Pico system, 10x Plan Fluor objective and FITC and DAPI channels. Images were processed using the Neurite Tracing analysis algorithm. Analysis masks on the right show the outgrowth (green), as well as cell bodies (blue), and branching points (pink). Disruption of neurite networks was measured for neurons treated with 1 µM of indicated compounds. EC50s defined by decrease in total outgrowth were 6.1 µM for rotenone and 0.07 µM for methyl mercury; EC50s defined for decrease of numbers of branches were 2.8 µM for rotenone and 0.071 µM for methyl mercury.

Assessing Compound Effects on Cardiomyocytes

To evaluate cytotoxicity effects, cells were treated with various cardiotoxic compounds for 24 hours and then live cells were stained with Hoechst nuclear stain, MitoTracker Orange dye and AF-488 Phalloidin for detection of cytoskeleton integrity. Images were acquired using the ImageXpress Pico system and were analyzed using the Cell Scoring algorithm for detection of total and average areas of cardiac cells, as well as the number and percentage of cells with intact cytoskeleton.

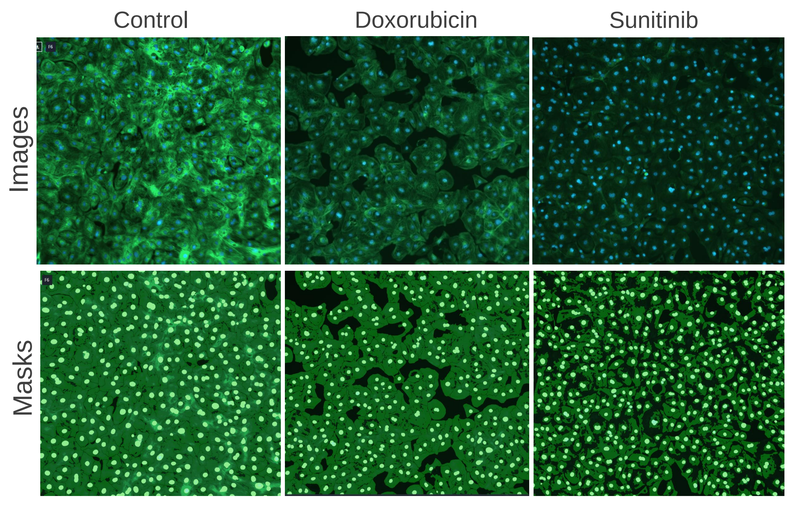


Figure 3. Cytoskeleton staining of cardiomyocytes for visualization of compound effects. Cardiomyocytes were plated and treated with compounds for 24h, and stained with a combination of Hoechst (2 µM), MitoTracker Orange (0.2 µM), and then fixed and stained with AF488-conjugated Phalloidin (1:100). Images were taken by ImageXpress Pico system, using a 10x Plan Fluor objective, and DAPI, TRITC, and FITC channels. Images were processed using the Cell Scoring analysis algorithm. Composite images of actin, nuclei, and mitochondria (top) and the analysis masks (bottom) are shown for the control and compound treated cells. Disruption of the cytoskeleton was observed for cardiomyocytes treated with 20 µM indicated compounds (EC50s defined by cell area: 5.4 µM for sunitinib, 22 µM for doxorubicin).

Phenotypic Changes in Hepatocytes

iPSC-derived human hepatocytes present a valuable cellular model that can closely resemble the phenotypes and functionality of primary hepatocytes, while minimizing variability and other limitations of primary cells. Automated imaging and analysis can be used for evaluation of hepatotoxic effects of compounds.

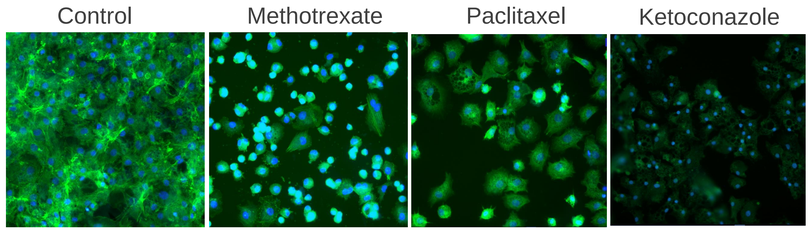


Figure 4. Assessment of changes in hepatocyte morphology after treatment with hepatotoxic compounds. Hepatocytes were plated and treated with compounds for 72h, stained with a combination of Hoechst (2 µM), MitoTracker Orange (0.2 µM), and then fixed and stained with AF488-conjugated Phalloidin (1:100). Images were taken by ImageXpress Pico system, using a 10x Plan Fluor objective, and DAPI, TRITC, and FITC channels. Images were processed using the Cell Scoring analysis algorithm. Composite images of actin, nuclei, and are shown for the control and compound treated cells. Disruption of the cytoskeleton, variations in cell spreading, and cell death were observed for hepatocytes treated with 30 µM of indicated compounds.

Mitochondria Potential and Autophagy Assays

Dysregulation of autophagy and mitochondria integrity have been established to play a role in various neurodegenerative diseases and cancers, therefore discovery of novel therapeutic agents targeting those process has emerged as a promising new approach for drug therapies. PC12 neuroblastoma cell line was used as a model for assay development.

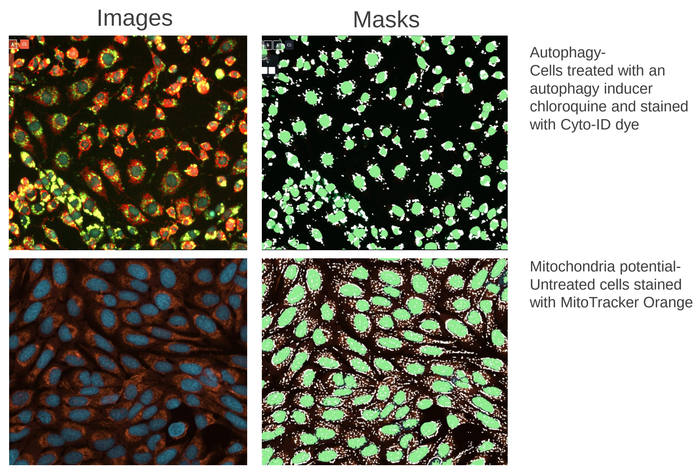


Figure 5. PC12 cells (ATCC) were plated into 384-well plates at 6,000 cells/well and incubated for 48h. To evaluate effects on autophagy, cells were treated with various compounds for 24-48 h, and then live cells were stained with the Cyto-ID® Autophagy Detection Kit for tracking authophagosomes (ENZO Life Sciences), MitoTracker Orange dye for detection of healthy mitochondria, and Hoechst to identify nuclei (0.2 µM, 0.2 µM and 1 µM, respectively, ThermoFisher). Images were taken using ImageXpress Pico Automated Cell Imaging System, with 20x or 40x magnification and detection channels for the appropriate dyes (FITC, TRITC, and DAPI, respectively). Images were analyzed using the Granularity application module in CellReporterXpress software. The Granularity algorithm detects and characterizes small objects, such as autophagosomes, and mitochondria in the cytoplasm of cells, while using the nuclear marker to segment cells. Readouts include total numbers and total area of "granules" (subcellular objects). Top: Representative images and analysis traces for cells treated with chloroquine (30 µM). Bottom: Images and analysis masks of control cells stained with MitoTracker Orange. Nuclei shown in blue, mitochondria in red, and autophagosomes in green.

Counting Cells Using Transmitted Light and Apoptosis Assay

The analysis software allows image segmentation and cell counting without using dyes. This application is especially useful for monitoring cell proliferation and cell death over time. In addition we describe an assay for apoptosis detection using a fluorescent marker.

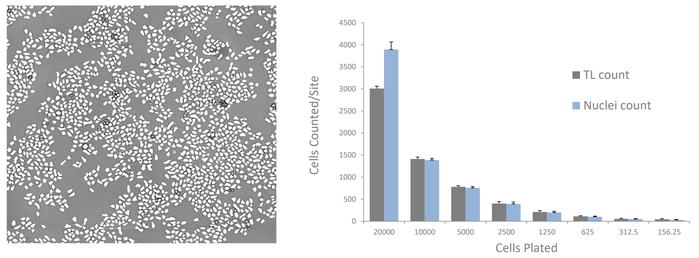


Figure 6. HeLa cell images in transmitted light (10x objective, 2ms exposure) with white analysis masks (left), and a comparison of cell quantitation using transmitted light segmentation vs. fluorescent nuclear count (right). CellReporterXpress software contains a pre-configured application module for quantifying cell count in transmitted light.

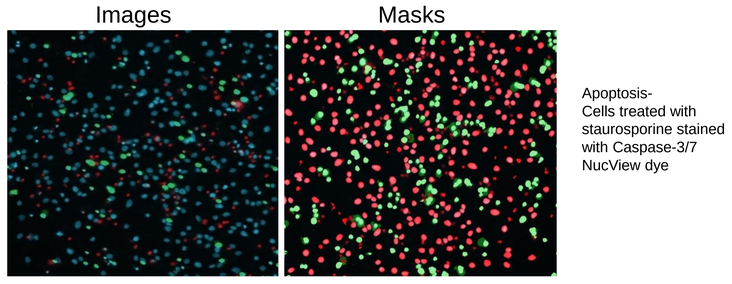


Figure 7. Images of Hela cells treated with 0.3 µM of staurosporine. Cells were stained for 30min with EarlyTox Caspase-3/7 NucView 488 dye (Molecular Devices), Ethidium Homodimer III (ThermoFisher), and Hoechst 33342 nuclear dye. Live cells were imaged using the 10x objective. The Hoechst nuclear stain is shown in blue, apoptotic nuclei indicated by green, and nuclei of dead cells labeled in red. Apoptosis protocol was performed for the analysis. Masks of apoptotic cells indicated in green, live cells in red.

SUMMARY

- We presented several examples of multi-parametric assays utilizing different cell types, including iPSC-derived human cardiomyocytes, neurons, and hepatocytes. Multiple readouts allows better discrimination between different cell phenotypes and compound effects.
- The assays demonstrates the breadth of imaging assays possible using the ImageXpress Pico Automated Cell Imaging System and CellReporterXpress software for evaluation of various biological effects.

MOLECULAR DEVICES