APPLICATION NOTE

Multi-parametric assessment of cell phenotypes using ImageXpress Pico Automated Cell Imaging System

Matthew Hammer and **Oksana Sirenko, PhD** | Applications Scientists | Molecular Devices

Benefits

- **Learn about efficiency of automated imaging by capturing various cell phenotypes**
- **Use preconfigured analysis modules to obtain numeric data**
- **Assess a variety of morphological changes in response to compound treatment**

Introduction

Automated cellular imaging is an efficient method for analyzing compound effects on cell phenotypes including morphology, viability, and marker expression. Here, we demonstrate how the ImageXpress® Pico Automated Cell Imaging System and CellReporterXpress Automated Imaging and Analysis Software is applied for phenotypic analysis of compound effects. Imaging and analysis methods provide tools for characterization of multiple readouts including the assessment of cell viability, characterization of cell shape, cell adhesion and spreading, cytoskeleton integrity, and mitochondria potential.

Materials

- HeLa cells (ATCC P/N: CCL-2)
- HeLa media
  - DMEM: CellGro, with L-glutmaine (Corning)
  - 10% FBS (BenchMark™; Gemini P/N 100-106)
  - 1% penicillin/streptomycin
- Staurosporine (Sigma P/N S5921)
- Mitomycin C (Sigma P/N M4287)
- Paclitaxel (Sigma P/N T7402)
- Etoposide (Sigma P/N E1383)
- Doxorubicin (Sigma P/N D1515)
- Calcein AM
- EarlyTox Live Cell Assay Kit (Explorer Kit, Molecular Devices P/N R8342)
- Hoechst 33342 (Thermo Fisher, Carlsbad, CA P/N H3570)
- MitoTracker Orange cMTMRos (Thermo Fisher, Carlsbad, CA, M7510)
- AF488-conjugated Phalloidin (ThermoFisher Scientific, Carlsbad, CA PN A12379)
- 384-well black, clear-bottom microplates (Corning Falcon P/N 62406-490)
- ImageXpress Pico Automated Cell Imaging System and CellReporterXpress Automated Imaging and Analysis Software

Method

To monitor the effects of compounds on changes in cell phenotype, HeLa cells were treated with drugs known to inhibit cell proliferation and induce cell death. HeLa cells were plated at a density of 2,000 cells per well for a total volume of 25 µL of media in a 384-well black, clear-bottom microplate, and incubated at 37°C, 5% CO_2 for 24 hours. The cells were then treated with serial dilutions of compounds, including staurosporine, mitomycin C, paclitaxel, etoposide and doxorubicin for 72 hours. Compound treatments were performed in triplicates or quadruplicates.

After 72 hours of compound treatment, cells were stained with a combination of several dyes: nuclear dye, Hoechst 33342, viability dye, Calcein AM, and MitoTracker Orange™ CMTMRos reagent for detection of mitochondria with intact membrane potential (MMP). A 2x staining solution was added directly to the medium. The final concentrations of stains in all wells were 3 µM Hoechst 33342, 0.2 µM MitoTracker Orange CMTMRos, and 1 µM Calcein AM. The cells were incubated with staining solution at 37°C, 5% CO_2 for 30 minutes.

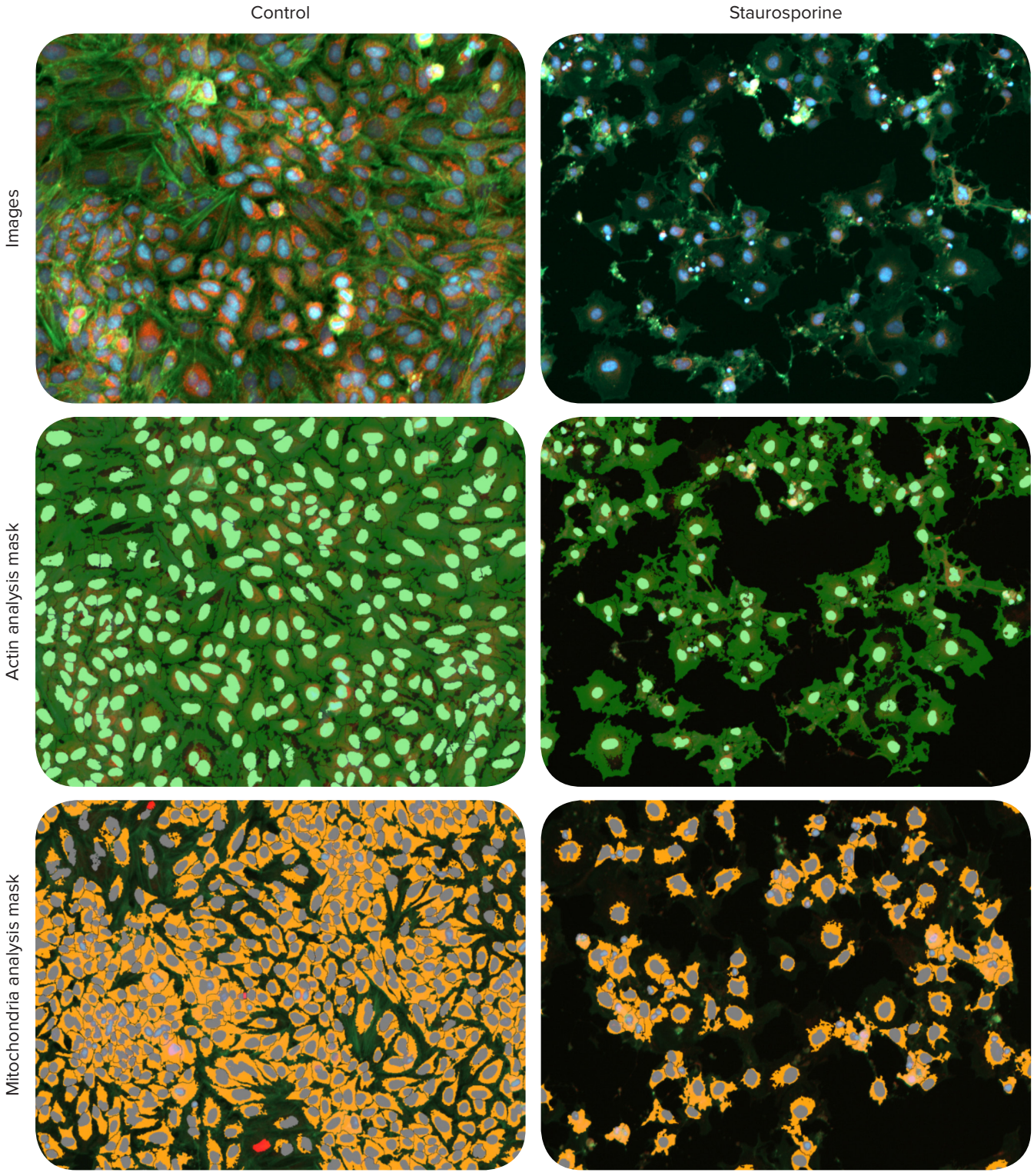

Figure 1. Image and analysis masks for multi-parametric analysis of cell viability and morphology. HeLa cells were treated with several compounds for 72 hours and then stained with a nuclear stain (Hoechst 33342) actin cytoskeleton stain (AlexaFluor 488 (AF488) labeled phalloidin) and a mitochondrial stain (MitoTracker Orange CMTMRos). Representative images and analysis masks show a comparison of control cells and cells treated with 0.1 µM staurosporine. The cells were imaged with DAPI, FITC, and TRITC channels using the ImageXpress Pico system with a 10X Plan Fluor objective. The images **(top)** are labeled for nuclei (blue), actin cytoskeleton (green), and mitochondria (orange). Images were analyzed with CellReporterXpress software using the Cell Scoring analysis modules optimized for the quantitation of phalloidin-positive cells **(middle)** and MitoTracker Orange-positive cells **(bottom)**. The resulting analysis masks are shown (light green = actin-positive nuclei, dark green = actin cytoskeleton, blue = mitochondria-positive nuclei, red = mitochondria-negative nuclei, orange = intact mitochondria).

Cells can be imaged live using DAPI, FITC, and TRITC channels for the Hoechst, Calcein AM and MitoTracker Orange stains, respectively. A 10X objective was used to acquire a single image of one site per well. Exposure times were set at 20 ms for DAPI, 10 ms for FITC, and 200 ms for TRITC channels. Cells may also be fixed for imaging later or for further processing with additional cellular markers. Note: Calcein AM is used for assessment of live cells and is not detectable after fixation (data not shown). MitoTracker Orange and Hoechst 33342 nuclear stains are stable after fixation.

To assess actin cytoskeleton integrity, cells were fixed for further processing. After staining with Mitotracker Orange and Hoechst 33342 stains, cells were fixed with a 4% formaldehyde solution and subsequently washed twice with PBS. Cell membranes were then permeabilized with DPBS + 0.01% saponin + 1% FBS for 10 minutes. An AlexaFluor® 488 (AF488) labeled phalloidin stain was used to assess cytoskeleton integrity. Phalloidin was diluted in permeabilization buffer (1:50) and incubated with cells for two hours at 37°C. The cells were washed with PBS, and then imaged and analyzed using a Cell Scoring analysis module, optimized to quantitate cells with AF488-stained cytoskeleton, from the CellReporterXpress software.

Live or fixed cells were imaged with the ImageXpress Pico system and analyzed with CellReporterXpress software. Cell images were analyzed with two Cell Scoring protocols: one detecting FITC as the marker to quantitate the number of Calcein AM- or phalloidin-positive cells, and a second protocol for detecting TRITC as the marker for analyzing cells positive for mitochondrial stain. In both analysis protocols, the nuclear stain was used for cell count and nuclear characterization.

Quantitation of phenotypic effects with automated imaging and analysis

A typical automated cell-based assay workflow includes plating cells, treatment with compounds, and automated imaging. For multi-color, multi-parameter assays, Calcein AM can be used to define the number of live or intact cells, characterize cell spreading and identify cell morphology. Nuclear dyes are used to count cells,

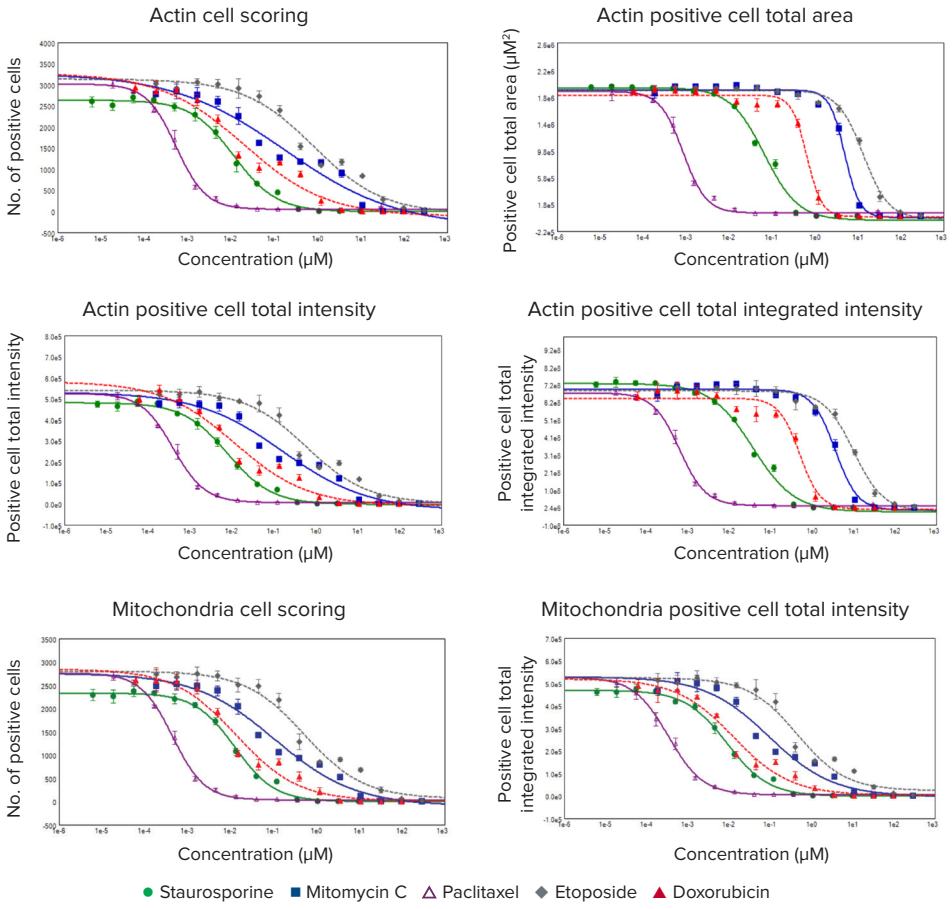

Figure 2. Dose-response curves demonstrate the effects of compound treatments on various parameters. HeLa cells were imaged with the ImageXpress Pico system and analyzed with CellReporterXpress software. The Cell Scoring module was optimized to measure cells with intact actin cytoskeleton stained with AlexaFluor 488 Phalloidin, and intact mitochondria labeled with MitoTracker Orange. The 4-parameter logistic curve plots were created using SoftMax® Pro Software, using CellReporterXpress-generated data points. The dose-response curves illustrate the effects of each compound for several readouts. Corresponding EC_{50} values are located in Table 1.

assess proliferation, and measure nuclear intensity and shapes. MitoTracker dyes can be used to define mitochondria integrity or membrane potential. Cells can be imaged live, or they can be fixed and stained with additional reagents (e.g. phalloidin) to provide information about cytoskeleton or other readouts. Phalloidin staining of actin cytoskeleton can be used for assessment of cytoskeleton organization, cell spreading, morphology, and to quantitate the number of intact cells.

CellReporterXpress software streamlines the production of assay results with the ability to perform simultaneous image acquisition and analysis with preconfigured analysis modules. Cell Scoring was optimized for analyzing cell viability, mitochondrial health, or cytoskeleton integrity. The analysis generated multiple readouts including, but not limited to, total cell counts, the quantification of marker specific cell numbers, intensity and integrated intensity values for the markers, total and average cell area, and nuclei characterization of average area and intensity.

Figure 1 represents the composite images of control and staurosporine-treated cells stained with three dyes: nuclear dye Hoechst, actin cytoskeleton dye AlexaFluor 488 (AF488) labeled phalloidin, and MitoTracker Orange dye for assessment of mitochondria potential. The corresponding analysis masks for detected nuclei, intact actin cytoskeleton, and intact mitochondria are displayed under both treatment conditions.

Concentration dependencies for the different readouts are shown in Figure 2. Viable cells, as determined by the total number of phalloidin-stained cells, decreased in response to increasing concentration of compound (Figure 2). A similar trend was observed for MitoTracker-positive cells. Decreases in the intensity of the phalloidin and MitoTracker stain were also consistent with increased concentrations of cytotoxic compounds. The impact on cell spreading can be characterized by assessing cell area. EC_{50} values for different readouts are summarized in Table 1.

Analyses readouts	EC_{50} (µM) ± Standard Deviation				
	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 intensity	$0.008 \pm 7.45 \times 10^{-4}$	0.164 ± 0.093	$3.95 \times 10^{-4} \pm 2.82 \times 10^{-5}$	0.603 ± 0.239	0.012 ± 0.007
Actin positive cell total integrated intensity	0.038 ± 0.005	3.614 ± 0.378	$6.33 \times 10^{-4} \pm 4.21 \times 10^{-5}$	10.10 ± 1.301	0.501 ± 0.078
Number of mitochondria positive cells	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
Mitochondria positive cell total intensity	$0.009 \pm 9.81 \times 10^{-4}$	0.094 ± 0.039	$3.35 \times 10^{-4} \pm 3.00 \times 10^{-5}$	0.451 ± 0.147	0.013 ± 0.005

Table 1. EC_{50} values for analysis readouts. The Cell Scoring module analyzes multiple readouts that describe compound effects on multiple phenotypic changes. The EC_{50} values correspond to 4-parameter logistic curve plots in Figure 2.

Conclusion

Automated cellular imaging is an efficient tool for testing the effects of compounds on toxicity, cell viability, or morphology. The ability to investigate a variety of cellular responses provides important information about mechanism of action. The ImageXpress Pico Automated Cell Imaging System and CellReporterXpress software allows the user to analyze a variety of measurements and enable multiple readouts to better characterize complex phenotypic effects.

Contact Us

Phone: +1.800.635.5577
Web: www.moleculardevices.com
Email: info@moldev.com
Check our website for a current listing of worldwide distributors.

The ImageXpress Pico system features optics by Leica Microsystems.