Novel High Content Stem Cell-based Assays for Screening

Oksana Sirenko, Pierre Turpin, Jayne Hesley, H. Roger Tang, and Evan F. Cromwell*
Molecular Devices, Inc. Sunnyvale, CA

Introduction

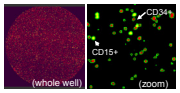
The biopharma industry is continuing to adapt more cell-based assays for primary and secondary screening because of higher biological relevance and increased value of information. As part of this evolution, there is a desire to move from immortalized, stable cell lines to primary cells and stem cells. Stem cells offer the advantages of providing better clinical relevance of information compared to cell lines, being available in larger quantities, and having higher assay reproducibility than primary cells. Accordingly, there is a great interest in automated stem cell assays to use as screening tools in early drug development, and to evaluate potential toxic effects of new compounds.

The workflows for assays involving stem cells are similar to other cell-based assays, but there are critical differences. Differentiation is often involved in stem cell assays because of the need to start with undifferentiated cells, or because the assay is studying the differentiation process. Monitoring the type and number of cells is often important when more than one lineage is present, or when both mature and undifferentiated cells are present. Accordingly, the data analysis and visualization requirements become more complex as multiple readouts from multiple cell types is required.

We have developed high content imaging methods based upon automated image acquisition and multiplexed analysis to perform assays on three types of stem cells: hematopoietic, neuronal, and induced pluripotent stem cell (iPS) derived cardiomyocytes. These methods in combination with optimized cell culture protocols will allow efficient screening and validation of drug candidates in three key areas of drug development: neurogenesis, hematopoiesis, or cardioprotection.

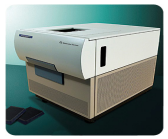
Instrumentation

Fluorescence & Label-Free Imaging by IsoCyte Cytometer
Fast and simple analysis of expression levels of markers on single cells.



- FI: 488nm laser excitation + 2 channels detection
  - Ch1 510-540 nm; Ch3 560-610 nm
- Label-free: 488nm laser scatter
- Images acquired at 5 μm x 5 μm sampling
- Single cells are identified in a specific ROI in each image (Ch1 & Ch3)
- Cells are filtered by region of interest, area, intensity, etc.
- Cell-by-cell results are generated for each well and exported to FCS Express®
- Total Scan Time w/Analysis: 5 minutes

High Content Imaging of Neuronal Stem Cells and Cardiomyocytes by ImageXpress® Micro System and MetaXpress® Software

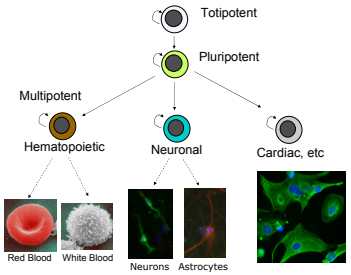


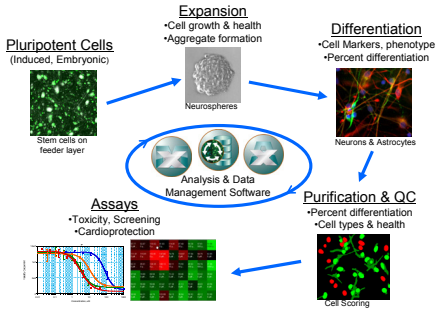
- Images acquired with ImageXpress Micro System using 20X objective and three exposures
  - AF488 anti-b-tubulinIII: 488nm Ex, 520nm Em
  - AF647 anti-GFAP: 640nm Ex, 670nm Em
  - Hoechst dye label for nuclei: 405nm Ex, 450nm Em
- Images were analyzed using standard algorithms from MetaXpress Software
  - Cell Scoring – identify number of Neurons & Astrocytes
  - Neurite Outgrowth – Identify number of neurons, neuron length, branching, etc.

High Throughput SpectraMax® Paradigm Fluorescence Plate Reader

- User configurable cartridges for flexible operation
- FI Mode: LED excitation with dual PMT detectors
  - Calcein AM: 485nm Exc., 535nm Em.
- On-the-fly Read Mode: 1 min for 384 well plate
- Sub-picomolar sensitivity
- Automation ready for Screening environments

Stem Cell Hierarchy & Workflow





EXPANSION:
Neuronal Progenitors into Neurospheres

The assay can be used to monitor expansion of neuronal progenitors and evaluate the effect of growth factors on neurosphere development. Cryo-preserved neurospheres (StemCell Technologies, mouse cells) were cultured in 6 well plates in media supplemented with growth factors. Growth was characterized automatically with IsoCyte cytometer using label-free detection and evaluating Total Area of neurospheres. The results from this study are shown below.

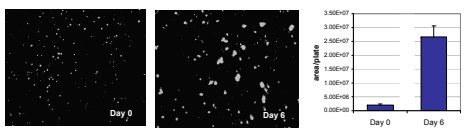
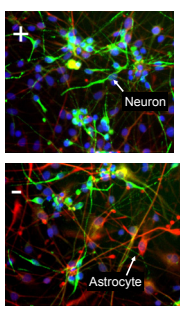


Fig. 1. Images of iPS cells in undifferentiated and differentiated state. Images were acquired with ImageXpress Micro system using a 20X objective.

DIFFERENTIAION:
Neuronal Progenitors into Neurons & Astrocytes

Neuronal progenitors (Lonza) were expanded in media with EGF and bFGF for 1-2 days and then grown in presence of differentiating media for 12-14 days. The NPCs were cultured into neurospheres with or without EPO to study the effect of EPO on differentiation. Neurons and astrocytes were visualized using ImageXpress Micro System; 20 images per well were acquired. Representative images from the two differentiation conditions are shown in Figure 2.



The images were analyzed using the Cell Scoring (CS) module and the Neurite Outgrowth (NO) module. Results for two outputs from these modules are shown to the left: 1) Total number of neurons (CS) and total neurite outgrowth (NO). A clear increase in both parameters was observed for cells cultured with EPO

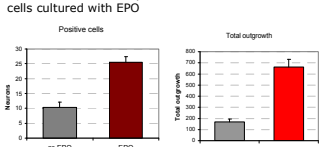


Fig. 2. Results of neuronal stem cell differentiation analysis. Left: Representative images of cells grown in the presence (+) and absence (-) of EPO. Above: Image analysis results for total number of neurons (Left) and total neurite outgrowth (Right). Images were acquired with ImageXpress Micro system using a 20X objective.

DIFFERENTIAION:
Hematopoietic progenitors into Myeloids & Erthyroids

Human BM CD34+ cells were cultured in 96w format with different hematopoietic cytokine combinations. The IsoCyte cytometer was used to scan and analyze the different cell types based on fluorescently labeled markers. Cell population results were furthered analyzed using FCS Express® 4 Software (De Novo Software) to quantify percentage of each cell type. Results for two cytokine combinations that promote self-renewal and myeloid differentiation are shown below in Figure 3

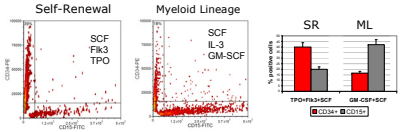


Figure 3. The combination SCF, Flk3, TPO resulted in greater expansion of CD34+ cells (hematopoietic progenitors, self-renewal, SR). GM-CSF promoted differentiation of cells toward myeloid cell phenotype (CD15+, Myeloid Lineage, ML). Percent and numbers of CD34+ or CD15+ cells were measured by IsoCyte cytometer.

STEM CELL ASSAY:
Lineage Specific Hematopoietic SC Toxicity

The use of IsoCyte cytometer and FCS Express software can provide information on lineage specific effects of cytotoxic drugs on hematopoietic stem cell differentiation and cell health. Cell-by-cell results are plotted by marker intensities and then populations are analyzed by quadrant gating. Results for differences between three anti-cancer drugs on myeloid and erythroid lineages are shown in Fig 4.

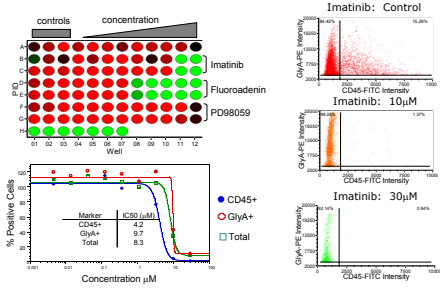


Figure 4. Effect of three compounds on expansion of hematopoietic progenitors toward myeloid and erythroid cells. **Top Left:** Heat map showing cytotoxicity does response as measured by total cell count. **Right:** 2D plots of cell marker intensities for Imatinib dose response at three concentrations. Notice the CD45+ population (myeloid) decreases before the GlyA population (erythroid). **Bottom Left:** Imatinib dose response curves after quadrant analysis showing difference in IC50s for myeloids and erythroids.

DIFFERENTIAION:
Cardiomyocytes into Cardiac Cells

iPS progenitors (Celprogen) were expanded in culture with expansion media for 3-7 days. Seven different markers were measured to determine extent of differentiation and maturity of cardiomyocytes. Representative images of Oct4 expression, a stem cell marker, and a-actinin expression, a cardiomyocyte marker, are shown in Figure 5. The markers were identified using mouse or rabbit primary antibodies (Novus) with anti-mouse AF555 or anti-rabbit AF488 (Invitrogen) labeled secondary antibodies. Nuclei were identified with Hoechst dye.

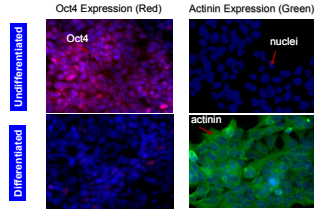
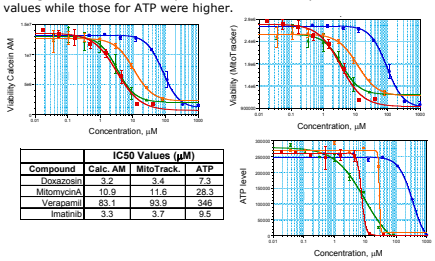


Fig. 5. Images of iPS cells in undifferentiated and differentiated state. Images were acquired with ImageXpress Micro system using a 20X objective.

STEM CELL ASSAY:
High Throughput Cardiotoxcity

A number of cardiotoxic drugs can effect general cell viability, integrity of mitochondria, and metabolic oxidative activity. These parameters were evaluated by SpectraMax Paradigm plate reader in high throughput compatible assays. In one assay, cells were stained with Calcein AM and MitoTraker in multiplexed assay after 72h incubation with cardiotoxic drugs and viability was determined by total fluorescence intensity. In a second, metabolic oxidative activity was evaluated by an ATP detection assay (ViaLight, Lonza). Results are shown in Figure 6. The cell viability and MitoTracker assays showed similar IC50 values while those for ATP were higher.



	IC50 Values (μM)		
Compound	Calc. AM	MitoTrack.	ATP
Doxazosin	3.2	3.4	7.3
MitomycinA	10.9	11.6	28.3
Verapamil	83.1	93.9	346
Imatinib	3.3	3.7	9.5

Fig. 6. Dose response curves and IC50 vlues for toxic effect of four drug compounds on cardiomyocytes. Cells were evaluated by SpectraMax® Paradigm either by viability assay (Calcein AM), mitochondrial integrity (MitoTracker) or ATP detection assay (ViaLight, Lonza).

Summary

- The stem cell workflow demands a variety of complex assay types that monitor all phases from initial growth and expansion, to differentiation, to quality control, and finally predictive assays for therapeutic research and drug discovery.
- Molecular Devices offers a suite of hardware and software solutions that enable automated analysis of stem cells for many of the steps in the workflow.
- We are continuing to develop new techniques to help further condense and automate the workflow for stem cell assays.

Molecular Devices