Novel Assays for Drug Discovery and Toxicology Using Human iPS Cell-derived Neurons and Cardiomyocytes

Susan DeLaura (CDI)
Oksana Sirenko, Ph.D. (Molecular Devices)

February 7, 2012
A major goal of the pharmaceutical industry has been to reduce late stage compound attrition.

One area given significant focus in hopes of optimizing this process is the development of more relevant *in vitro* disease models.

Stem cells provide a biologically relevant model useful for disease modeling, drug discovery and toxicity testing.

Present demands from the drug discovery process have propelled a shift in focus from high-throughput screening to high content analysis in the assay and drug discovery technology arena.

When joining these two technologies, (high content analysis and stem cells) together, a powerful opportunity arises to develop more physiologically relevant biochemical and cell-based assays for compound screening.
iPS Cell Technology

Cardiomyocytes
- Characterization (Genomic, Protein, Electrophysiological)
- Utility (Functional Screens, Mechanistic Insight)

Neurons
- Characterization (Protein, Electrophysiological)

High Content Screening and Assay Development
- iPSC-derived Neurons (Network development & neurotoxicity assays)
- iPSC-derived Cardiomyocytes (Cardiomyocyte beating assay)
The Need for Better Systems to Predict Toxicity

- **Scope of the problem is large**
  - 10,000’s of untested chemicals in current commerce

- **Problem cannot be solved by animals**
  - Practical constraints; cost, time, logistics
  - Ethical constraints

- **Societal and regulatory pressures in US and Europe stimulating rapid move to animal-free systems**
  - Stem Cells for Safer Medicine (SC4SM), IMI, REACH, ToxCast, Tox21,

- **In vitro cell systems are a critical part of the solution**
  - ToxCAST, Tox21, IMI, NCGC, 9 NIH screening centers (Burnham, Stanford, etc)
Current Cell Models are an Inadequate Solution

Cell models derived from cadavers and animals, and engineered cells, are minimally effective

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Genetic Stability</th>
<th>Phenotypic Stability</th>
<th>Quantity</th>
<th>Availability</th>
<th>Cellular Variability</th>
<th>Gene Targeting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transformed Cells</td>
<td>Aneuploid</td>
<td>Stable</td>
<td>Unlimited</td>
<td>Available on demand</td>
<td>Change as culture</td>
<td>Yes</td>
</tr>
<tr>
<td>Primary Human Cells</td>
<td>Diploid</td>
<td>Rapidly dedifferentiate (\textit{in vitro})</td>
<td>Restricted</td>
<td>Restricted and highly variable</td>
<td>Highly donor dependent</td>
<td>No</td>
</tr>
</tbody>
</table>

**Primary Cells**
- Limited and sporadic supply
- Variable phenotype and quality (donor genetics, handling time at harvest, ischemia/reperfusion injury)
- Gain or loss of function (rapidly de-differentiate, change proliferation state)

**Transformed Cells**
- Proliferating, potentially aneuploid, do not closely recapitulate in vivo biology
iPS cells are uniquely useful stem cells
- Derived from adult tissue via non-invasive methods
- Can be expanded indefinitely
- Can be differentiated into any cell type in the body
- Fully pluripotent

iPS cells offer distinct advantages to ES cells
- Can be created via streamlined & non-invasive methods
- Eliminates political/social issues regarding tissue source
- Enables diversity of genotype and phenotype
ISO and GMP processes ensure highest quality
Highly pure populations of differentiated cell types
Industrial quantities of iPS cells and differentiated cell types
Manufacturing process is translatable, scalable and IP protected
Scaling to include parallel simultaneous reprogramming and differentiation from multiple lines

Large Scale Manufacturing
- High Quality
- High Purity
- Billions of Cells
● iPS Cell Technology

● Cardiomyocytes
  – Characterization (Genomic, Protein, Electrophysiological)
  – Utility (Functional Screens, Mechanistic Insight)

● Neurons
  – Characterization (Protein, Electrophysiological)

● High Content Screening and Assay Development
  – iPSC-derived Neurons (Network development & neurotoxicity assays)
  – iPSC-derived Cardiomyocytes (Cardiomyocyte beating assay)
iCell Cardiomyocytes

- Human iPS cell-derived
- 99% pure, cryopreserved, ready to use.
- Available in virtually unlimited quantities
- Full product solution; cells, media, protocols
- Demonstrate normal human cardiac biology, electrophysiology, and toxicity responses
- Broad platform utility for discovery and preclinical development

Sarcomeric α-actinin

(cTNT / MLC)
(cTNT / Cx-43)
(cTNT / MHC)

Sarcomeric α-actinin (Vala Sciences)
Transcriptome Analysis

Gene Category

Stem cell

Transition

Cardiomyocyte

Comparative Analysis

Primary Heart cultures

Gene expression analysis by Roche Pharmaceuticals.

250 cardiac genes identified from the Novartis GNF expression atlas were plotted in rank-order along X axis.

‘Primary’ cells do not necessarily recapitulate in vivo biology.

iCell Cardiomyocytes show stable expression pattern similar to adult mRNA.

“More human than human”
iCell® Cardiomyocytes
Electrophysiology Characterization

### Ionic Currents

- $I_{Na}$
- $I_{Ca-L}$
- $I_{to}$
- $I_{Kr}$
- $I_{funny}$
- $I_{K1}$

### Spontaneous Action Potentials

- Atrial like
- Nodal like
- Ventricular like

### Elicited Action Potentials

<table>
<thead>
<tr>
<th>Gαs – β1</th>
<th>Gαq – α1</th>
<th>Gαi – m2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoproterenol</td>
<td>Phenylephrine</td>
<td>Carbachol</td>
</tr>
</tbody>
</table>

Ma et al., AJP 2011 In press
iCell® Cardiomyocytes
Utility – Functional Screens

**Electrophysiology**

- **IKr Block**
  - APD prolongation

- **PatchXpress Current Block**

**Mechanical Activity**

- **A. Contraction**
  - Control
  - 10 mM nifedipine
  - 30 mM nifedipine
  - 100 mM nifedipine
  - 500 mM nifedipine

- **B. Ca²⁺ Transient**

**Energetics**

- **% Oxygen Consumption Rate**

**Cytotoxicity**

- Healthy
- Unhealthy

**GPCR**

- FLIPR® Tetra System

**Label-Free**

- xCELLigence RTCA Cardio
Cardiac Hypertrophy; Increase in heart mass, due to increase in size of cardiomyocytes

Key Levers
- Cell density
- Pre-plate duration
- Incubation media
- Application duration
- Endpoint Assay
● **LQT2 Phenotype**
  - Delayed repolarization
  - Prolonged QT interval
  - Polymorphic ventricular tachycardia

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**Multi-Electrode Array (MEA) Field Potential Recordings**

- **Control**
- **LQT2**

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**MEA FPD**

- **LQT2**: 2000 ms
- **Control**: 500 ms

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**MEA Frequency**

- **LQT2**: 0.7 Hz
- **Control**: 0.1 Hz
iPS Cell Technology

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Neurons
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High Content Screening and Assay Development
- iPSC-derived Neurons (Network development & neurotoxicity assays)
- iPSC-derived Cardiomyocytes (Cardiomyocyte beating assay)
• **Morphology**
  Bipolar or multi-polar neurite outgrowths

• **Molecular Markers Analysis Including:**
  - βIII-Tubulin
  - Map2
  - Neuron-specific Enolase
  - Synapsin
  - Synaptophysin
  - vGAT (GABAergic)
  - vGLUT (Glutamatergic)
  - Tyrosine Hydroxylase (Dopaminergic)

• **Functional Tests**
  - **Electrophysiology:** Evoked and spontaneous action potentials; Inhibitory and excitatory post-synaptic currents; Ion channel blocks (Na⁺ and K⁺)
  - Cytotoxicity
  - Neurite outgrowth
  - Mitochondrial integrity

5 ml Pellet of Pure Neurons = ~4 Billion Neurons (~4% of total neurons in brain)
Post-thaw iCell Neurons provide efficient cell recovery, high purity and morphologically display mature processes.
iCell® Neurons Characterization

Neuron Subtype Immunocytochemistry

ICC data demonstrates the presence of a mixed neuronal cell population consisting largely of:

- **GABAergic** (*inhibitory*) neurons
- **Glutamatergic** (*excitatory*) neurons
- small percentage of **Dopaminergic** neurons (<1%)

Beta III Tubulin/Nestin/Hoechst

vGAT / vGLUT2

MAP2 / GABA / Hoechst
iCell® Neurons exhibit co-localization of pre-synaptic and post-synaptic markers.
iCell Neurons exhibit typical neuronal electrical responses as demonstrated by:

- Evoked action potentials
- Spontaneous action potentials
- Inhibitory post-synaptic currents
- Excitatory post-synaptic currents
- Ion channel blocks

Methods used:

- Single-cell current patch clamps
- Single-cell voltage patch clamps
- Multi-electrode arrays
- Automated patch clamping
iPS Cell Technology

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Novel Assays for Drug Discovery and Toxicology using Human iPS Cell-Derived Neurons and Cardiomyocytes

Oksana Sirenko, Ph.D.
February 7, 2012
Assays for Predicative Toxicology

- Cardio Safety & Toxicity
  - Live contracting cardiomyocytes

Cardiac Beating assay

- Neuronal development & toxicity
  - Stem cell derived neurons

Neurotoxicity
## Impact of Drug Toxicity

### What if we can predict failure?

<table>
<thead>
<tr>
<th>Drug Indication</th>
<th>Cardiovascular Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antibiotic</strong></td>
<td></td>
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<tr>
<td>Erythromycin</td>
<td>Sudden Cardiac Death</td>
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<tr>
<td><strong>Antihypertensive Drugs</strong></td>
<td></td>
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<tr>
<td>Clonidine</td>
<td>Brady卡dia</td>
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<tr>
<td><strong>Appetite Suppression</strong></td>
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<tr>
<td>Dexfenfluramine</td>
<td>Pulmonary Hypertension</td>
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<tr>
<td>Fenfluramine</td>
<td>Pulmonary Hypertension, Valvular Heart Disease</td>
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<tr>
<td>Phenylpropanolamine</td>
<td>Valvular Heart Disease</td>
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<tr>
<td>Phenteramine</td>
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<tr>
<td><strong>Cancer Drugs</strong></td>
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<td>Anthracycline</td>
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<tr>
<td>Cisplatin</td>
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<tr>
<td>Cyclophosphamide</td>
<td></td>
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<tr>
<td>5-Fluorouracil</td>
<td>Cardiomyopathy &amp; Heart Failure</td>
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<tr>
<td>Gemcitabine</td>
<td>Atrial Fibrillation</td>
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<td><strong>HIV Medications</strong></td>
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<td>Protease Inhibitors</td>
<td>Metabolic Syndrome</td>
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<tr>
<td><strong>Neurological Drugs</strong></td>
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<tr>
<td>Cabergoline</td>
<td>Valvular Heart Disease</td>
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<tr>
<td>Donepezil</td>
<td>Atrial Fibrillation</td>
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<tr>
<td>Pergolide</td>
<td>Valvular Heart Disease</td>
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<tr>
<td>Sumatriptan</td>
<td>Atrial Fibrillation</td>
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<tr>
<td><strong>Urological &amp; Erectile Dysfunction</strong></td>
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<tr>
<td>Alpha₁ adrenergic receptor antagonists</td>
<td>Hypotension</td>
</tr>
<tr>
<td>PDE5 Inhibitors</td>
<td>Hypotension</td>
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<tr>
<td>Yohimbine</td>
<td>Hypertension</td>
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<tr>
<td><strong>Miscellaneous Drugs</strong></td>
<td></td>
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<tr>
<td>Neostigmine</td>
<td>Bradycardia</td>
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<tr>
<td>Pseudopropylidine</td>
<td>Hypertension</td>
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<tr>
<td>Pioglitazone</td>
<td>Heart Failure</td>
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<tr>
<td>Rosiglitazone</td>
<td>Heart Failure, Myocardial Infarction</td>
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<tr>
<td><strong>Rheumatological Drugs</strong></td>
<td></td>
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<tr>
<td>Bisphosphonates</td>
<td>Atrial Fibrillation</td>
</tr>
<tr>
<td>Corticosteroids</td>
<td>Atrial Fibrillation</td>
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<tr>
<td>Tumor necrosis factor antagonists</td>
<td>Heart Failure</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>Hypertension, Heart Failure</td>
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Rofecoxib: Myocardial Infarction

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Olanzapine: Metabolic Syndrome

Tricyclic Antidepressants: Sudden Cardiac Death

Venlafaxine: Tachycardia

Bisphosphonates: Atrial Fibrillation

Corticosteroids: Atrial Fibrillation

Tumor necrosis factor antagonists: Heart Failure

NSAIDs: Hypertension, Heart Failure

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Future of Predictive Toxicology

• Prediction of drug toxic effects remains one of the industry’s greatest challenges\(^1\)
  • Overall success rate from “first-in-man” is \(\sim 11\%\)
  • \(\sim 30\%\) of compounds fall out for safety reasons
• The important trend in drug discovery is to adopt \textit{in vitro} assays\(^2\)
  • More predictive, disease relevant
  • Reduce use of animals
  • Efficiently identify adverse effects
• What is needed?
  • Improved cell based models, more complex biology
  • Advancements in assay techniques & instrumentation

\(^1\)Kola & Landis, Nat. Rev. Drug Discov., 3, 711, Aug. 2004
\(^2\)Toxicity Testing in the 21\textsuperscript{st} Century, National Academies Press, 2007
High Throughput Cell-Based Cardiotoxicity Assays

Control

Epinephrine

Verapamil

Measures rate and beating pattern of live iCell® Cardiomyocytes

![Graphs of Calcium 5 Intensity vs Time for Single Wells from FLIPR Tetra](image)

Positive Chronotropes:
- Isoproterenol
- Epinephrine
- Dopamine

Negative Chronotropes:
- Doxazosin
- Verapamil
- Propranolol

Concentration, μM

4-P Fit: $y = \frac{A - D}{1 + (x/C)^B} + D$

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration vs MeanValue</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iso (Iso)</td>
<td>32.1</td>
<td>1.51</td>
<td>0.0075</td>
<td>87.3</td>
<td>0.998</td>
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</tr>
<tr>
<td>Doxa (Dox)</td>
<td>31.6</td>
<td>2.28</td>
<td>0.626</td>
<td>11.4</td>
<td>0.826</td>
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<tr>
<td>Vera (Ver)</td>
<td>28.9</td>
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<td>0.0534</td>
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<td>0.983</td>
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<td>Epi (Epi)</td>
<td>30.6</td>
<td>0.734</td>
<td>0.0155</td>
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</table>

Beats/min

96w format

Molecular Devices products are for Research Use Only. Not for use in diagnostic procedures.
High Throughput Cell-Based Cardiotoxicity Assays

**Control**

**Epinephrine**

**Verapamil**

Measures rate and beating pattern of live cardiomyocytes

ScreenWorks® Peak Pro™ Software for the FLIPR® Tetra System

- Characterize Ca\textsuperscript{2+} oscillations from beating cardiomyocytes
- Fast, automated peak detection
- Enhanced analysis features
  - Frequency, Width, Rise & Decay times
iCell® Cardiomyocytes Beating
Analysis of Beat Rate from Imaging

Intensity

Differential

Derivative vs Time

Epinephrine

Isoproterenol
Analysis of Beat Rate from Ca²⁺ Flux

- Ca²⁺ levels fluctuate with contraction events
- Provide surrogate assessment of beat rate and sarcolemmal activity
- Cell contractions visualized with Ca²⁺ sensitive dye
  - Calcium 5 Kit from Molecular Devices
Predictive Assay for Cardio Effects

Positive Chronotropes

Concentration, μM

Epinephrine (Epinephrine: Concentration vs Mean Value) 9.47 0.53 0.0077 20.5 0.949
Plot#2 (verapamil: Concentration vs Mean Value) 9.89 1.81 0.00876 0.367 0.982

Negative Chronotropes

Beats/23 sec

epinephrine

verapamil

Weighting: Fixed

Molecular Devices products are for Research Use Only. Not for use in diagnostic procedures.
FLIPR Tetra System delivers a HT solution for cardiomyocyte assays

- HTS mechanics increases assay speed and throughput
  - 96-, 384-, and 1536-well simultaneous reads
  - Camera captures 8 images per second

- Assay simplicity
  - FLIPR Calcium Dye measures transient calcium signals
  - Signal patterns of beating cardiomyocytes

- Enables early risk assessment
  - Pre-screen to remove cardio-toxic compounds from lead generation
  - Quickly direct SAR and Med Chem efforts on a larger scale

NEW: ScreenWorks® Peak Pro™ Software delivers on the fly analysis of important peak characteristics, enabling assessment of compound effects on cardiomyocytes earlier in the drug discovery process.
Cardiac Toxicity and Long QT syndrome

- Cardiotoxic drugs often cause Long QT syndrome or Torsades de Pointes
- TdP is a form of ventricular tachycardia, which can lead to sudden death
  - TdP is associated with long QT syndrome observed on the ECG
- Most drugs that cause long QT are the IKr current blockers (hERG)
  - erythromycin, terfenadine, and ketoconazole
**Predictive Assays for Cardiac Toxicity**

- Electrophysiology measures activities of **individual** channels (e.g. hERG)
- Measurements of contractions allows a more **holistic** approach
  - Measure FLIPR Calcium 5 Assay signal

![Diagram showing electrophysiology and calcium signal during cardiomyocyte contraction](image)

- **K⁺, Cl⁻ (out)**
- **I_{h1.2}** (transient outward)
- **Ca²⁺ (in)**, **K⁺ (out)**
- **I_{Ca-L}** (Ca long)
- **I_{Ks}** (K slow delayed rect.)
- **Na⁺ (in)**
- **I_{Na}** (rapid)
- **K⁺ (out)**
- **I_{Ks}** (K slow delayed rect.)
- **I_{Kr}** (K rapid delayed rect.)
- **I_{K1}** (inward rect.)

![Graph showing Calcium 5 Signal During Cardiomyocyte Contraction](image)

- **Control**
- **Isoproterenol**

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Cardiac Beating Assay Work Flow

1. Plate iCell® Cardiomyocytes into gelatin coated plates
2. Observe cardiomyocyte beating after 3-5 days in culture
3. Load the cells with FLIPR Calcium 5 dye (2x solution)
4. Add compounds and read calcium signal
5. Analyze data using ScreenWorks software

- 20k 96; 4k 384
- PBS + 20mM HEPES
- 37°C +5%CO2 for 1 hour
- Post 5-15min, 37°C
## FLIPR Tetra Protocol

<table>
<thead>
<tr>
<th>Parameter</th>
<th>384-Well Settings</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Read Mode</strong></td>
<td>Fluorescence</td>
</tr>
<tr>
<td><strong>Excitation/Emi</strong></td>
<td>470-495 / 515-575</td>
</tr>
<tr>
<td><strong>LED Power</strong></td>
<td>40%-70%</td>
</tr>
<tr>
<td><strong>Camera Gain</strong></td>
<td>2,000</td>
</tr>
<tr>
<td><strong>Exposure Time</strong></td>
<td>0.05 sec</td>
</tr>
<tr>
<td><strong>Gate Open</strong></td>
<td>6%-100%</td>
</tr>
<tr>
<td><strong>Read Interval</strong></td>
<td>0.125 sec</td>
</tr>
<tr>
<td><strong>Reads Before</strong></td>
<td>80-180</td>
</tr>
<tr>
<td><strong>Reads During</strong></td>
<td>300-800</td>
</tr>
</tbody>
</table>

![Graph showing the addition of a compound](image_url)

**Graph Description:**
- The graph illustrates the response to the addition of a compound to the system.
- The x-axis represents time in seconds, ranging from 0 to 120.
- The y-axis indicates relative light units, with values ranging from 0 to 100.
- The time of addition is marked with an arrow labeled "Addition of compound."
High Throughput Cardiac Beating Assay: FLIPR® Tetra system

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
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<td>epinephrine</td>
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<td>digoxin</td>
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<td>dopamine</td>
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<tr>
<td>verapamil</td>
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</tr>
<tr>
<td>propranolol</td>
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<td>doxazosin</td>
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</tr>
</tbody>
</table>

| concentration    |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |

control
FLIPR Tetra System Assay Quality

**Excellent Reproducibility of Beat Rates**

Comparison of Beat Rates for Control Wells

CV’s < 10%

(n=24)
ScreenWorks Peak Pro Software: Analysis Output Parameters

- Average and standard deviation of all important parameters
- Identifies irregular spacing for finding missing peaks, extra peaks, and arrhythmias

Calcium 5 Response on FLIPR: Epinephrine High Dose (C06)

- Peak Count & Frequency
- Peak Position (time) and Amplitude
- Peak Width (FWHM)
- Rise Time (10% to 90%)
- Decay Time (90% to 10%)
New ScreenWorks Peak Pro Software Features

- Configure Kinetic Reduction
- Show Kinetic Values
- Auto-Export Data

Measure critical peak parameters associated with beating Cardiomyocytes, within ScreenWorks Software
Development of Predicative Cell-Based Assays: Positive and negative chronotrope effects

Graphs of Calcium 5 Intensity vs Time for Single Wells from FLIPR Tetra

Positive Chronotropes:
- Isoproterenol
- Epinephrine
- Dopamine

Negative Chronotropes:
- Doxazosin
- Verapamil
- Propranolol
Prospective Cell-based Model for Testing Cardiac Drugs

Control

Epinephrine (speeds up)

Dose with epinephrine followed by cardiac drugs: alpha- and beta- blockers

Verapamil

Propranolol

Cardiac drugs slow down beat rate

- Measure effect on beating rate

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration, μM</th>
<th>R²</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>propranolol</td>
<td>32.7 0.692 1.27 -9.03</td>
<td>0.989</td>
<td></td>
</tr>
<tr>
<td>doxasozin</td>
<td>31.3 1.5 2.51 -0.557</td>
<td>0.993</td>
<td></td>
</tr>
</tbody>
</table>

4-P Fit: $y = \frac{A - D}{1 + (x/C)^B} + D$

Weighting: Fixed
FAIL EARLY: Identification of compounds that affect beat rate and rhythm of iPSC cardiomyocytes

Ion Channel Blockers

- 4-P Fit: $y = (A - D)/(1 + (x/C)^B) + D$
- Concentration, µM
- IC50, µM
- Cisapride 0.07
- Nifedipine 0.12
- Tetrodotoxin 4.02

hERG Channel Blockers

- 4-P Fit: $y = (A - D)/(1 + (x/C)^B) + D$
- Concentration, µM
- IC50, µM
- Astemisole 0.07
- Cisapride 0.04
- Pimoside 0.29
- Terfenadine 0.54

Weighting: Fixed
Prolongation of Repolarization Stage by hERG Blockers

**Astemizole 1μM**

**Cisapride 0.3μM**

Overlap of beating patterns leading to high frequency low amplitude oscillations
Effect of hERG inhibitors

Cisapride

Astemizole

Concentration
Software identifies peak irregularity

Control: OK

Lidocaine: Irregular

Isoproterenol: Missing

Astemizole: Extra peaks
Surrogate Markers for Cardiac Toxicity Assay

- Measuring peak width, peak spacing and other parameter may predict compounds inducing long QT syndrome, arrhythmia and other potentially dangerous events.
Comparing Cardiomyocyte Response to Various Compounds

**Isoproterenol**
- Peaks = 19
- Freq = 21.5 bpm
- Rise = 0.18 sec
- Decay = 0.38 sec

**Control**
- Peaks = 12
- Freq = 12.0 bpm
- Rise = 0.39 sec
- Decay = 0.76 sec

**Propranolol**
- Peaks = 6
- Freq = 7.2 bpm
- Rise = 0.53 sec
- Decay = 1.81 sec
## Variability of Cardiac Beating Assay

- **Variability of different parameters across 384w plate**

<table>
<thead>
<tr>
<th>Statistics</th>
<th>Exp1 24 wells</th>
<th>Exp2 200 wells</th>
<th>Exp3 24 wells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak Count</td>
<td>8.8 ± 0.7</td>
<td>8.5 ± 1.4</td>
<td>15.9 ± 2.5</td>
</tr>
<tr>
<td>Average Peak Width</td>
<td>1.0 ± 0.2</td>
<td>2.3 ± 0.3</td>
<td>3.0 ± 0.4</td>
</tr>
<tr>
<td>Average Peak Amplitude</td>
<td>18.0 ± 5.0</td>
<td>2249.3 ± 530.3</td>
<td>195.1 ± 31.8</td>
</tr>
<tr>
<td>Average Peak Baseline</td>
<td>59.4 ± 5.5</td>
<td>2805.6 ± 199.7</td>
<td>265.1 ± 23.4</td>
</tr>
<tr>
<td>Average Peak Spacing</td>
<td>4.1 ± 0.3</td>
<td>6.9 ± 1.0</td>
<td>6.4 ± 1.0</td>
</tr>
<tr>
<td>Average Peak Rise Time</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>Average Peak Decay Time</td>
<td>1.0 ± 0.1</td>
<td>1.4 ± 0.2</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>Average Peak Width at 10% Amplitude</td>
<td>1.7 ± 0.2</td>
<td>2.7 ± 0.4</td>
<td>3.6 ± 0.5</td>
</tr>
</tbody>
</table>
FLIPR Tetra system and ScreenWorks Peak Pro Software

- FLIPR Tetra system HTS mechanics dramatically increases safety throughput
- ScreenWorks Peak Pro expands peak detection for beating cardiomyocytes and other primary cells with Ca\(^{+2}\) oscillations
  - Enables analysis of samples with multiple peaks per response
- ScreenWorks Peak Pro speeds up peak detection analysis
  - Easy to use analysis methods, performed directly within ScreenWorks
  - Built on proven ScreenWorks Software platform
- Fail poor compounds early = Large cost savings in clinical trials and product liability
Neuronal Toxicity Assays Using iPSC Derived Neurons: iCell® Neurons

• Nervous system is a target organ for the toxic effects of chemical compounds & environmental agents
  • HTS assays are in high demand

• There is a great interest in developing more predictive cell-based models and efficient screening tools
  • Neurodegenerative diseases Alzheimer’s or Parkinson’s
  • Excitotoxicity

• We present here several assays for assessment of neural toxicity
  • Neurite outgrowth
  • Integrity of mitochondria
  • Live cell assays
Neuronal Cell Imaging & Analysis

- Imaging: ImageXpress® Micro XL
- Image analysis: MetaXpress® software, Neurite Outgrowth module
ImageXpress® Micro XL Automated Imaging System

- High sensitivity and dynamic range improves ability to quantify features

Cells: ~500

Cells: ~120
Neurite Outgrowth: 
Fast and Biologically Relevant Assay for Neurotoxicity

<table>
<thead>
<tr>
<th>Compound</th>
<th>Total neurite outgrowth</th>
<th>% cells with outgrowth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antimycin A</td>
<td>13</td>
<td>14</td>
</tr>
<tr>
<td>Staurosporine</td>
<td>0.9</td>
<td>1.7</td>
</tr>
<tr>
<td>MK571</td>
<td>4.1</td>
<td>3.9</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>NMDA</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>SNAP</td>
<td>68</td>
<td>84</td>
</tr>
<tr>
<td>PD98059</td>
<td>31</td>
<td>46</td>
</tr>
</tbody>
</table>

Acquisition Time = 4:00
Analysis Time = 7 min
Z-prime ~ 0.7
Effect of Growth Factors on Neural Development

- Multi-parametric profiling
  - Multiplexed analysis to maximize information
  - Outgrowth, branches, processes, cell number, process length, etc.

![Graph showing log change in cell number, processes, and branch number with control, NFG, BDNF, and EPO treatments.]

![Control and BDNF images showing neural tissue under microscopic conditions.]
Neural Regeneration: Live Cell Real Time Neurite Outgrowth

Untreated Cells

Staurosporine

- Re-growth of neurons after injury is mediated by neurotrophic factors that stimulate cell adhesion, migration, and extension of neurites
  - Measured in real time using time lapse acquisition in transmitted light
Real Time Neurite Outgrowth Assay

- Inhibition of neurite outgrowth by staurosporine
  - Transmitted light
  - Time-lapse acquisition (14h)
  - Automated analysis

![Graph and images showing neurite outgrowth over time with different treatments and staurosporine inhibition.]
Integrity of Mitochondria

- Mitochondrial depolarization is an early signal for excitotoxicity, hypoxic damage or oxidative stress
  - Mitochondria membrane potential monitored with the mitochondria active dye JC-10
  - Analyzed using the Granularity module
  - End-point or real time assay

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antimycin A</td>
<td>46</td>
</tr>
<tr>
<td>Valinomycin</td>
<td>0.15</td>
</tr>
</tbody>
</table>
HTS Assay for Predicative Toxicology

Methyl Mercury

Neurite outgrowth

Hoechst – Nuclear condensation

CalceinAM - Neurite outgrowth

Concentration, uM

0.01 0.1 1 10 100 1000

0 100 200 300

4-P  Fit: y = (A - D)/( 1 + (x/C)^B ) + D: A B C D R^2

dex (dex: Concentration vs MeanValue) 270 1.62 152 -10.4 0.972

ret acid (ret acid: Concentration vs MeanValue) 255 6.54 168 5.06 0.988

MK (MK801: Concentration vs MeanValue) 255 3.33 295 11.1 0.984

mer (mercury: Concentration vs MeanValue) 266 2.14 4.44 12.2 0.998

valp acid (kain acid: Concentration vs MeanValue) 285 1.33 1.79e+03 1.79 0.938

Weighting: Fixed

Dexamethasone  152 uM
Retinoic acid   168 uM
Methyl Mercury  4.4 uM
MK801   295 uM
Valproic acid >1000 uM

live
dead

Nuclei
## Predictability of Neurotoxicity Assay

<table>
<thead>
<tr>
<th>Known Neurotoxins</th>
<th>IC50, uM</th>
<th><em>in vivo Neurotoxicity</em></th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl Mercury</td>
<td>4</td>
<td>+</td>
<td>metal</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>77</td>
<td>+</td>
<td>anti-inflammatory (steroid)</td>
</tr>
<tr>
<td>Retinoic acid</td>
<td>197</td>
<td>+</td>
<td>vitamin A, anti-cancer</td>
</tr>
<tr>
<td>MK801</td>
<td>270</td>
<td>+</td>
<td>anti-convulsant, NMDA rec.ant.</td>
</tr>
<tr>
<td>Kainic acid</td>
<td>723</td>
<td>+</td>
<td>neurostimulant, receptor agonist</td>
</tr>
<tr>
<td>Valproic acid</td>
<td>&gt;1000</td>
<td>+</td>
<td>anti-convulsant</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Anti-Proliferation</th>
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</thead>
<tbody>
<tr>
<td>Cytosine arabinoside</td>
<td>151</td>
<td>+</td>
<td>anti-cancer</td>
</tr>
<tr>
<td>Hydroxyurea</td>
<td>n/d</td>
<td>-</td>
<td>anti-cancer</td>
</tr>
<tr>
<td>5-Fluorouracil</td>
<td>n/d</td>
<td>-</td>
<td>anti-cancer</td>
</tr>
<tr>
<td>Ochratoxin A</td>
<td>n/d</td>
<td>-</td>
<td>mycotoxin</td>
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<table>
<thead>
<tr>
<th>Safe</th>
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<tbody>
<tr>
<td>Ascorbic acid</td>
<td>n/d</td>
<td>-</td>
<td>vitamin C</td>
</tr>
<tr>
<td>Aspirin</td>
<td>n/d</td>
<td>-</td>
<td>anti-inflammatory</td>
</tr>
</tbody>
</table>

n/d = not detectable
HCA for Predicative Toxicology

- We have demonstrated several automated neurotoxicity assays suitable for screening environments:
  - Neural network integrity (fixed or live cells)
  - Mitochondrial integrity (live cells)
  - Nuclear condensation (fixed or live cells)
- These assays can be used for:
  - Testing biologics or chemical compounds on neuronal development
  - Screening and validation of drug candidates
  - Evaluating potential neurotoxic effects of different agents
Acknowledgements

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- Monica Strathman
- Debra Gallant
- Grischa Chandy

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delaura@cellulardynamics.com