The Technical Merits Required to Develop High Content Imaging Applications for Cardiomyocytes Derived from Human Induced Pluripotent Stem Cells

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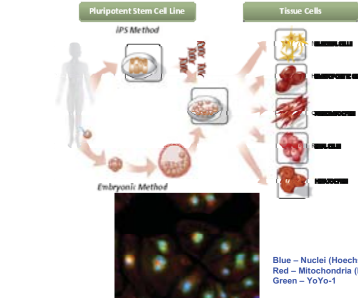
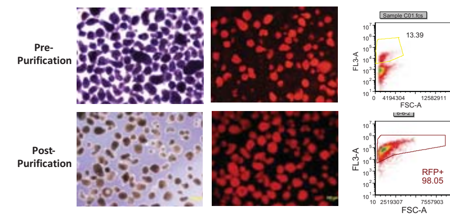
Abstract

The potential of utilizing human primary cardiomyocytes in early safety programs for compound assessment has been historically limited by cell availability and technical challenges. However, recently, human induced pluripotent stem (hiPS) cell-derived cardiomyocytes have been established in suitable scale sufficient for the conduct of characterization experiments. During bulk production of hiPS cell-derived cardiomyocytes, particular emphasis was placed upon defining appropriate quality controls for consistent manufacturing including cardiomyocyte purity and post-cryopreservation viability. From a functional utility purpose, initial sets of validation experiments assessed the hiPS cell derived cardiomyocytes for their suitability to profiling molecules for their safety pharmacological and toxicological properties. In doing so, a set of specific cytotoxic endpoints were identified. This presentation will highlight the method development for cell handling and provide select examples of the high-content screening (HCS) assays and feature measurements that have been adapted for such studies. Illustrated features include, but are not limited to, cell viability, cell purity, nuclear morphology, DNA damage, mitochondrial membrane potential, oxidative stress, reactive oxygen species, lysosomal integrity, and apoptotic events.

Introduction



Aggregates Pre and Post Selection huIPSC-Derived Cardiomyocytes



Protocol Optimization for Dissociation of Aggregates Based on Viability

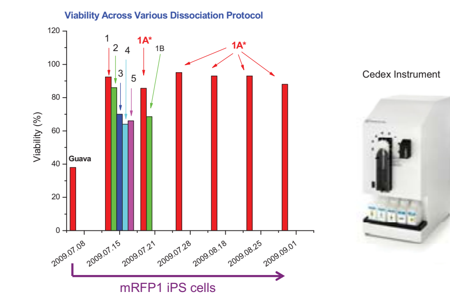
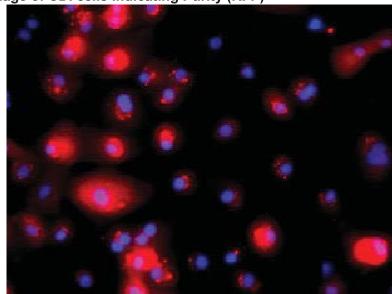
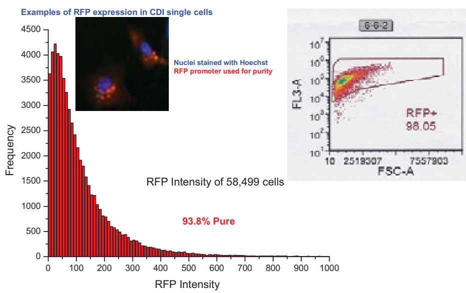


Image of CDI cells Indicating Purity (RFP)



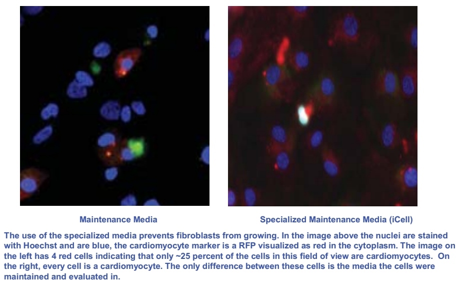
Methods for Determining Purity



Final Aggregate Dissociation Protocol

- Upon arrival, the aggregates were transferred to a low adhesion T25 flask and allowed to recover for 2-3 hours.
  (otherwise 37C incubated aggregates in TC flasks are the starting point)
- Transfer 37C aggregates to a conical 15ml tube and allow them to settle to the bottom of the tube
- Aspirate media; add 5mls prewarmed 0.25% Trypsin-EDTA
- Incubate for a total of 5 minutes in a 37C water bath; flick the tube after 2.5 minutes
- After the 5 minute incubation, invert the tubes 3x
- Allow the cells to gravity settle and pipet cells up/down 2x; wait ~45 seconds to allow aggregates to settle
- Transfer the solution containing single cells to a 50ml conical tube containing 30mls of prewarmed growth media
- Repeat the Trypsin step on aggregates as many times as needed to disrupt all of the aggregates as opposed to using longer Trypsin times (Usually 3-4 repeats)
- Once all of the aggregates have been dissociated and combined in the 50ml tube, centrifuge for 5 minutes at room temperature @ 200xg
- Aspirate; add growth media (13mls)
- Transfer 1ml of cell suspension to the Cedex tube and count cells for density and viability on Cedex

Specialized Media Minimizes Fibroblast Growth



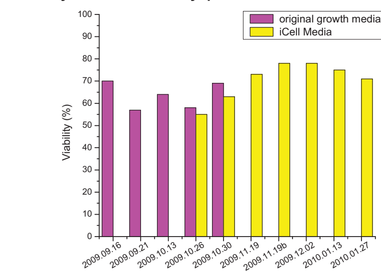
Maintenance Media — Specialized Maintenance Media (iCell)

The use of the specialized media prevents fibroblasts from growing. In the image above the nuclei are stained with Hoechst and are blue, the cardiomyocyte marker is a RFP visualized as red in the cytoplasm. The image on the left has 4 red cells indicating that only ~25 percent of the cells in this field of view are cardiomyocytes. On the right, every cell is a cardiomyocyte. The only difference between these cells is the media the cells were maintained and evaluated in.

Gentle Thawing and Handling of Cryopreserved Cells

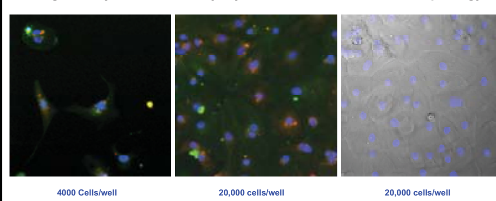
- 2-3 vials of cells can be thawed at the same time, if additional vials are required they should be thawed separately.
- Cells are thawed in the 37C water bath while gently and slowly shaking vials in hand.
- As soon as the cells are thawed, remove the cells from the incubator and wash them thoroughly with ethanol
- Using a 1ml pipet, the cells are transferred to a 50ml conical tube containing 8mls of cold media (per vial of cells being thawed, (all cells are mixed together)) Add the cell suspension dropwise while gently tapping the tube. It is very important not to quickly dispense the cells, viability will be lost.
- Using a 1ml pipet, use 1ml of cold media to rinse the cryo tube and lid to secure as many cells as possible. Add this solution dropwise to the 50ml tube.
- Once all of the cells from this round of thaw have been transferred to the 50ml tube, put the lid on the tube and gently invert the tube 3x. Transfer 1ml of cell suspension to a Cedex tube for cell counting and viability determination.
- Adjust the cell density to what is needed and plate cells by hand using a multi-channel pipet.
- Repeat the procedure for the next set of vials needing to be thawed.
- KEY NOTES: DO NOT PIPET CELLS UP/DOWN. This mechanical method will rupture cells and cause a lower viability. Also, DO NOT USE A AUTOMATED INSTRUMENT FOR PLATING (ex. multidrop) again, the mechanical processes of these instruments also rupture the cells.

Viability of Commercial Cryopreserved Cells



Gentle cell handling must be utilized to achieve a higher viability of cryopreserved cells out of thaw. Viability was determined using the Cedex.

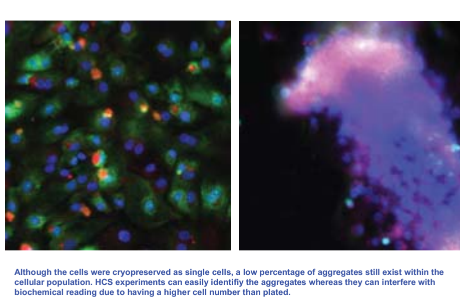
Plating Density of the Cardiomyocytes Can Alter the Cellular Morphology



4000 Cells/well — 20,000 cells/well — 20,000 cells/well

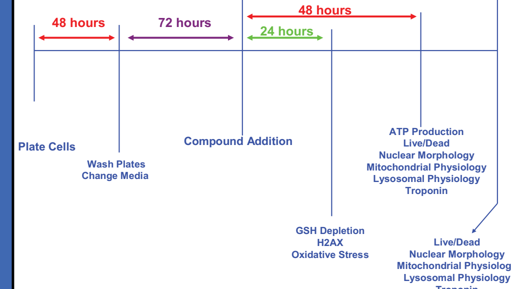
Original experiments were completed using the low density of cells. Then experiments were switched to have 20,000 cells/well so that the cardiomyocytes would be a complete monolayer mimicking the heart and beating in synchronization.

Aggregates Are Present Within The Cryopreserved Cells

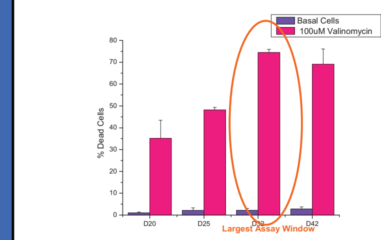


Although the cells were cryopreserved as single cells, a low percentage of aggregates still exist within the cellular population. HCS experiments can easily identify the aggregates whereas they can interfere with biochemical reading due to having a higher cell number than plated.

Assay Outline

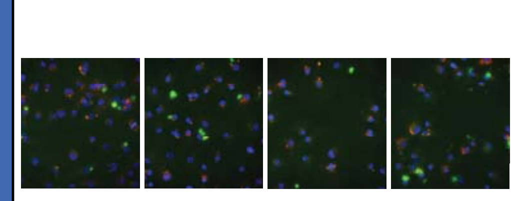


% Dead Cell Population Throughout Differentiation



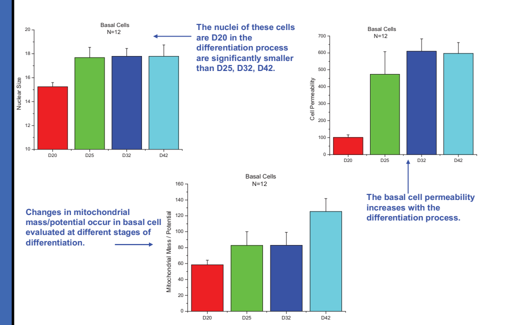
Dead cell number is determined by staining nuclei with Hoechst and treating the cells with Carboxyfluorescein which stains dead cells green.

Images of Different Stages of Differentiation



D20 — D25 — D32 — D42

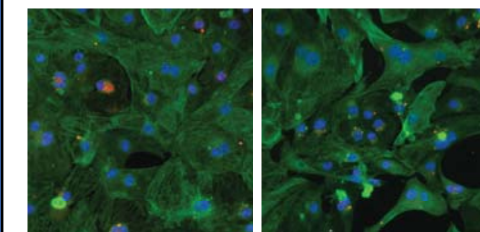
Differentiation Time Point Differences



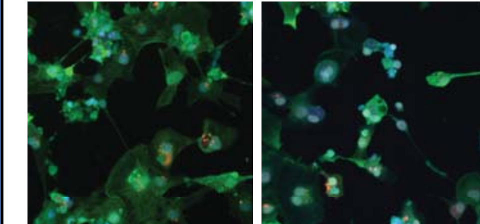
Doxorubicin Effects in CDI Cardiomyocytes

	Doxorubicin Concetration Resulting in a Toxic Result
GSH - 24hr	100uM
CT4 - 48hr	30uM, 100uM
CT8 - 24hr	10uM, 30uM, 100uM
CT3 - %Dead - 48hr	3uM, 10uM, 30uM, 100uM
CT3 - %Dead - 72hr	1uM, 3uM, 10uM, 30uM, 100uM
CT5 - Cell # - 48hr	30uM, 100uM (50% @ 10)
CT5 - Cell # - 72hr	30uM, 100uM (50% @ 10)
CT5 - Cell Perm - 48hr	10uM (2x), 30uM (3x), 100uM (3x)
CT5 - Cell Perm - 72hr	0.3uM (2x)
CT5 - MitoMassPot - 48hr	1uM, 3uM, 10uM, 30uM, 100uM
CT5 - MitoMassPot - 72hr	0.3uM, 1uM, 3uM, 10uM, 30uM, 100uM
CT5 - NucSize - 48hr	0.3uM, 1uM, 3uM, 10uM, 30uM, 100uM
CT5 - NucSize - 72hr	0.3uM, 1uM, 3uM, 10uM, 30uM, 100uM
LysoMass/pH - 48hr	30uM, 100uM
H2AX (DNA damage) - 24hr	0.3uM, 1uM, 3uM, 10uM, 30uM, 100uM

Doxorubicin Treated - Troponin Stained Cells

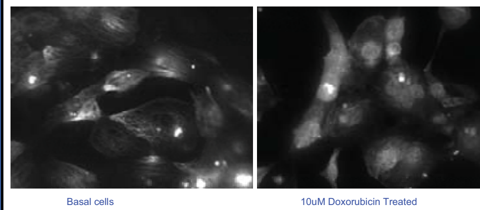


Basal — 0.1uM Doxorubicin



1uM Doxorubicin — 10uM Doxorubicin

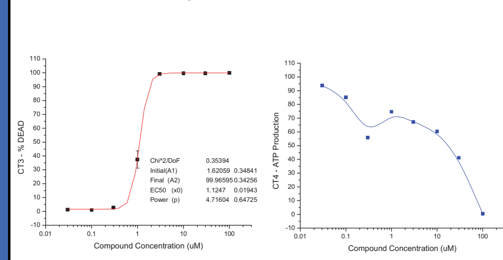
Phalloidin-488 Stained iPS cells



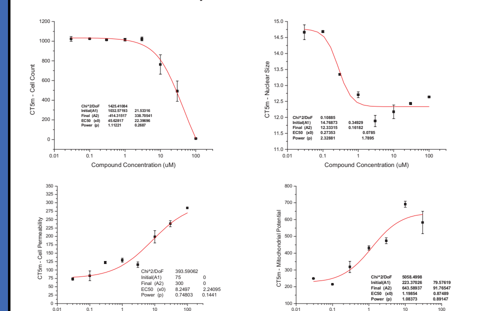
Basal cells — 10uM Doxorubicin Treated

Doxorubicin at 10uM disrupts the actin fibers throughout the cells.

Doxorubicin - 48hr Compound Incubation



Doxorubicin - 48hr Compound Incubation



Assay Development Differences: CDI iPS Cells vs Typically Used HCS Cell Lines

- **Differences**
  - Gentle Handling (especially cryopreserved cells)
  - Cell Density (non-proliferating cells)
  - Media (type and volume)
  - Pretreatment time to allow cardiomyocyte beating characteristics and homeostatsis
- **No Difference**
  - Basic Probe sets (modified due to RFP marker)
  - Assay Time Points
  - HCS Readouts
  - Assay Plate Extracellular Matrix
  - Standards and reference compounds

Conclusions

- Routine techniques for handling, plating, and using the human iPS derived cardiomyocytes have been established.
- An optimal protocol for the dissociation of aggregates was established resulting in consistently high cellular viability to allow compound evaluations.
- Gentle handling techniques are required to maintain good viability of the cryopreserved cardiomyocytes.
- Specialized media from CDI, iCell media, optimally maintains the cells and also prevents the growth of non-differentiated fibroblasts.
- High-Content Imaging Assays have been developed evaluating Doxorubicin, a known cardiotoxicant, to initiate the use of human iPS cells for preclinical in vitro safety monitoring. Results with Doxorubicin show that this treatment effected the human iPS cells causing:
  - Cell death
  - Changes in cell number
  - Mitochondrial potential
  - DNA damage