High Content Analysis of Cardiac Differentiation from iPS Cells

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Abstract

Human induced pluripotent stem (iPS) cells are a promising new tool for cardiovascular research and therapeutic applications. The ability of human iPS cells to differentiate into functional cardiomyocytes has been demonstrated in various reports. iPS derived cardiomyocytes express markers of mature cardiac cells, ion channels, and also demonstrate beating and action potentials similarly to primary cardiac cells. However, the high cost for purified iPS derived cardiomyocytes limits their utility for HCS. New methods are needed to enable efficient differentiation of cardiomyocytes to provide a low-cost source of mature cardiac-like cells.

Introduction

Monitoring cell differentiation can be used for development of standardized protocols and quality control procedures, and characterization of the impact of different agents on cardiac development. Here we described methods that can be used to monitor differentiation of fibroblasts-derived iPS to cardiomyocytes in automated fashion (see Fig 1). We demonstrate use of ImageXpress® Micro system and MetaXpress® software to simultaneously monitor expression of cardiac markers and stem cell markers. We show that expression of the pluripotency genes Sox2 and Oct4 is high in undifferentiated iPS cells, and becomes down-regulated during culture in differentiation media. At the same time, expression of the cardiac markers a-actinin and troponin T increases over time during differentiation process. In addition, a preliminary method for measuring beating cells will be presented.

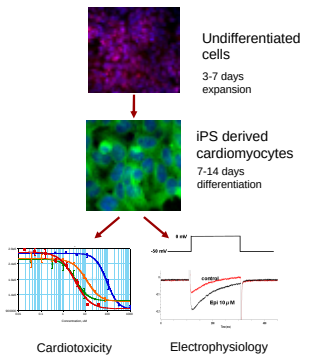

Fig 1. Steps in transformation of iPS progenitors into cardiomyocytes.

An emerging application for iPS derived cardiomyocytes is use as a model system for testing toxicity and cardioprotection effects of drug candidates and developing drugs. Cell viability and membrane integrity can be monitored simultaneously using HCA and staining with Calcein AM and MitoTracker. We have used this system to determine IC50s of several cardiotoxic drugs (doxozamine, imatinib, mitomycinA, verapamil). We have also demonstrated cytoprotection effect of sphingosine phosphate against cytotoxic damage.

Experimental

Cell Preparation

- iPS derived cardiomyocytes were obtained from Celprogen or Cellular Dynamics
- iPS progenitors (Celprogen) were expanded in culture for 3-7 days in the presence of expansion media
- Cells were plated on laminin coated 96w plates, in the presence of differentiating media for 7-14 days
- Cells were visualized using ImageXpress® Micro System and fluorescently labeled cardiomyocyte specific markers
- Images were analyzed using standard algorithms from MetaXpress® Software

High Content Image Acquisition & Analysis

- Images acquired with ImageXpress Micro System using 20X objective and three exposures
  - AF488 labeled markers: 488nm Ex, 520nm Em
  - AF555 labeled markers: 535nm Ex, 570nm Em
  - Hoechst dye label for nuclei: 405nm Ex, 450nm Em
- Images were analyzed using the Cell Scoring algorithms from MetaXpress® Software

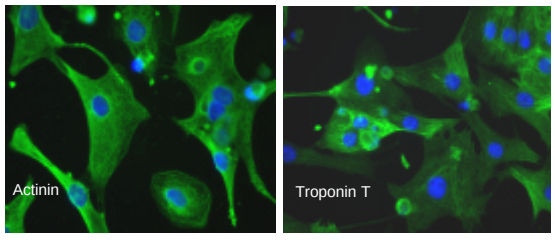

Plate Reader Acquisition & Analysis

- Fluorescence intensity data was acquired with SpectraMax® Paradigm System
  - Calcein AM: 485nm Ex, 535 nm Em
  - MitoTraker Green: 485nm Ex, 595 nm Em
- Data was analyzed using SoftMax® Pro

References

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Department of Medicine, University of Wisconsin, WiCell Research Institute, Madison, WI 53792-3248, USA.
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Wernig M, Meissner A, Foreman R, Brambrink T, Ku M, Hochedlinger K, Bernstein BE, Jaenisch R.
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Cardiomyocyte Differentiation

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 Fig 2. 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.

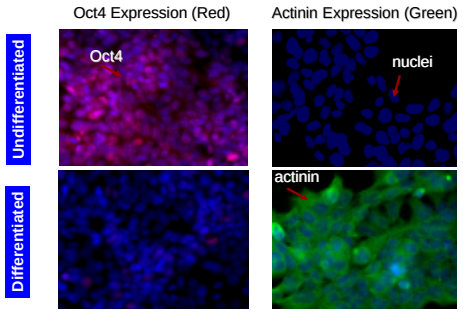


Fig. 2. Images of iPS cells in undifferentiated and differentiated state. Images were acquired with ImageXpress Micro system using a 20X objective.

Image analysis was done using the Cell Scoring module in MetaXpress Software, and data visualization and analysis was done using AcuityXpress™ Software. The Cell Scoring module determines a "positive" cell by presence of both nuclear and marker stains. Statistics on number and phenotype of cells in each well are then calculated. Examples of image analysis results are shown in Figure 3. A summary of the data for the seven different markers and an isotype control is shown in Figure 4.

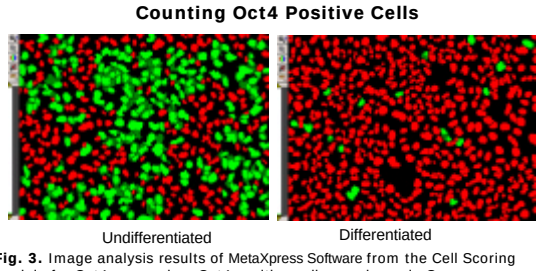


Fig. 3. Image analysis results of MetaXpress Software from the Cell Scoring module for Oct4 expression. Oct4 positive cells are shown in Green.

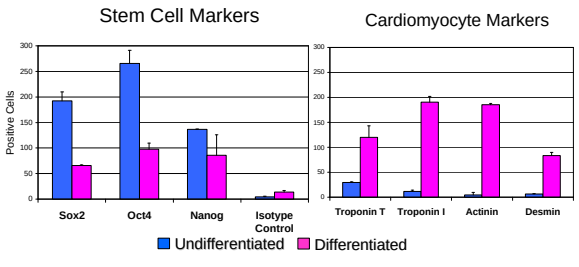


Fig. 4. Expression levels for seven different markers and an isotype control in undifferentiated and differentiated cells.

The rate of change of cardiomyocyte markers on differentiating iPS cells was measured in a time course study. The number of actinin positive cells was measured in nine wells (96 well plate) over a five day period as described previously. The results are shown in Figure 5. A clear increase is observed over the 5 day period.

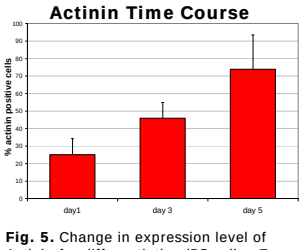


Fig. 5. Change in expression level of Actinin for differentiating iPS cells. Error bars represent 1 stdev; n=9.

Cardioprotection Assay

iPS derived cardiomyocytes are ideal for studies of cardioprotective compounds. In this assay such cells were cultured in the presence of Antimycin A and Ca Ionophore (hypoxia model). Cell viability was determined using Calcein AM and measuring fluorescence intensity with a Paradigm plate reader. Significant protective effect was observed with sphingosine phosphate.

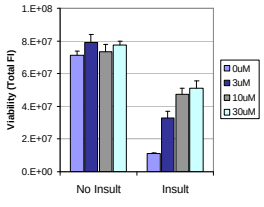


Fig. 6. Improvement in cell viability due to presence of sphingosine phosphate during hypoxic insult.

Spontaneous Beating of Cardiac Cells

Maturity of cardiac cells is assessed by the ability of a lawn of cells to spontaneously expand and contract (beating). This can be observed visually quite easily and also measured with standard electrophysiology tools. However, characterization of this activity via HCA remains a challenge. Here we present preliminary data on a method to measure beat rate and amplitude. Cardiomyocytes were treated with 1 μM epinephrine (EPI). Results from the time period of 10 to 20 min after addition of EPI are shown below.

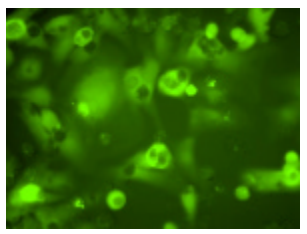


Fig. 7. Single image of cardiac cells that are spontaneously beating

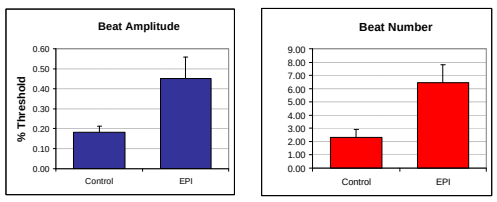
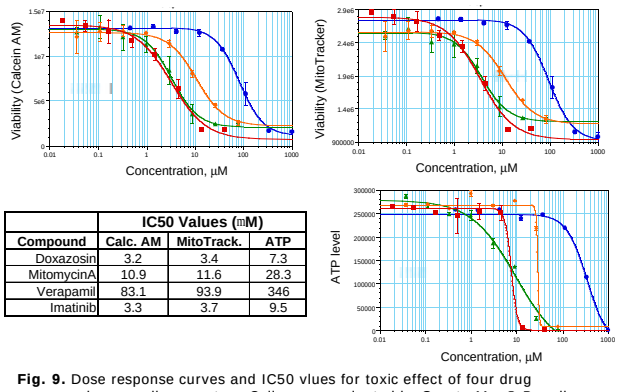


Fig 8. Average amplitude and number of beats of cardiomyocytes after dosing with EPI for 10 to 20 minutes. Contractions were measured over a 19 second period with images taken every 300msec. Average change in the cell lawn was determined by a differential threshold algorithm. Beat Amplitude represents the average amplitude of all beats. Beat Number is number of contractions observed during the 19 second periods.

Cardiotoxicity

A number of cardiotoxic drugs can effect general cell viability, integrity of mitochondria, and metaolic oxidative activity. In this assay these parameters were evaluated by SpectraMax Paradigm plate reader. 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 9. The cell viability and MitoTracker assays showed similar IC50 values while those for ATP were higher.



	IC50 Values (mM)		
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. 9. 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

We have developed high content imaging methods that allow automatic evaluation of differentiation of iPS derived cardiomyocytes

This method can be used to automate assays for:

- Testing biologics or chemical compounds on cardiac development
- Screening and validation of drug candidates
- Evaluating potential cardiotoxic and cardioprotective agents

We have demonstrated the effect of several cardiotoxic drugs on cell viability and membrane integrity

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