Modeling Cardiac Hypertrophy:
Endothelin-1 Induction with High Content Analysis

Introduction

Cardiac hypertrophy is characterized by several different cellular changes, including reactivation of the fetal gene program (1, 2). One of the genes in this group, *NPPB*, and the protein product for which it encodes, B-type natriuretic peptide (BNP), have been utilized as classical biomarkers for hypertrophy. Because cardiac hypertrophy is observed under many conditions, including myocardial infarction, ischemia, hypertension, valvular dysfunctions, and toxic side effects of small molecule/protein stimulation, there is a need for reproducible methods for its detection and quantification.

iCell® Cardiomyocytes are derived from human induced pluripotent stem cells (iPSCs) and have been shown to recapitulate the biochemical, electrophysiological, mechanical, and pathophysiological properties of native cardiac myocytes, including hypertrophic responses (3). Due to their human origin, high purity, and functional relevance, iCell Cardiomyocytes enable the interrogation of cardiac biology in basic research and drug discovery.

High content analysis (HCA) combines fluorescent microscopy with an automated digital imaging system to provide qualitative and quantitative analysis of changes in cellular phenotype. This Application Protocol describes how to utilize iCell Cardiomyocytes in an in vitro cellular model of cardiac hypertrophy that is induced by endothelin-1 (ET-1). The response can be quantified as early as 5 days post-plating via increased BNP expression using screening-compatible 96- and 384-well assay with high content analysis. Longer post-plating incubation times can be implemented to better fit end-user workflows or to increase the assay response window.

Required Equipment and Consumables

The following equipment and consumables are required in addition to the materials specified in the iCell Cardiomyocytes User's Guide.

Item	Vendor	Catalog Number
Equipment		
High Content Imaging System	Multiple Vendors	N/A
Multichannel Pipettor	Multiple Vendors	N/A
Consumables		
iCell Cardiomyocytes Kit	Cellular Dynamics International (CDI)	CMC-100-010-001 CMC-100-010-005
384-well Optical Imaging Flat Clear Bottom Black Polystyrene TC-treated Microplate (384-well Cell Culture Plate)*	Greiner Bio-One	781091
	Corning	3985
	BD Biosciences	353962
	PerkinElmer	6007550
96-well Optical Imaging Flat Clear Bottom Black Polystyrene TC-treated Microplate (96-well Cell Culture Plate)*	Greiner Bio-One	655090
	BD Biosciences	353219
Alexa Fluor 647-Labeled Donkey Anti-mouse IgG (Secondary Antibody)	Life Technologies	A31571
Anti-proBNP Mouse Monoclonal Antibody [15F11] (Detection Antibody)	Abcam	ab13115
Brefeldin A (BFA)	Sigma	B7651
Cocktail B from Hepatocyte Maintenance Supplement Pack (Supplement Pack)	Life Technologies	CM4000
Dulbecco's Phosphate Buffered Saline without Ca^{2+} and Mg^{2+} (D-PBS)	Invitrogen	14190
Endothelin-1 (ET-1)	Sigma	E7764
Fibronectin	Roche Applied Science	11051407001
Hoechst 33342, 10 mg/ml	Life Technologies	H3570
HPLC-grade Methanol	Multiple Vendors	N/A
Nonfat Dry Milk	Multiple Vendors	N/A
Triton X-100 Detergent Solution	Multiple Vendors	N/A
Ultra Pure Methanol-free Formaldehyde, 16%	Polysciences, Inc.	18814-20
William's E Medium	Life Technologies	A12176

* Order the format (96-well or 384-well cell culture plates) required for your experiment.

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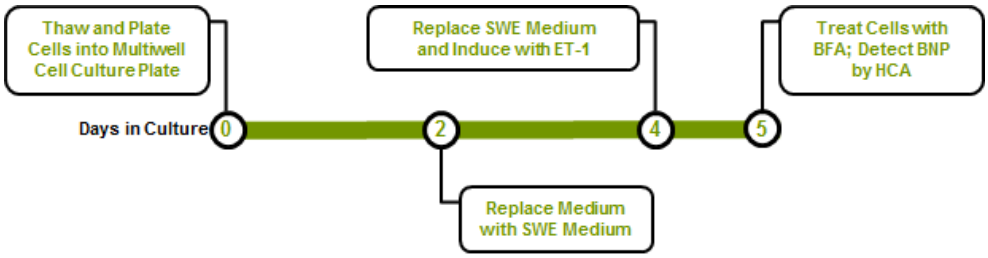
Workflow

iCell Cardiomyocytes are thawed and plated into either a 96- or 384-well cell culture plate previously coated with fibronectin. On day 2 post-plating, Plating Medium is replaced with supplemented William's E (SWE) medium. On day 4 post-plating, ET-1 is added in fresh SWE medium. On day 5 post-plating, cells are treated with BFA and then analyzed for BNP expression using HCA. The assay is optimized for a 5-day workflow but can be extended if larger signal-to-noise ratios are necessary. Contact CDI's Technical Support (support@cellulardynamics.com; +1 (877) 320-6688 (US toll-free) or (608) 310-5100) for instructions on extending the assay workflow.



Methods

Thawing and Culturing iCell Cardiomyocytes

1. Dilute 1 mg/ml fibronectin solution in sterile D-PBS to a final concentration of 5 µg/ml immediately before use. Approximately 10 ml are required to coat either a 96- or 384-well cell culture plate.

   Note: *Reconstitute the fibronectin in sterile water at 1 mg/ml according to the manufacturer's instructions. Aliquot and store at -20°C.*

2. Add 100 µl/well of 5 µg/ml fibronectin solution to a 96-well cell culture plate.

   Note: *For a 384-well cell culture plate, add 25 µl/well of 5 µg/ml fibronectin solution.*

   Note: *If necessary, store fibronectin-coated plates at 4°C for up to 1 week.*

3. Incubate at 37°C for at least 1 hour.

4. Thaw iCell Cardiomyocytes according to the iCell Cardiomyocytes User's Guide.

5. Dilute the cell suspension in iCell Cardiomyocytes Plating Medium (Plating Medium) to 200,000 plated cells/ml. Refer to the User's Guide for instructions to calculate the *Target Plating Density* based on *Plating Efficiency*.

6. Aspirate the fibronectin solution. Immediately add 100 µl/well of the cell suspension (20,000 plated cells/well).

   Note: *For a 384-well cell culture plate, add 25 µl/well of the cell suspension (5,000 plated cells/well).*

7. Centrifuge the cell culture plate at 100 x g for 1 minute.

8. Incubate in a cell culture incubator at 37°C, 7% CO_2 for 48 hours.

   Note: *CDI recommends leaving the cells undisturbed for maximum attachment.*

9. Prepare the supplemented William's E (SWE) medium by diluting Cocktail B to 1:25 in William's E Medium immediately before use. Invert to mix. Do not filter.

   Note: *Cocktail B is provided in the Supplement Pack and supplies the cardiomyocytes with a source of energy in the serum-free SWE medium. Also provided in the Supplement Pack is dexamethasone, which is not used for this protocol.*

   Note: *Stored separately, William's E Medium and Cocktail B are stable at 4°C for 1 year according to the manufacturer. CDI recommends preparing fresh 50 ml aliquots of SWE medium before performing this protocol.*

10. Aspirate or quickly decant the Plating Medium from the 96-well cell culture plate. Wash 2 times with 200 µl/well of SWE medium.

    Note: *For a 384-well cell culture plate, wash 2 times with 50 µl/well of SWE medium.*

    Note: *CDI recommends replacing the Plating Medium with SWE medium in the morning on day 2 post-plating to maximize the time that the cardiomyocytes are maintained in culture in serum-free medium before inducing a hypertrophic response.*

11. Aspirate or quickly decant the SWE medium. Add 100 µl/well of fresh SWE medium.

    Note: *For a 384-well cell culture plate, add 25 µl/well of fresh SWE medium.*

12. Centrifuge the cell culture plate at 100 x g for 1 minute.

13. Incubate in a cell culture incubator at 37°C, 7% CO_2 for 48 hours.

    Note: *Contact CDI's Technical Support (support@cellulardynamics.com; +1 (877) 320-6688 (US toll-free) or (608) 310-5100) for instructions on extending the assay workflow beyond 5 days.*

Inducing a Hypertrophic Response in iCell Cardiomyocytes

iCell Cardiomyocytes are suitable for inducing a hypertrophic response with ET-1 on day 4 post-plating when cultured according to this protocol. CDI recommends inducing a hypertrophic response with ET-1 in iCell Cardiomyocytes in the afternoon on day 4 post-plating. This timing allows for data acquisition and analysis in the morning on day 5 post-plating at 15 hours post-treatment.

1. Reconstitute the ET-1 in sterile water to a stock concentration of 10 µM.

2. Prepare a serial dilution of ET-1 in SWE medium at 5X the final concentrations to allow for assessment of a 10-point dose-response curve of induced BNP expression during data analysis.

   Note: *CDI recommends preparing a 4-fold titration with the top concentration of 10 nM. EC_{50} values in the low pM range have been observed for ET-1.*

3. Remove the cell culture plate containing iCell Cardiomyocytes from the incubator.

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4. Aspirate the spent SWE medium. Immediately add 80 µl/well of fresh SWE medium.

   Note: *For a 384-well cell culture plate, add 32 µl/well of fresh SWE medium.*

5. Add 20 µl/well of serially diluted ET-1.

   Note: *For a 384-well cell culture plate, add 8 µl/well of serially diluted ET-1.*

6. Centrifuge the cell culture plate at 100 x g for 1 minute.

7. Incubate in a cell culture incubator at 37°C, 7% CO_2 for 15 hours.

Data Collection and Analysis

The following sections detail how to prevent BNP secretion**,** how to label cells for data acquisition, and then how to perform data analysis.

Preventing Secretion of the BNP Protein

BNP is a secreted protein. Incubation of cardiomyocytes in BFA prevents BNP secretion, increasing cellular BNP levels and thus experimental signal. The optimal time to prevent BNP secretion is 15 hours post-ET-1 treatment.

1. Dilute the BFA solution in SWE medium to a 5X concentration of 50 µg/ml immediately before use.

   Note: *Reconstitute the BFA in HPLC-grade methanol to prepare a 10 mg/ml stock solution. Aliquot and store at -20°C.*

2. Remove the cell culture plate containing ET-1-stimulated iCell Cardiomyocytes from the incubator.

3. Add 25 µl/well of the 5X BFA solution without removing the spent medium to achieve a final concentration of 10 µg/ml in a final volume of 125 µl.

   Note: *For a 384-well cell culture plate, add 10 µl/well of the 5X BFA solution for a final volume of 50 µl.*

4. Centrifuge the plate at 100 x g for 1 minute.

5. Incubate at 37°C, 7% CO_2 for 3 hours.

Labeling of iCell Cardiomyocytes: Day 1 - Fixation, Permeabilization, and Primary Antibody Incubation

The optimal time to detect ET-1-induced BNP expression in iCell Cardiomyocytes is 18 hours post-ET-1 treatment.

1. Prepare the fixative solution by diluting a stock solution of formaldehyde to 4% in D-PBS.

2. Remove the cell culture plate containing ET-1-stimulated and BFA treated iCell Cardiomyocytes from the incubator.

3. Aspirate or quickly decant the spent medium. Wash the cells with 200 µl/well of D-PBS.

   Note: *For a 384-well cell culture plate, wash the cells with 50 µl/well of D-PBS.*

4. Aspirate or quickly decant the D-PBS. Add 100 µl/well of fixative solution.

   Note: *For a 384-well cell culture plate, add 20 µl/well of fixative solution.*

5. Incubate at room temperature for 15 minutes.
6. Prepare the permeabilization buffer by diluting nonfat dry milk to 3% (w/v) and Triton X-100 to 0.1% (v/v) in D-PBS.
7. Prepare the primary antibody solution by diluting the detection antibody to 1:500 in permeabilization buffer. Approximately 5 ml are required for a 96-well cell culture plate or 8 ml for a 384-well cell culture plate.
8. Aspirate or quickly decant the fixative solution. Wash 2 times with 200 µl/well of D-PBS.

   Note: *For a 384-well cell culture plate, wash 2 times with 50 µl/well of D-PBS.*
9. Aspirate or quickly decant the D-PBS. Add 50 µl/well of primary antibody solution.

   Note: *For a 384-well cell culture plate, add 20 µl/well of primary antibody solution.*
10. Incubate at 4°C overnight.

Labeling of iCell Cardiomyocytes: Day 2 - Secondary Antibody Incubation and Nuclei Staining

1. Remove the cell culture plate containing fixed iCell Cardiomyocytes from 4°C.
2. Aspirate or quickly decant the primary antibody solution. Wash 2 times with 200 µl/well of D-PBS.

   Note: *For a 384-well cell culture plate, wash 2 times with 50 µl/well of D-PBS.*
3. Prepare the secondary antibody solution by diluting the secondary antibody to 1:1,000 in permeabilization buffer. Approximately 5 ml are required for a 96-well or 8 ml for a 384-well cell culture plate.
4. Aspirate or quickly decant the D-PBS. Add 50 µl/well of secondary antibody solution.

   Note: *For a 384-well cell culture plate, add 20 µl/well of secondary antibody solution.*
5. Incubate at room temperature for 1 - 2 hours, protected from light.
6. Prepare a 2X nuclei staining solution by diluting the Hoechst 33342 to 1:2,500 in D-PBS. Approximately 5 ml are required for a 96-well or 8 ml for a 384-well cell culture plate.
7. Add 50 µl/well of nuclei staining solution directly to the 96-well cell culture plate containing 50 µl of secondary antibody solution.

   Note: *For a 384-well cell culture plate, add 20 µl/well of nuclei staining solution.*
8. Incubate at room temperature for 20 minutes, protected from light.
9. Aspirate or quickly decant the secondary antibody and nuclei staining solution. Wash 2 times with 200 µl/well of D-PBS.

   Note: *For a 384-well cell culture plate, wash 2 times with 50 µl/well of D-PBS.*

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10. Aspirate or quickly decant the D-PBS. Add 100 µl/well of D-PBS and prepare for HCA.

    Note: *For a 384-well cell culture plate, add 25 µl/well of D-PBS.*

    Note: *If necessary, store the plate at 4°C and perform the analysis within 2 weeks.*

Data Analysis

Refer to the guide for the high content imaging system for data analysis instructions.

1. Use the area of BNP signal (red, Figure 1) to calculate BNP expression.

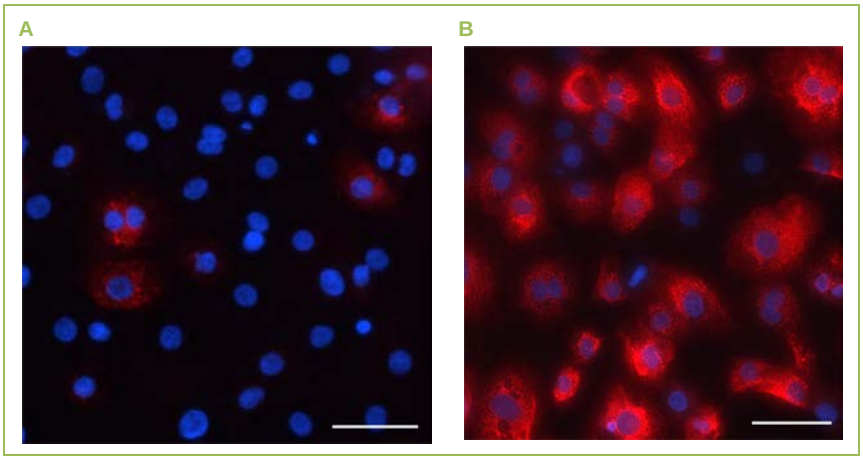

Figure 1: BNP Expression in iCell Cardiomyocytes before and after ET-1 Addition and Incubation
These images show iCell Cardiomyocytes in SWE medium (A) untreated or (B) stimulated with ET-1 (10 nM) for 18 hours. Cells were labeled with antibodies to detect BNP expression (red), and nuclei were stained with Hoechst 33342 (blue). Scale bar = 50 µm.

2. Generate a dose-response curve for the hypertrophic response of iCell Cardiomyocytes induced by ET-1 by plotting the area of BNP signal per cell (Y-axis) against the log concentration of ET-1 treatment (X-axis).
3. Analyze the data further by fitting the curve to generate an EC_{50} value from the ET-1 dose-response (Figure 2).

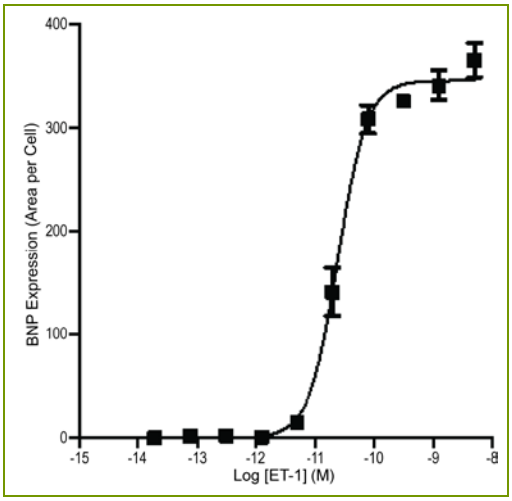

Figure 2: HCA Detection of BNP Expression in iCell Cardiomyocytes after ET-1 Addition and Incubation

In this representative 96-well experiment, a dose-dependent increase in BNP expression occurred after stimulation with the indicated concentrations of ET-1 as detected by HCA. Acquisition and analysis were performed using the ImageXpress Micro System and MetaXpress Software with Multi Wavelength Cell Scoring Module (Molecular Devices). iCell Cardiomyocytes were assayed on day 5 post-plating. The EC_{50} value for ET-1 was 24 pM (mean ± SEM; n = 4 for each point on the curve).

Summary

iCell Cardiomyocytes are derived from human iPSCs and provide an in vitro cellular system for modeling cardiac hypertrophy. The methods and data presented here highlight a reproducible cell culturing protocol coupled with a screening-compatible 96- or 384-well assay format for monitoring the expression of BNP after stimulation of a hypertrophic response with ET-1.

References

1. Swynghedauw B. (1999) Molecular Mechanisms of Myocardial Remodeling. Physiol Rev 79.
2. Chien KR, Knowlton KU, Zhu H, Chien S. (1991) Regulation of Cardiac Gene Expression during Myocardial Growth and Hypertrophy: Molecular Studies of an Adaptive Physiologic Response. FASEB J **15**:3037.
3. Zhi D, Irvin MR, et al. (2012) Whole-exome Sequencing and an iPSC-derived Cardiomyocyte Model Provides a Powerful Platform for Gene Discovery in Left Ventricular Hypertrophy. Front Genet **3**:92.

Notes

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