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Use of Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes (hiPSC-CMs) to Monitor Compound Effects on Cardiac Myocyte Signaling Pathways

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Abstract

There is a need to develop mechanism-based assays to better inform risk of cardiotoxicity. Human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) are gaining rapid acceptance as a biologically relevant *in vitro* model for use in drug discovery and cardiotoxicity screens. Utilization of hiPSC-CMs for mechanistic investigations would benefit from confirmation of the expression and activity of cellular pathways that are known to regulate cardiac myocyte viability and function. This unit describes an approach to demonstrate the presence and function of signaling pathway(s) in hiPSC-CMs and the effects of treatments on these pathways. We present a workflow that employs protocols to demonstrate protein expression, functional integrity of signaling pathway(s) of interest and that characterize biological consequences of signaling modulation. These protocols utilize a unique combination of structural, functional and biochemical endpoints to interrogate compound effects on cardiomyocytes.

Keywords

human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs); cell signaling pathways; protein expression; nanofluidic proteomic immunoassay (NIA); real-time impedance and field potential-based cell assay (RTCA) CardioECR system; multiparameter imaging

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INTRODUCTION

Human cardiomyocytes, derived from either embryonic stem cells or induced pluripotent stem cells, are increasingly being used for basic cardiac biology research and in drug discovery and toxicology investigations (Eldridge et al., 2014; Force and Kolaja, 2011; Ivashchenko et al., 2013; Khan et al., 2013; McGivern and Ebert, 2014; Pointon et al., 2013; Scott et al., 2013; Zeevi-Levin et al., 2012). Human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) are viewed as a biologically relevant model of the human myocardium because they exhibit many of the same characteristics, including cellular and subcellular structures, ion channels, calcium cycling, bioenergetics, receptors, contractility, electrophysiology, gene expression and responses to exogenous stimuli (Babiarz et al., 2012; Guo et al., 2011; Hoekstra et al., 2012; Ivashchenko et al., 2013; Khan et al., 2013; Lundy et al., 2013; Ma et al., 2011; Puppala et al., 2013; Rana et al., 2012; Sharma et al., 2013; Shinozawa et al., 2012; Sirenko et al., 2013a; Sirenko et al., 2013b). Utilization of this cardiomyocyte system to evaluate targeted agents, in particular tyrosine kinase inhibitors, requires confirmation that the cells have the functional signaling pathways with which compounds are expected to interact. Moreover, a mechanistic evaluation of cytotoxicity and other compound-mediated effects will likely require interpretation of on-target or off-target effects. Experimental strategies should therefore start with the application of flexible methods that confirm target expression and their regulation in these cells. Our laboratory is exploring the role of several cell-signaling pathways in hiPSCCMs. We have developed a preclinical drug-testing strategy (using hiPSC-CMs) that enables mechanistic studies of investigational anticancer drugs. This multifunctional approach permits the evaluation of target regulation and analysis of signaling pathway activities. We integrated multiple approaches to formulate a strategy, namely: (1) multiparameter imaging and selected biochemical endpoints as a measure of cell viability; (2) real-time monitoring of cardiomyocyte function by impedance and electric activity by field potential measurements; and (3) modified versions of capillary electrophoresis and isoelectric focusing with immunodetection to identify targets of interest and their activation state, including a measure of cellular consequences of protein loss using siRNA knockdown of key signaling proteins.

Herein, we submit the ErbB2 pathway as an example of the application of this strategy (Figure 1). The ErbB2 pathway is known to play a pivotal role in regulating cardiac myocyte function and response to targeted anti-cancer agents (Azim et al., 2009; Chen et al., 2008; Curigliano et al., 2010; Fedele et al., 2012; Force and Kolaja, 2011; Mellor et al., 2011; Senkus and Jassem, 2011; Zambelli et al., 2011). Through mediating the adaptive response of myocardia to physiological or pathological stresses, the ErbB2 signaling plays a critical role in maintaining the essential function of the human heart (Fuller et al., 2008). Trastuzumab, a humanized monoclonal antibody targeted against the ErbB2 receptor, exhibited a significant improvement in the treatment of ErbB2 overexpressing breast tumors; however, it also increased the incident of cardiotoxicity, particularly when patients were also treated with anthracyclines, such as doxorubicin (Bowles et al., 2012). We selected ErbB signaling pathway to start with as we believe a biologically relevant *in vitro* model system should be able to recapitulate this well-described and in-depth investigated phenomenon.

ErbB signaling is activated by its natural ligand, neuregulin-1 β (NRG), and regulates a large body of protein kinases and nuclear transcription factors both in cytoplasm and in nuclei via two key mediators of activation cascade, AKT and Erk1/2 (Figure 1). AKT and Erk1/2 are key mediators of the downstream cascades in the ErbB signaling pathway (Wadugu and Kuhn, 2012). Post-translational modification of proteins, such as phosphorylation, is a mechanism of modulation for many pathways (Wang et al., 2014). The levels of phosphorylated AKT or Erk1/2 can be utilized to assess functionality of ErbB signaling. Upon activation, Erk1/2 translocates to the nucleus where it phosphorylates a variety of transcription factors regulating gene expression (Mebratu and Tesfaigzi, 2009). For instance, activated AKT or Erk1/2 in the cytosol, or translocation into the nucleus, phosphorylates FOXO3a (Forkhead box O3a) and CREB (cAMP response element-binding protein) directly or indirectly through RSK (ribosomal S6 family kinases) activation to promote cell survival and cardiac hypertrophy (Brunet et al., 2001; Mebratu and Tesfaigzi, 2009; Takaishi et al., 1999). Therefore, we focused on characterization of expression, translocation and phosphorylation of AKT, Erk1/2, FOXO3a and CREB.

In this unit, we present four Basic Protocols that are further subdivided into procedures and/or endpoints measured. Basic Protocol 1 provides procedures for preparing and maintaining the hiPSC-CM cell cultures, and confirming the purity and basic functionality of the cardiomyocytes prior to further experimental utilization. Basic Protocol 2 describes several biochemical and imaging assays used to evaluate cell viability, mitochondrial membrane potential, caspase activation, ATP content, and LDH and cardiac troponin release. Real-time monitoring of cardiomyocyte contractility and electrophysiology function is described in Basic Protocol 3. Finally, Basic Protocol 4 details our approach to interrogate ErbB2 pathway activation and modulation in hiPSC-CMs.

BASIC PROTOCOL 1 – PREPARATION, MAINTENANCE AND CHARACTERIZATION OF HUMAN INDUCED PLURIPOTENT STEM CELL-DERIVED CARDIOMYOCYTE CULTURES

In order to successfully apply human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) as an *in vitro* model system in cardiac biology and in drug discovery (e.g. cardiotoxicity testing), it is essential that the cell system recapitulate the native physiological functional characteristics of mature myocardial cells. Although hiPSC-CMs are increasingly becoming available from various sources, we have been utilizing cells obtained from Cellular Dynamics International (CDI). These cells are a reliable source of highly purified mixture of spontaneously electrically active atrial, nodal, and ventricular human myocytes. They demonstrate phenotypic, electrophysiological and functional characteristics of mature cardiomyocytes (Khan et al., 2013; Sirenko et al., 2013a). Before these cells may be used experimentally, they must be properly thawed, plated, cultured and assessed for adequate qualification for application. Therefore, Basic Protocol 1 describes the basics necessary to establish the foundation for the remaining protocols. The complete “iCell Cardiomyocytes User's Guide” is conveniently provided on the CDI website (<http://www.cellulardynamics.com/>). Here, this protocol is subdivided to include cell culture

conditions under (a) plate coating and (b) cell plating, and characterization methods under (c) cell quality control, (d) cardiomyocyte purity, and (e) cardiomyocyte contractility.

Materials

Cells—Human induced pluripotent stem-cells cardiomyocytes (iCell® Cardiomyocytes, Cellular Dynamics International).

Cell culture media—Plating media; maintenance media (Cellular Dynamics International).

Buffers and reagents—Phosphate buffered saline (PBS) with or without $\text{Ca}^{2+}/\text{Mg}^{2+}$ (Lonza, catalog #17-513 or 17-512F); gelatin (Sigma catalog #G1890); fibronectin (Sigma, catalog #F1141-1 mg); paraformaldehyde (Electron Microscopy Sciences, catalog# 15714); Odyssey blocking buffer (LI-COR, catalog #927-40003); Triton-X 100 (Sigma, catalog #T8787).

Antibodies—cardiac troponin I (Abcam, catalog # ab52862); myomesin (clone B4) (University of Iowa Developmental Studies Hybridoma Bank); anti-rabbit antibody conjugated with FITC (Life Technologies, catalog # A11008); anti-mouse antibody conjugated with FITC (Life Technologies, catalog # A11029).

Consumables from various suppliers—Sterile 15 and 50 mL centrifuge tubes; 50 mL reagent transfer reservoirs; 250 mL polystyrene bottles and Bottle-Top Filtration Units; 100 μL to 1 mL low-adhesion pipette tips; sterile tissue culture plates (6, 12, 24 or 96-wells).

Instruments— CO_2 incubator (set to 37°C , 5% CO_2 , 95% humidity); biosafety cabinet hood certified for handling of Level I Biohazard Materials; water bath with adjustable temperature setting (37 to 60°C); inverted phase contrast microscope; automated cell counter (Vi-CELL™ XR Cell Viability Analyzer, Beckman Coulter) or hemocytometer with trypan blue (Sigma, catalog #T8154); 100 μL to 1 mL single- channel pipettes and a 300 μL 8-channel pipette; Nikon ECLIPSE Ti fluorescence microscope equipped with Nikon DS-Ri1 digital camera (Nikon Instrument); IN Cell analyzer 1000 (GE Healthcare).

(a) Plate coating

1. To use 0.1% gelatin, set water bath temperature to 60°C ; weigh 250 mg of gelatin to a sterile 250 mL polystyrene bottle and add 250 mL of deionized and filtered Milli-Q water; dissolve the gelatin in the 60°C water bath for 10–15 minutes, swirling every 5 minutes; cool at room temperature then sterile filter using 0.22 μM Bottle-Top filtration units; store at room temperature.

The 0.1% gelatin may be used for up to 3 months.

2. To use fibronectin, dilute the stock (1 mg/mL as provided) in PBS with Ca^{2+} and Mg^{2+} at 1:100, i.e. 60 μL fibronectin to 5.94 mL PBS, to make a 10 $\mu\text{g}/\text{mL}$ solution; mix the dilution gently.

3. To coat culture plates, dispense 1 mL/well gelatin or fibronectin solution to 6-well plates, 0.5 mL/well to 12-well plates, 0.25 mL/well to 24 well plates or 50 μ L/well to 96-well plates; incubate at a 37°C/5% CO₂ incubator for 1 to 3 hours.

(b) Cell plating

1. Thaw the provide media at 4°C overnight or in a 37°C water bath quickly without over-heat; equilibrate the plating or thawing medium to room temperature right before use.

Store thawed media at 4°C for use within 4 weeks; if not used within 2 weeks, aliquot and re-freeze for one future thaw and use.

2. Thaw cells in cryovials no more than 3 vials at one time; place each vial on a floating microcentrifuge tube rack in 37°C water bath for 4 minutes (avoid extended-heat); spray/wipe the vial with 70% ethanol and transfer the vial to the biosafety cabinet hood; gently transfer the cells to a sterile 50 mL centrifuge tube using a 1 mL pipette; add 1 mL plating/thawing medium to the empty vial to rinse, then transfer the medium to the 50 mL centrifuge tube containing the cells drop-wise (a few seconds per drop over 90 seconds) while gently swirl the tube for quick mix; add 8.5 mL plating/thawing medium to the cell suspension drop-wise over 1–2 minutes while gently swirling the tube.

Avoid vigorous shaking or vortexing of the cell suspension.

3. To determine the cell viability and density, gently invert the tube 2–3 times, take 500 μ L cell suspensions from the middle part of the tube, add it to a sample cup pre-filled with 500 μ L PBS; mix by gently pipetting and load the cup on Vi-CELL counter.
4. Use the plating efficiency (% viable cells plated eventually attach and grow) provided by the vendor, or assume 50% plating efficiency if not provided, to calculate the viable cells needed to achieve the target cell density with the equation:

$$\text{viable Cells/Well (to plate)} = \frac{\text{Target Cells/Well}}{\% \text{ Plating Efficiency}}$$

5. Adjust the volume of cell suspension in the 50 mL-centrifuge tube by adding more plating medium or, if needed, centrifuge cells at $\sim 200 \times g$ for 5 minutes then re-suspend cells at target volume to achieve the appropriate viable cell density, i.e. 150k to 200k/mL after correction of Plating Efficiency, for plating. For example, a vial of total 5×10^6 viable cells with 50% Plating Efficiency needs to be suspended in 16.7 or 12.5 mL plating medium to a density of 150k or 200k/mL to achieve that 15k

or 20k cells attach and grow in each well of 96-well plates when 100 μ L/well cell suspension is dispensed.

6. To plate cells to a 384- or 96-well plate, transfer the cell suspension to a sterile 50-mL reservoir, remove the coating solution from each well and dispense 25 or 100 μ L/well cell suspensions immediately with a multi-channel pipette.

Rock the reservoir from side to side gently during dispensing to prevent cell from sedimentation.

7. Use 1 mL-pipette to plate cells to a 12- or 24-well plate, or a 5 mL-pipette to plate cells to a 6-well plate; remove the coating solution from each well and immediately dispense 0.6, 1.2 or 3 mL/well cell suspensions to 12-, 24- or 6-well plates.

Withdraw cells from the middle layer of cell suspension in the 50 mL centrifuge tube; gently invert the tube twice prior to each withdrawal.

8. After cell plating, place the plate in the biosafety cabinet hood for 30 minutes at room temperature, then transfer to a 37°C/5% CO₂ incubator.
9. 48 hours after cell plating remove the plating medium from each well with a multi-channel pipette while placing the plate on a tilting plate holder; immediately add 200 μ L/well pre-warmed Maintenance Medium; return the plate to the incubator.

Avoid touching cells with pipette tips while withdrawing the medium; perform medium change every 24 or 48 hours per vendor's recommendation.

(c) Cell quality control

In vitro differentiation of human stem cells to cardiomyocytes is a complex process which often results in a mixture of several cellular populations (Burridge and Zambidis, 2013; Mummery et al., 2012). To ensure that this cell culture model system truly reflects cardiac biology, the purity and basic functionality of cardiomyocytes in the culture need to be characterized at the time when the culture is ready for interrogation of pharmacological and toxicological interventions. We use positive staining for cardiac troponin I (cTnI), a key component of thin filaments in cardiomyocytes to regulate force generation, as a biomarker of cardiomyocytes, and myomesin, a core component of functional sarcomere structure, to assess the sarcomere organization. We use the presence of rhythmic contractions to evaluate cardiac function.

(d) Cardiomyocyte purity

1. Plate cells at low density (5,000 or 10,000 cells/well) in 96-well plates; culture for at least 10–12 days.
2. Add 20 μ L of paraformaldehyde stock solution to each well containing 200 μ L culture media (final paraformaldehyde concentration is approximately 4%);

incubate the plate in the incubator for 10 minutes for fixation; remove media and rinse cells twice with 100 μ L/well PBS.

Take videos prior to fixation to assess cardiomyocyte contraction.

3. Add 100 μ L/well of 0.1% Triton X-100 in PBS, store at room temperature for 10 minutes for membrane permeabilization; remove Triton X-100 and rinse cells with 100 μ L/well PBS twice.
4. Add 100 μ L/well Odyssey Blocking Buffer; incubate at room temperature for 60 minutes to block non-specific binding.
5. Dilute the primary antibodies anti-cTnI at 1:100 and anti-myomesin (clone B4) at 1:50 in Odyssey Blocking Buffer; add 30 μ L/well of primary antibody solution; incubate the plate at room temperature for 2 hours; rinse cells twice with 100 μ L/well PBS.
6. Dilute the secondary antibody conjugated with FITC (anti-rabbit) or Cy5 (anti-mouse) at 1:1000 in PBS; add 50 μ L/well and incubate at room temperature for 1 hour while protected from light; rinse cells twice with 100 μ L/well PBS.
7. Add 50 μ L/well of Hoechst (2.5 μ g/mL in PBS) and incubate in dark at room temperature for 20 minutes to stain nuclear DNA; rinse cells twice with 100 μ L/well PBS, then fill each well with 100 μ L PBS.
8. Take images using a 10X or 20X objective lens with Nikon ECLIPSE or IN Cell Analyzer 1000.
9. To assess the cardiomyocyte purity, count at least 100 nuclei, calculate the % nuclei staining positive with cTnI or myomesin in cytosol.

Figure 2 shows representative microscopic images of hiPSC-CMs labeled with antibodies specific for cardiac troponin I (cTnI), myomesin and F-actin.

(e) Cardiomyocyte contractility

1. Play the videos taken prior to cell fixation (see protocol above); count at least 100 cells randomly and calculate the % of beating cells.
2. If the microscope is not equipped with a video recorder, counting beating cells may be performed directly under the microscope. However, a heated plate holder is required to maintain the temperature of culture plate as the temperature drop results in a reduction in beat rate and contraction force, or arrests the beating completely.
3. Alternatively, assess the contractility using any instrument capable of analyzing cellular impedance, field potentials, intracellular Ca^{2+} cycling or cytoplasm membrane potential with Ca^{2+} or voltage sensing dyes, etc. at high resolution (with a sampling rate in the mini-second range).

BASIC PROTOCOL 2 – MULTIPARAMETER BIOCHEMICAL AND IMAGING ASSAYS TO EVALUATE CELL VIABILITY, MITOCHONDRIAL MEMBRANE POTENTIAL, CASPASE ACTIVATION, ATP, LDH AND CARDIAC TROPONIN

High-content image analysis (HCA) coupled with biochemical cell viability assays such as the measurement of cellular ATP depletion have been used in human iPSC-derived cardiomyocytes to reveal drug-induced structural cardiotoxicity (Pointon et al., 2013; Schweikart et al., 2013). Here we focus on analysis of nuclear morphology and mitochondrial membrane potential together with caspase 3/7 activation, attempting to capture increased apoptotic events, nuclear counts, ATP measurements, and release of lactate dehydrogenase (LDH) and cardiac troponin T (cTnT) to reveal cell death and compromised integrity of cytoplasmic and mitochondrial membranes. The assessment of ATP, caspase 3/7, LDH and cTnT is integrated routinely with xCELLigence® RTCA or CardioECR recording as endpoint readouts. The measurement of cTnT is performed along with LDH since quantification of cTnT in blood serum has been conducted routinely in the clinic for diagnosis of myocardial injury (Reichlin et al., 2009).

Although there are numerous methods for assessing cell viability using a wide variety of cellular and biochemical markers, the assays we describe here allow for multiple parameters to be measured within a single experiment. This approach saves time and expense by maximizing the amount of information gained from the relatively costly hiPSC-CMs. For example, culture media may be collected from plates used in experiments described in Basic Protocols 3 and 4 to measure LDH and cardiac troponin release. For multiparameter imaging assays, cells are cultured and treated in 96-well optical-bottom plates at 18,000 cells/well. These plates are used for assessing mitochondrial membrane potential and cell viability using live cell stains described below. Alternatively, cells may be fixed and stained for biomarkers of interest using standard immunocytochemical methods. If live cell stains are used, the culture media may be collected for determining LDH and/or cardiac troponin release, and cells may be lysed for biochemical assays to measure ATP content and caspase activation. Basic Protocol 2 describes the use of high-content analysis and multiparameter biochemical and immunocytochemical assays to assess cardiomyocyte injury. The protocol is subdivided by individual endpoint measurements: (a) assessment of nuclear morphology and cell count with nuclear staining, (b) assessment of mitochondrial membrane potential using image analysis, (c) measurement of cellular ATP content using a plate reader format, (d) measurement of LDH release using a plate reader format, (e) measurement of caspase 3/7 activation using a plate reader format, and (f) measurement of cTnT release using the MSD platform.

Materials

DMEM, no glucose (Life Technologies, catalog# 11966-025)

Sodium pyruvate (Life Technologies, catalog# 11360-070)

D-(+)-Galactose (Sigma-Aldrich, catalog# G5388-100G)

Paraformaldehyde (Electron Microscopy Sciences, catalog# 15714)

96-well Acoustic uClear Flat-Bottom Black-Wall Plates (Greiner, catalog #655090)

Adhesive plate seals (VWR, catalog # 60941-126)

Hoechst 33258 (Enzo Life Sciences, catalog #ENZ-52402)

Cell Meter Meter™ JC-10 Assay kit (AAT Bioquest, catalog #22800)

CytoTox 96® Non-Radioactive Cytotoxicity Assay Lactate Dehydrogenase (LDH) kit (Promega, catalog #G1780)

Dimethylsulfoxide (DMSO, Sigma, catalog #2650)

Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (Tocris, catalog #0452)

CellTiter-Glo® Luminescent Cell Viability Assay (ATP) kit (Promega, catalog #G7570)

Caspase-Glo® 3/7 Assay kit (Promega, catalog #G8090)

MSD® 96-Well MULTI-ARRAY® Human Cardiac Troponin T Assay Kit (Meso Scale Discovery, catalog #K151EFC)

Instruments

IN Cell Analyzer 2000 (GE Healthcare)

TECAN Infinite® M1000 Plate Reader (TECAN)

MSD® SECTOR Imager 6000 (Meso Scale Discovery)

- (a) Assessment of nuclear morphology and cell count with nuclear staining
1. To stain nuclear DNA in live cells with Hoechst 33258, dissolve 5 mg Hoechst in 1 mL Milli-Q water to make a 5 mg/mL stock; dilute this stock in the culture medium at 1:100 to 50 µg/mL, then add 10 µL of the dilution to each well containing 200 µL medium to a final concentration of 2.5 µg/mL; incubate the plate in a 37°C/5% CO₂ incubator for 20 minutes.
 2. To stain nuclear DNA in cells fixed with 4% paraformaldehyde, refer to the procedures for assessment of the cardiomyocyte purity in Basic Protocol 1.
 3. Use the filter set of 325/455 nm (excitation/emission) to image Hoechst-stained nuclei; refer to procedures described below for nucleus analysis, set the appropriate threshold for nucleus area to identify shrinking nuclei, or use granularity algorithm to locate DNA condensation areas.

A nuclear DNA stain using Hoechst, DAPI or other dyes is included in all multiparameter imaging analysis assays to

capture cell counts and to set as a reference for cellular measurements. Figure 3 demonstrates doxorubicin-induced nuclear shrinkage modulated by ErbB2 signaling.

- (b)** Assessment of mitochondrial membrane potential using image analysis
1. To assess the mitochondrial membrane potential using Cell Meter™ JC-10 assay kit, thaw all 3 kit components at room temperature before use.
 2. Add 50 µL of provided 100X JC-10 reagent into 5 mL of Assay Buffer A to make JC-10 dye-loading solution and mix well.
 3. Add 50 µL dye-loading solution into each well containing 100 µL medium and incubate the plate in a 37°C/5% CO₂ incubator for 30 minutes.
 4. Add 10 µL of 50 µg/mL Hoechst solution to each well 20 minutes after incubation with dye-loading solution to stain nuclear DNA.
 5. Add 50 µL Assay Buffer B then image the wells immediately.
 6. To take images using IN Cell Analyzer 2000, turn on the power switch and wait for approximately 5 minutes until the “Green light” on the front of instrument is “on”, then click IN Cell Analyzer 2000 icon to start the system software.
 7. Click “Arrow” icon on the main toolbar to load the plate.
 8. Open “Assay Development” in the “Mode” pull-down list from the main menu bar; in “Protocol Designer” window, enter a name and a short description for the protocol to be created; select the type of plate used from the pull-down list; click anywhere in “Plate/slide View” window to complete the plate loading, then click “Grid” icon to select the wells and define the number and the placement of fields in each well (typically 6 fields in the middle of each well) to be scanned.
 9. Click the “Bulb” icon to turn the lamp, wait for approximately 3 minutes until the “Bulb” icon turns yellow; in “Protocol Designer” window, click “Objective lens” to select 20X or other magnification as needed.
 10. In “Protocol Designer” window, click on the microscope “Channel Setting” to select 3 wavelengths with excitation/emission filters set to DAPI, FITC and Cy3, add a “Brightfield” imaging if needed; set the imaging modality to “2-D”; set an appropriate exposure time for each wavelength; click on “Digitize” button to view the images of the selected wavelength in “Image Preview” window; select “Focus” to set focus offset manually or enable “Laser Autofocus” to obtain a good focus on the selected wavelength;
 11. Select “Focus” in “Protocol Designer” window to adjust the exposure time on each selected wavelength.

Exposure times are generally less than 100 ms for bright field images and up to 1 second for fluorescent images.

12. To adjust the image appearance for visualization, click “Palette” icon in the “Image Preview” window to visualize the dynamic range plot, adjust the exposure time to give a dynamic range of approximately 1500 to 2000 grey levels for fixed cells, or 2–3 fold of signal/background ratio for the live stain cells.

Image data are not affected by the adjustment of image appearance.

13. Click “Acquisition Options” to set the path and destination folders to store the scanned images.
14. Save the protocol from the main menu bar or the “Save” icon in the main toolbar.
15. Click “Run” the protocol from the “Protocol Designer > Acquisition Options” to start the image acquisition.
16. For image analysis, click “IN Cell Analyzer Workstation” icon to launch the software; select “Assay Development Mode”, then load the image stack files.
17. Create the analysis protocol using “Multi Target Analysis”: assign “DAPI” channel to “Nucleus”, “FITC” channel to “Cell” and “Cy3” channel to “Reference” to “Cell” defined in “FITC” channel.
18. Use “Top-hat” method for segmentation of nuclei; set the minimum area to 100 μm^2 , detection sensitivity to 50%.
19. Use “Multiscale top-hat” method for segmentation of cells; set the characteristic area to 1000 μm^2 , detection sensitivity to 50%.
20. Click “Sampling” button to run the segmentation on the selected well(s) to test settings; adjust the area and/or detection sensitivity, or use “Global Threshold” with the manually determined background intensity value to optimize segmentation of nuclei and cells.
21. Select output parameters, such as “area” and “intensity”, from the selection list.
22. Select “Excel” as output format, click “Save” button to save the protocol.
23. Click “Analyze” button to analyze the whole plate or the selected wells.

We recommend including a positive control compound (CCCP) for the JC-10 assay. Dissolve CCCP in DMSO to make a 10 mM stock, dilute it to 10 or 100 μM in the culture medium and add 10 μL to untreated cells in wells containing 90 μL media to a final concentration of 1 or 10 μM CCCP, respectively, 10 minutes prior to addition of dye-loading buffer.

Figure 4 shows an example of changes in mitochondrial membrane potential following drug treatments. The cationic, lipophilic JC-10 dye selectively enters mitochondria where it changes its color from green to orange/red as the membrane potential increases due to reversible formation of J-aggregates upon mitochondrial membrane polarization. JC-10 changes reversibly from green (monomeric form) to orange/red (aggregate form) as the mitochondrial membrane becomes more polarized. The compound carbonyl cyanide 3-chlorophenylhydrazone (CCCP) is a proton ionophore that destroys the membrane potential across the mitochondrial membrane. As demonstrated in Figure 4, with increasing CCCP drug concentration the ability of JC-10 dye to form aggregates and emit orange/red fluorescence is diminished.

(c) Measurement of cellular ATP content using a plate reader format

1. To measure cellular ATP content using the CellTiter-Glo® Luminescent Cell Viability Assay kit, thaw the provided Assay Buffer and equilibrate the lyophilized Substrate to room temperature overnight with light-protection.
2. Add 10 mL Buffer to one bottle of Substrate; mix by gently inverting to obtain a homogeneous solution.
3. Add 100 µL the Substrate solution to each well containing 100 µL medium and gently pipette 3 times to induce cell lysis.
4. Transfer 150 µL cell lysate from each well to an opaque-walled 96-well plate; store with light-protection at room temperature for 10 minutes.
5. Read the luminescent signals on a plate reader; for data analysis, use the readings relative to the vehicle control wells to assess the level of ATP content in the treatment groups.

Figure 5A shows an example of depletion of ATP content following treatment with anti-cancer agents, trastuzumab and doxorubicin. The CellTiter-Glo® Luminescent Cell Viability Assay is based on quantitation of ATP present in cells as a measure of metabolically active viable cells. This assay is simple to perform as it is a homogenous method that results in cell lysis and generation of a luminescent signal proportional to the amount of ATP present, which is directly proportional to the number of cells present in culture. The unique homogenous format is advantageous over other ATP assays that involve multiple steps that may introduce pipetting errors. Furthermore, the luminescent signal is stable for more than five hours.

(d) Measurement of LDH release using a plate reader format

1. To measure LDH release using the CytoTox 96® Non-Radioactive Cytotoxicity Assay, thaw the provided Assay Buffer to room temperature overnight with light-protection or in 37°C water bath immediately before use.
2. Add 12 mL Assay Buffer to a bottle of Substrate Mix and invert/shake gently to dissolve the substrate.
3. Collect 50 µL medium from each well and transfer to a fresh, flat-bottom 96-well plate; add 50 µL of the reconstituted Substrate Mix to each well and incubate with light-protection for 30 minutes at room temperature.
4. Add 50 µL Stop Solution to each well, read the absorbance at 490 nm on a plate reader within one hour after addition of the Stop Solution.
5. For data analysis, use the readings relative to the vehicle control wells to assess the level of comprised membrane integrity within treatment groups.

Figure 5B shows an example of LDH release following treatment with anti-cancer agents, trastuzumab and doxorubicin. The CytoTox 96® Non-Radioactive Cytotoxicity Assay has the distinct advantage over the standard ⁵¹Cr release assay in that it is nonradioactive. Stable cytosolic lactate dehydrogenase (LDH) is released upon cell lysis. Released LDH in the cell culture media is determined by a coupled enzymatic reaction resulting in conversion of the tetrazolium salt INT to a red formazan product, which is proportional to the number of lysed cells. Generation of formazan is monitored by measuring absorbance at 490 nm using a plate reader. To determine the maximum LDH activity, cells in control wells with vehicle alone are completely lysed with the provided lysis solution. The only variables that need to be addressed in performing this assay are the number of cells plated and the background absorbance correction using a media only sample.

(e) Measurement of caspase 3/7 activation using a plate reader format

1. To measure the caspase 3/7 activation using the Caspase-Glo® 3/7 Assay kit, thaw the provided Caspase 3/7 Buffer and equilibrate the lyophilized Caspase 3/7 Substrate to room temperature overnight with light-protection.
2. Transfer 10 mL Buffer to one bottle of Substrate, mix by gently inverting to obtain a homogeneous solution.
3. Add 100 µL the Substrate solution to each well containing 100 µL medium and gently pipette 3 times to induce cell lysis.
4. Transfer 150 µL cell lysate from each well to an opaque-walled 96-well plate; store with light-protection at room temperature for 30 minutes.

5. Read the luminescent signals once every 30 minutes up to 3 hours using a plate reader to determine the maximal signal; for data analysis, use the readings relative to the vehicle control wells to assess the level of caspase 3/7 activity in the treatment groups.

Figure 5C shows activation of caspase 3/7 following drug treatment. The Caspase-Glo® 3/7 Assay is more sensitive than fluorescence-based caspase assays with maximum sensitivity typically reached within one hour. This luminescent assay avoids potential interference from fluorescent signals of test compounds, resulting in high signal to noise ratios. Adding a single Caspase-Glo® 3/7 reagent, results in cell lysis followed by caspase cleavage of the substrate reagent, which is selective for caspase 3 and 7, and generation of a luminescent signal produced by luciferase. Luminescence is proportional to the amount of caspase activity present.

(f) Measurement of cTnT release using MSD platform

1. To measure cardiac troponin release; plate and culture cardiomyocytes in 96-well E-Plates or optical-bottom plates as described in Basic Protocol 1 for culturing hiPSC-CMs.
2. On day 14 post-plating, replace the Maintenance Medium with the serum-free DMEM, and equilibrate for 3 hours; pretreat cells with trastuzumab or NRG for 4 hours, then expose cells to doxorubicin for 24 hours.
3. At the end of drug treatment, transfer 180 μ L/well of the serum-free medium to a blank 96-well plate; centrifuge the plate at $500 \times g$ for 10 minutes at 4°C; transfer 150 μ L/well supernatant to another blank 96-well plate, seal it with the adhesive plate seal and store at –80°C.
4. For quantification of the cardiac troponin T (cTnT) using MSD® 96-Well MULTI-ARRAY® Kit Calibrator, prepare the 2X standard concentrations of cTnT (0, 0.004, 0.012, 0.048, 0.196, 0.782, 3.02 and 12.50 pg/mL) by diluting the Calibrator (25 ng/mL human cTnT) in serum-free DMEM; store the diluted Calibrator on ice before use.
5. Add 60 μ L of 50X SULFO-TAG™ anti-human cTnT Antibody to 2.94 mL of Diluent 22 to make the 1X Detection Antibody Solution
6. Dilute 5 mL of 4X Read Buffer T in 15 mL of Milli-Q water to make 1X Read Buffer.
7. Add 250 μ L Tween 20 to 499.75 mL PBS to make the 0.05% Tween 20 PBS Wash Buffer (PBS-T).
8. In a MULTI-SPOT® 96-well 4 Spot Human cTnT plate, add 25 μ L/well of Detection Antibody Solution, then add 25 μ L/well of diluted Calibrators and the collected culture media in triplicate.

9. Seal the plate with an adhesive plate seal; incubate for 1 hour with vigorous shaking at room temperature.
10. Wash plate 3 times with 150 μ l of PBS-T, decant plate, and blot onto paper towel.
11. Add 150 μ l of 1x Read buffer T per well (avoid bubbles).
12. Analyze the plate immediately with MSD® Imager 6000.
13. Double-click the "MSD® Discovery Workbench" icon to launch the application software; click "SECTOR Imager" icon on the toolbar to run the SECTOR Imager 6000 Reader.
14. Load the MSD® human cTnT plate into the single plate adaptor.
15. In "Setup" window, select "Read From Barcode" from the "Plate Type" drop box; check the "Return Plates to Input Stack" box; Check the "Read" box and enter "1" plate, then click on the green "Run" button.
16. In the "Run Options" window, enter a name for the file to be created and define the output path; click "OK" button to start reading the plate.
17. For the plate data analysis, select "File > New Plate Layout in Library" to create a new plate layout.
18. In "Plate Layout Editor" window, click "Assign Assay (A)" button, then right click the spot "A1" and select "Troponin T" from the dropdown lists of "Clinical Markers" and "Human"; click "Assign Standards to Wells (S)" button and select "Standards" in the group name box "3" in the replicates box; check on "Apply to all assay" box, then enter the well ID, standards name, concentration values and a short description in each boxes; click "OK" button to exit; click "Assign Controls to Wells (C)", "Assign Unknowns to Well (U)" and "Assign Blanks to Well (B)" buttons to define attributes of the controls, samples and blanks in each well; click on "Unit" button to define the unit of concentrations, i.e. pg/mL; save the created plate layout to Library.
19. Click "Plate Data History" button, select the plate from the list then select "Analyze Plates" from the file menu; a new experiment file named by the date and time stamp is created automatically once the data analysis is done.

Figure 6 shows the effect of drug on cardiac troponin T release as a measure of cardiotoxicity using the MSD® platform. The unique spot patterns of the MSD® MULTI-ARRAY® technology allows for detection of biomarkers in single or multiplex formats using immunoassay detection methods. This platform requires small sample volumes, exhibits high sensitivity and a large dynamic range as shown in Figure 6A. Levels of cardiac troponin T released by hiPSC-CMs after

treatment with doxorubicin were increased, and attenuated by ErbB2 signaling activation with neuregulin (Figure 6B). Trastuzumab treatment did not potentiate doxorubicin-induced cardiac troponin T release as demonstrated in Figure 6B.

BASIC PROTOCOL 3 – REAL-TIME MONITORING OF CARDIOMYOCYTE FUNCTION BY IMPEDANCE-BASED CONTRACTILITY AND ELECTRICAL ACTIVITY BY FIELD POTENTIAL MEASUREMENT

Monitoring of cardiomyocyte function in real-time is achievable using an impedance-based instrument that enables continuous, label-free measurements of cardiomyocyte beat rate and contractility (xCELLigence® RTCA Analyzer). This is accomplished by culturing the cells in specially designed 96-well plates containing electrodes that affect the local ionic environment at the electrode/solution interface, leading to an increase in impedance. The more cells attached to the electrodes, the larger the increase in electrical impedance. Impedance also changes with respect to the quality of the cell interaction with electrodes and is measured as a cell index (CI) value. CI values reflect cell viability, cell number, cell morphology, and cell adhesion. This dimensionless unit is the relative change in measured impedance and provides a continuous monitoring of physiologically relevant cellular responses for cardiotoxicity testing. The high-resolution impedance-based xCELLigence® RTCA Cardio system provides a powerful tool to monitor both the viability and contractility of beating cardiomyocyte continuously in a label-free, non-invasive manner. A successful application of this real-time cellular analysis (RTCA) system to interrogate both structural and functional toxicity of known cardiotoxicants using hiPSC-CMs has been well demonstrated (Doherty et al., 2013; Eldridge et al., 2014; Guo et al., 2011; Schweikart et al., 2013).

Recently, a new system “xCELLigence® RTCA CardioECR” that combines both the high-resolution impedance and multielectrode array (MEA) measurement has been developed to enable simultaneous analysis of all three key attributes, i.e. viability, contractility and electrophysiological function of beating cardiomyocytes. Monitoring the field potential directly with the added MEA feature is important since the field potential represents the integrated cardiac ion channel activity and has been identified recently as a key biomarker in “the Comprehensive In Vitro Proarrhythmia Assay (CiPA) initiative” to assess the proarrhythmic liability of compounds earlier in the drug discovery process (Sager et al., 2014).

The newly developed xCELLigence® RTCA CardioECR system possesses multiple advantages over other analytic platforms that employ Ca^{2+} or voltage sensing fluorescence dyes to monitor intracellular Ca^{2+} cycling or cytoplasm membrane potential, or have only a single readout of either impedance or MEA signals. (1) It provides comprehensive readouts simultaneously for viability, contractility and field potential electrophysiology. (2) It allows for long-term continuous monitoring (days to weeks) of compound effects since the plate docking station is placed inside a standard CO_2 incubator. (3) Its label-free and non-invasive features allow incorporation with other end-point biochemical assays, such as the measurement of ATP, caspase 3/7 activity, LDH, etc. Since there are only two electrodes in

each well to record the field potential signal, this system is not able to analyze the conduction pattern or calculate the conduction velocity of excitation waveforms like a standard MEA system does.

Simultaneous monitoring of cardiomyocyte contraction and electrophysiology function helps elucidate mechanisms underlying drug-induced arrhythmia or alterations in contractility. For example, using tool molecules E-4031, an hERG channel blocker, and blebbistatin that binds myosin II preventing contraction, have demonstrated a clear distinction between impedance and electrical field potential using the RTCA and MEA platforms (Guo et al., 2011). Arrhythmia induced by E-4031 is observed with both impedance (RTCA) and electrical field measurements (MEA), whereas blebbistatin reduces cardiomyocyte contraction dramatically without a measurable effect on the electrical activity detected by MEA (Guo et al., 2011).

Basic Protocol 3 describes the use of impedance and field potential to analyze cardiomyocyte function in real-time. The protocol is subdivided into two sections based on platform used to measure endpoints: (a) RTCA system to measure impedance-based contractility and beat rate, and (b) RTCA/MEA system to simultaneously measure cellular impedance and electrical field potential.

Materials and Instruments

Bovine serum albumin (BSA, Sigma, catalog #A9418)

Phosphate-Buffered Saline without $\text{Ca}^{2+}/\text{Mg}^{2+}$ (PBS, Lonza, catalog #17-517Q)

Dimethylsulfoxide (DMSO, Sigma, catalog #2650 -5X5ML)

E-Plate cardio 96 (ACEA Biosciences, catalog #6417035001)

E-Plate CardioECR 48 (ACEA Biosciences, catalog #300600950)

xCELLigence® RTCA Cardio instrument (ACEA Biosciences, catalog #380601060)

xCELLigence® RTCA CardioECR system (ACEA Biosciences, catalog #380601210)

Test Articles

E-3041 dihydrochloride (Tocris, catalog #1808)

Blebbistatin (Sigma, catalog #B0560)

Doxorubicin-HCl (Enzo Life Sciences, catalog #BML-GR319)

Lapatinib (National Cancer Institute compound repository, NSC 727989)

Neuregulin-1 β (NRG) [Recombinant human neuregulin-1 β (NRG1- β 1/HRG1- β 1 EGF domain), R&D Systems, catalog #396-HB-050]

Trastuzumab (Genentech, Inc.)

(a) Monitor cardiomyocyte contraction and cell health in real-time

1. Plate cardiomyocytes in the E-Plate cardio 96-well plate at 18,000 cells/well as described in Basic Protocol 1; culture cells at 37°C, 5% CO₂ for at least 10–12 days before testing.

Measure the background cellular impedance immediately prior to plating the cells.
2. Turn on xCELLigence® RTCA Cardio system; double-click on “RTCA Software” icon to launch the application; remove coating solution and immediately add 100 µL/well of 37°C plating medium; load the E-Plate onto docking station; wait for 5 minutes; click on “Exp Notes” button to type in the experiment identification; in “Layout” page, select all 96-wells and fill in “Cell Type” and “Cell Number”/well; in “Schedule” page, add several undefined “Steps” as a place holder for later editing, then click “Run” icon to take the background measurement (default Step 1).
3. Dislodge the E-Plate after the background reading; remove the medium and plate cells immediately; leave the E-Plate in the biosafety cabinet for 30 minutes before re-loading it in the docking station.
4. To monitor the cell attachment and growth, set up Step 2 in “Schedule” page to 999 Sweeps and record 20 seconds/sweep at 1 hour sweep intervals. Click “Start” button to run Step 2.

Cell impedance rises rapidly during 48 hours post-plating.
Synchronized beating occurs as early as 24 hours post-plating.
However, a minimum of 10 days is needed to achieve stable beating across all wells in the plate.
5. When cells are ready to test, abort Step 2; set the sweep duration to 30 seconds/sweep and Step 3 to 60 sweeps at 1 minute sweep intervals; set Step 4 to 24 sweeps at 5 minute sweep intervals; set Step 5 to 12 sweeps at 15 minute sweep intervals, and Step 6 to 34 sweeps at 1 hour sweep intervals; check “Auto” on Step 4, 5 and 6 so all steps will be executed automatically sequentially.
6. Dissolve doxorubicin in DMSO at 1 mM and NRG in PBS containing 0.1% BSA at 100 µg/mL to make stock solutions.
7. For pre-treatment, dilute NRG in Maintenance Medium to 200 ng/mL; dissolve trastuzumab directly in Maintenance Medium at 2 µM; replace 100 µL media from each well with 100 µL NRG or trastuzumab to a final concentration of 100 ng/mL or 1 µM, respectively; Run Step 3 and record for 24 hours.
8. For treatment with doxorubicin, dilute 1 mM doxorubicin stock in Maintenance Medium to 10 µM; replace 20 µL medium from each well with 10 µM doxorubicin medium to achieve 1 µM; continue on Step 3 to record for 40 hours; dislodge the E-Plate and proceed to quantification of

cellular ATP content, LDH release, caspase-3/7 activity, and cardiac troponin release (see Protocol 3).

9. To analyze cellular impedance, click on “Plot” button, select wells from the plate map and the time period from the axis scale; select Y axis in plot selection to “Normalized Cell Index”; normalize time to the last sweep prior to addition of drug; copy data in list format from the Cell Index Window and paste to an Excel spreadsheet or other software for further analysis.
10. To continue analysis of the beat parameters such as rate, amplitude and rhythm irregularity, set Threshold to 12 in the Axis Scale Box in Plot Window; click on Data Analysis button; select Beating Rate or Amplitude in Parameter Box, Positive or Negative Peak Counts in Method Box, Beat Rate or Amplitude in a Time Period in Curve Type; select the Measurement Time Period in Multiple Time Selection Box, then click on “Draw a New Curve” button; once completed, click “Copy Data on Chart” to copy and paste the measurement to an Excel spreadsheet or other software for further analysis.

(b) Monitor cardiomyocyte field potential and cellular impedance in real-time

1. Plate cardiomyocytes in the E-Plate cardioECR 48-well plate at 20,000 cells/well as described in Basic Protocol 1; culture cells at 37°C, 5% CO₂ for at least 10–12 days before testing.

Measure the background cellular impedance immediately prior to plating the cells.

2. Turn on xCELLigence® RTCA CardioECR system; double-click on “RTCA CardioECR Data Acquisition” icon to launch the application; remove coating solution and immediately add 50 µL/well of 37°C plating medium; load the E-Plate onto docking station; wait for 5 minutes; click on “Exp Notes” button to type in the experiment identification; in “Layout” page, select all 48-wells and fill in “Cell Type” and “Cell Number”/well; in “Schedule” page, select “CI+ECR” and all electrodes in “Test Mode” box to record both cell impedance (cardio) and field potential (ECR) signals from all electrodes; add several undefined “Steps ” for later editing, then click “Run” icon to take the background measurement of cell impedance (default Step 1).
3. Make one copy (or more when needed) of the data file after the background reading, save as the 2nd data file for drug testing (since a single data file has a limit of 10 GB in capacity).
4. Dislodge the E-Plate; remove media and plate cells immediately; leave the E-Plate in the biosafety cabinet for 30 minutes before re-loading it in the docking station.

5. To monitor the cell attachment and growth, set up Step 2 in “Schedule” page to 60 Sweeps and record 20 seconds/sweep at 6 hour sweep intervals; set the sampling rate for impedance (cardio speed) to 12 ms and for field potentials (ECR speed) to 0.1 ms (10 KHz); click “Start” button to run Step 2.

Synchronized beating in cell index (CI) signal and measurable field potential (FP) occur as early as 24 hours post-plating.

However, a minimum of 10 days is needed to achieve stable beating CI and FP signals across all wells in the plate.

6. When cells are ready to test, stop and close the data file; use the 2nd data file to continue on recording of both CI and FP signals; set the sampling rate of CI or ECR to 12 ms or 0.1 ms (10 KHz), the sweep (block) duration to 120 seconds; set Step 2 to 7 sweeps at a sweep (block) interval (SI) of 5 minutes (total 30 minutes) for the baseline measurement; set Step 3 to 13 sweeps at a SI of 5 minutes for the first 60 minutes post-dose measurement; set Step 4 to 5 sweeps at a SI of 1 hour; set Step 5 to 20 sweeps at a SI of 3 hours; check “Auto” on Step 3, 4 and 5 to allow each step to be executed automatically one after another.
7. Perform drug treatment using the protocols described in the previous section.
8. For off-line data analysis, double-click on “RTCA CardioECR” icon to launch the application; open the raw data file, click on “Plot” in “Exp Studio”, select the CI as “Display Signal”; refer to the protocol step 9 of the previous section or select function buttons from “IMP Analysis” for the global CI analysis; select function buttons from “Cardio Analysis”, “ECR Analysis” or “Cardio + ECR Analysis” modules to perform measurement of beat parameters in either CI or FP signals, or both, such as beat rate, amplitude, peak-to-peak interval and beating rhythm irregularity, etc.
9. The field potential duration (FPD) is measured in the averaged FP waveform: click on “Block Workshop”, select ECR as “Display Signal”, select the sweep, well and electrode to be analyzed; click “+” button to add the displayed data trace to the “Block Workshop” window; select another data sweep from a different time point of the same electrode and add to the “Block Workshop” window; click “Single Wave” button to average all detected beating waves at two different time points; click “Traces” button to remove all individual waveforms and leave only the averaged one; click “Peak” button to apply automatic detection of “sodium” spike and “T” waveform, adjust the detected point manually by moving the representing symbols of each turning point, such as the peak or end of “T” waveform; click “Data Table” button to show the measured value, copy/paste the averaged waveform and data table to an Excel book for statistical analysis.

Figure 7 demonstrates a data output for real-time monitoring of cardiomyocytes function by impedance-based contractility and electric field potential. Figure 8 represents the results of a typical run of E-Plate CardioECR 48, showing doxorubicin-induced changes in both CI and FP measurements that are modulated by ErbB2 signaling.

BASIC PROTOCOL 4 – ANALYSIS OF ErbB, AKT and Erk1/2 PROTEIN EXPRESSION AND PATHWAY MODULATION IN HUMAN INDUCED PLURIPOTENT STEM CELL-DERIVED CARDIOMYOCYTES

The rationale for selecting the ErbB signaling pathway as an example for the protocols offered here is presented in the Introduction to this unit. This final Basic Protocol encompasses several subsections spanning methods for cell lysate collection through measurements of protein expression and modulation with compounds and siRNA approaches while applying novel platforms for analysis.

Cell lysate preparation and antibody quality are both key to successful protein expression analysis. Lysate preparation is particularly meaningful because techniques must be employed that allow evaluation of native (non-denatured) or denatured protein sources. Additionally, methods are presented for preparing non-denatured protein from cell lysates to distinguish between nuclear and cytoplasmic proteins.

For analysis of protein expression, we describe methods to demonstrate protein expression in cell lysates using a size-based or charge-based capillary electrophoresis immunoassay systems. These unique systems are gel-free, blot-free, capillary-based, automated western blotting platforms. Capillary electrophoresis (CE) represents a novel technique of protein separation with high resolution and reproducibility. Proteins either in a denatured state or a non-denatured, native conformation are used in these CE-based assays, and once loaded into capillaries, proteins are separated based on either size (for denatured proteins as equivalent to the conventional western blot with an SDS-PAGE gel) or charge [for non-denatured proteins via isoelectric focusing (IEF)]. The separated proteins are immobilized directly on the capillary walls by UV-exposure, then immunoprobed with target-specific primary antibodies followed by HRP-conjugated secondary antibody for detection of amplified chemiluminescent signals. Wes™ Simple Western recently developed by ProteinSimple has been shown to provide absolute quantitation of endogenous proteins with precision and accuracy (Chen et al., 2013a).

To measure activation of signaling molecules, a novel charge-based capillary isoelectric focusing automated immunoassay system (NanoPro™ Simple Western) is described. Proteins and their respective phosphorylated isoforms are separated by charge in this nanofluidic proteomic immunoassay (NIA) that enables multiple protein phosphorylation isoforms to be resolved and detected simultaneously with the non-phosphorylated protein using the same primary antibody. NanoPro™ Simple Western (ProteinSimple) is an automated capillary isoelectric focusing immunoassay in which proteins and their respective isoforms (e.g., phosphorylated forms) are separated by charge, followed by target-specific

antibody probing (Aspinall-O'Dea et al., 2015; Bradley et al., 2014; Chen et al., 2013b; Eldridge et al., 2014; Fan et al., 2009; O'Neill et al., 2006). Thus, NanoPro™ can simultaneously separate, detect and quantify multiple protein phosphorylation isoforms allowing a more sensitive and accurate analysis of cell signaling events.

Basic Protocol 4 is divided into subsections: (a) lysate collection for both denatured proteins and native, non-denatured protein, and separation of nuclear and cytoplasmic proteins; (b) measurement of protein expression using novel platforms to examine modulation with compounds and siRNA approaches.

Materials

Dulbecco's Phosphate-Buffered Saline without Ca^{2+} and Mg^{2+} (DPBS, Lonza, Catalog# 17-512F)

DMEM, no glucose (Life Technologies, catalog# 11966-025)

Sodium pyruvate (Life Technologies, catalog# 11360-070)

D-(+)-Galactose (Sigma-Aldrich, catalog# G5388-100G)

Paraformaldehyde (Electron Microscopy Science, catalog #15714)

Sytox red (Life Technologies, catalog #34859)

Goat serum (Rockland, catalog #D204-00-0050)

96-well Acoustic uClear Flat-Bottom Black-Wall Plates (Greiner, catalog #655090)

10X RIPA Lysis Buffer (0.5M Tris-HCl, pH 7.4, 1.5M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10 mM EDTA) (Millipore, catalog #20-188)

Sodium Dodecyl Sulfate (SDS, Sigma, catalog #L6026)

Protease Inhibitor Cocktail (Sigma, catalog #P8340)

Phosphatase Inhibitor Cocktail (Sigma, catalog #P0044)

Halt Protease Inhibitor Cocktail, EDTA-free (Thermo Scientific, catalog #87785)

M-PER® Mammalian Protein Extraction Reagent (Thermo Scientific, catalog #78501)

NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, catalog #28833)

XDR Charge Separation Master Kit (ProteinSimple, catalog #CBS2001)

pI Standard Ladder 3 (ProteinSimple, catalog #040-646)

Premix G2, Pharmalyte pH 5-8 Separation Gradient (ProteinSimple, catalog #040-973)

DMSO Inhibitor Mix (ProteinSimple, catalog #040-510)

Wes™ 12-230 kDa Master Kit (ProteinSimple, catalog #PS-MK01 or PS-MK02)

Bicine/CHAPS Lysis Buffer and Sample Diluent (ProteinSimple, catalog #040-764)

Pierce BCA Protein Assay Kit (Thermo Scientific, catalog #23225)

Lipofectamine RNAiMAX transfection reagent (Life Technologies, catalog #13778)

Opti-MEM I Reduced Serum Medium (Life Technologies, catalog #31985-062)

ON-TARGETplus SMARTpool Human ErbB2 siRNAs (GE Dharmacon, catalog #L-003126-00-0005)

Non-targeting pool control siRNAs (GE Dharmacon, catalog #D-00180-10-05)

Lapatinib (National Cancer Institute compound repository, NSC 727989)

Neuregulin-1 β (NRG) [Recombinant human neuregulin-1 β (NRG1- β 1/HRG1- β 1 EGF domain), R&D Systems, catalog #396-HB-050]

Trastuzumab (Genentech, Inc.)

Antibodies

EGF/ErbB1 Receptor (D38B1) XP rabbit monoclonal antibody (Cell Signaling, catalog #4267S)

ErbB2 Receptor (M45) rabbit polyclonal antibody (Cell Signaling, catalog #3250S)

ErbB4 Receptor (111B2) rabbit monoclonal antibody (Cell Signaling, catalog #4795S)

AKT (pan; C67E7) rabbit monoclonal antibody (Cell Signaling, catalog #4691S)

Phospho-AKT (Ser473) rabbit polyclonal antibody (Cell Signaling, catalog # 9271)

p42/44 MAPK (Erk1/2) rabbit polyclonal antibody (Cell Signaling catalog #9102)

Phospho- p42/44 MAPK (Erk1/2) (Thr202/Tyr204) rabbit polyclonal (Cell Signaling, catalog #9101)

Alpha-tubulin (11H10) rabbit monoclonal antibody (Cell Signaling, catalog #2125)

Lamin B1(D4Q4Z) rabbit monoclonal antibody (Cell Signaling, catalog #12586)

FOXO3a (D19A7) rabbit monoclonal antibody (Cell Signaling, catalog #12829)

Phospho-FOXO3A (S253) rabbit polyclonal antibody (Abcam, catalog #ab47285)

CREB (48H2) rabbit monoclonal antibody (Cell Signaling, catalog #9197)

Phospho-CREB (Ser133) (87G3) rabbit monoclonal (Cell Signaling, catalog #9198)

Amplified Rabbit or Mouse Secondary Antibody Detection Kit (ProteinSimple, catalog #041-126 or 041-127, respectively)

Instruments

Sonic Dismembrator (Fisher Scientific™, model 100)

Infinite® M1000 plate reader (TECAN)

Wes™ size-based electrophoresis immunoassay Simple Western system (ProteinSimple)

NanoPro™ 1000 charge-based nanofluidic isoelectric focusing proteomic immunoassay (NIA) Simple Western system (ProteinSimple)

IN Cell Analyzer 2000 (GE Healthcare)

(a) LYSATE COLLECTION

(i) Cell lysate collection for denatured proteins

1. Rinse cells cultured in 6-well plates with 2 mL/well ice-cold DPBS twice.
2. Aspirate DPBS and add 100 or 150 μ L/well ice-cold, complete RIPA lysis buffer, depending on desired final protein concentration.
3. Use a sterile scraper with a plate stand at full tilt to collect all cell lysate and debris into a 1.5 mL ice-cold microcentrifuge tube and store the tube on ice.
4. Combine lysates of duplicate or triplicate wells of the same treatment group.
5. Transfer lysate to a 1.5 mL ice-cold microcentrifuge tube and keep the tube on ice.
6. Sonicate the lysate for 3 cycles $\times$ 10 seconds each while on ice, with the power of sonicator or dismembrator setting at 1.
7. Centrifuge the lysate at $15,000 \times g$ for 15 minutes at room temperature.
8. Carefully transfer the supernatant to a fresh, ice-cold microcentrifuge tube.
9. Aliquot into desired sample tubes and store at -80°C for up to 1 year.

(ii) Cell lysate collection for native, non-denatured proteins

1. Rinse cells with ice-cold DPBS twice (2 mL/well for 6-well plates or 1 mL/well for 12-well plates).

2. Aspirate DPBS and add 125 μL /well for 6-well plates or 100 μL /well for 12-well plates, respectively, ice-cold complete M-PER® lysis buffer into each well.
 3. Store the plates on ice for 5 minutes.
 4. Use a sterile scraper with plate stand at full tilt to collect all lysate and debris into a 1.5 mL ice-cold microcentrifuge tube and store the tube on ice.
 5. Combine lysates from duplicate or triplicate wells of the same treatment group.
 6. Transfer lysate to a 1.5 mL ice-cold microcentrifuge tube and keep the tube on ice.
 7. Sonicate the lysate for 3 cycles $\times$ 10 seconds each while on ice, with the sonicator power setting at 1.
 8. Centrifuge the lysate at $15,000 \times g$ for 15 minutes at 4°C .
 9. Carefully transfer the supernatant to a fresh, ice-cold microcentrifuge tube.
 10. Aliquot into desired pre-cooled sample tubes and store at -80°C for up to 1 year.
- (iii) Cell lysate collection for separating nuclear and cytoplasmic proteins (non-denatured, native proteins)
1. Place the Cytoplasmic Extraction Reagent I, II (CER I, II) and Nuclear Extraction Reagent (NER) on ice.
 2. Wash cells that have been cultured in a 6-well plate with 2 mL per well DPBS twice removing as much DPBS as possible after last wash.
 3. Add 4 μL of both phosphatase inhibitor cocktail and Halt protease inhibitor to 392 μL CER I; then add 100 μL of this solution to one sample well at a time; tilt the plate and scrape to collect lysate; transfer all lysate to the second corresponding sample well and again scrape to collect lysate; transfer the lysate to a 1.5 mL microcentrifuge tube; vortex the tubes vigorously on the highest setting for 15 seconds; incubate tubes on ice for 10 minutes.
 4. Add 5.5 μL of ice-cold CER II to each tube; vortex on the highest setting for 5 seconds; incubate on ice for 1 minute; vortex the tube again on the highest setting for 5 seconds; centrifuge at maximum speed ($21,000 \times g$) for 5 minutes; immediately transfer the supernatant to a fresh ice-cold centrifuge tube and store on ice.

The supernatant from this step is the cytoplasmic extract; aliquot and store at -80°C until use.

5. Add 2 μL of both phosphatase inhibitor cocktail (#P0044) and Halt protease inhibitor (#87785) to 196 μL NER; then add 50 μL NER to the tube from the previous step and re-suspend the pellet by pipetting.
6. Vortex the tube vigorously on the highest setting for 15 seconds; incubate on ice for 10 minutes; repeat the vortex/incubation cycle 3 more times.
7. Centrifuge the tube at maximum speed ($21,000 \times g$) for 10 minutes; immediately transfer the supernatant to a fresh, ice-cold centrifuge tube.

The supernatant from this step is the nuclear extract; aliquot and store at -80°C until use.

8. Determine the protein concentration or purity in the cytoplasmic and nuclear extracts using the BCA or Wes™ Simple Western assay as described below.

(iv) Protein concentration determination in cell lysates

1. Dilute cell lysates in Milli-Q water at 1:2 and 1:5 ratios for a final volume of 25 μL .
2. Follow the procedures described in the Pierce BCA protein assay kit (https://tools.lifetechnologies.com/content/sfs/manuals/MAN0011430_Pierce_BCA_Protein_Asy_UG.pdf).
3. Analyze the data with built-in plate reader analysis software or export the measurements to Excel for manual calculations; subtract the average of the blank from all individual standard and diluted lysate measurements; fit the blank-corrected data of standards to generate the standard curve and then calculate the protein concentration of each sample.

Typically, the protein yield after 14 days in culture is approximately $500 \mu\text{g}/10^6$ cells plated. The protein concentration achieved from an entire 6-well plate is approximately 2 mg/mL using the method described for collecting denatured proteins, and approximately 0.7 mg/mL from 2 wells of a 12-well plate using the method described for non-denatured protein collection. Figure 9 represents a typical standard curve of protein concentration with standards and cell lysates collected from 4 wells of a 6-well plate. The average yield for cytoplasmic and nuclear extracts collected from 2 wells (approximately 10^6 cells) of a 6-well plate is

approximately 2.5 mg/mL in 0.1 mL and 3.5 mg/mL in 0.05 mL, respectively.

(b) MEASUREMENTS OF PROTEIN EXPRESSION

(i) Confirm protein expression using a size-based electrophoresis immunoassay (Wes™ Simple Western system)

- 1.** Prepare the standard pack reagents provided in the Wes™ 12–230 kDa master kit:
 - a.** Add 40 µL deionized water to DTT (clear tube) to make 400 mM DTT solution.
 - b.** Add 20 µL 10X sample buffer and 20 µL DTT solution to the fluorescent 5X master mix (pink tube); store the mix on ice.
 - c.** Add 16 µL deionized water, 2 µL 10X sample buffer and 2 µL DTT solution to make the biotinylated ladder (white tube).
 - d.** Transfer the entire volume of the biotinylated ladder to a fresh microcentrifuge tube and store on ice.
- 2.** Combine 150 µL luminol-S and 150 µL peroxide in a microtube; vortex and store on ice.
- 3.** Dilute the lysate with 0.1X sample buffer (1:100 dilution of 10X sample buffer with water) to 0.25 or 0.5 mg/mL protein, depending on the expression level of target proteins; mix gently by pipette 1 part (µL) 5X fluorescent master mix with 4 parts (µL) lysate to the final 0.2 or 0.4 mg/mL protein concentration.
- 4.** Denature the sample and biotinylated ladder by vortexing the microtubes and heating at 80° to 100°C for 10 minutes; vortex and briefly centrifuge; store on ice.

We use a small table-top centrifuge that has no settings.
- 5.** Dilute primary antibodies in antibody diluent II at 1:50 or 1:25 for target protein, at 1:50 or 1:100 for loading control proteins.

The supplied secondary antibody is ready to use without dilution.
- 6.** Pipette the above-prepared solutions into the Wes™ pre-filled microplate that is included with the Wes™ master kit as follows:
 - a.** Load 5 µL biotinylated ladder in well A1.

6-well plates at 2 mL media/well; dilute 22.5 μ L Lipofectamine in 277.5 μ L Opti-MEM Medium in a 1.5 mL microcentrifuge tube; dilute 20 μ L SMARTpool Human ErbB2 siRNA and 20 μ L non-targeting pool control siRNA in two 1.5 mL microcentrifuge tubes labeled with ErbB2 siRNA or control siRNA and pre-loaded with 80 μ L Opti-MEM medium; load the 3rd microcentrifuge tube labeled as Lipofectamine control with 100 μ L Opti-MEM medium; add 100 μ L diluted Lipofectamine/Opti-MEM into each of three labeled microcentrifuge tubes; mix gently by pipetting and incubate at room temperature for 5 minutes.

3. Dispense 200 μ L transfection complex mixtures from each labeled microcentrifuge tube drop-wise into each of 3 wells of a 6-well plate, which yields 100 nM per well of control or ErbB2 siRNAs; rock the plate gently back-and-forth and from side-to-side to equally distribute the mixtures in the well; continue to incubate the cell culture at 37°C/5% CO₂ for 72 hours.
4. Collect cell lysates using the method described above for denatured proteins.
5. Confirm protein expression using the Wes™ Simple Western system as described above.

Figure 12 demonstrates reduced protein expression of ErbB2 by siRNA knockdown using Wes™ Simple Western system.

(iii) Modulation of AKT and Erk1/2 phosphorylation detected using an antibody-based nanofluidic isoelectric focusing proteomic immunoassay (NanoPro™ 1000 Simple Western)

1. To prepare the stock of ErbB pathway modulating compounds, dissolve lapatinib in DMSO at 1 mM, NRG in PBS containing 0.1% BSA at 100 μ g/mL; aliquot the stock solutions and store NRG at -80°C and lapatinib at -20°C.

NRG and lapatinib may be stored for approximately 3 months once reconstituted.

2. To prepare the 10x dosing solution, dilute lapatinib or NRG stock in the culture medium at 10 μ M or 1 μ g/mL, respectively; dissolve trastuzumab in the culture medium at 10 μ M.
3. To pre-treat cells with trastuzumab or lapatinib, remove 200 μ L medium from each well of a 6-well plate containing 2 mL media/well; add 200 μ L 10x dosing solution to achieve a final concentration of 1 μ M for both drugs; add 200 μ L medium

containing 1% DMSO to one well as the vehicle control; incubate the plate at 37°C/5% CO₂ for 30 minutes.

4. To treat the plates with NRG, remove 200 µL medium from each well of a 6-well plate pre-treated with trastuzumab, lapatinib or vehicle; add 200 µL of 1 µg/mL NRG to each well, then incubate the plate 37°C/5% CO₂ for 30 minutes.
5. Collect cell lysates using the methods described above for non-denatured proteins.
6. Dilute the pan- or phosphorylated AKT and Erk1/2 primary antibodies 1:50 by adding 5 µL primary antibody to 245 µL antibody diluent in 1.5 mL microcentrifuge tubes.
7. Dilute both the goat anti-rabbit biotinylated secondary antibody and the streptavidin-HRP conjugated reagent 1:100 by adding 2.5 µL to 247.5 µL antibody diluent in 1.5 mL microcentrifuge tubes.
8. Combine a 1:1 ratio of detection reagent by adding 125 µL luminol and 125 µL peroxide-XDR.
9. Gently vortex each of the four reagents (primary and secondary antibodies, conjugated tertiary reagent, and detection reagent); place microcentrifuge tubes on ice until use.
10. Prepare the master mix and test samples in 1.5 mL microcentrifuge tubes as follows:
 - a. Dilute the pI standard ladder 3 at 1:40 in premix G2 pharmalyte pH 5-8 separation gradient. Formula: $X(40) = n(\mu\text{L})(1.3X)$. Example to make 600 µL master mix: add 19.5 µL of pI Standard ladder 3 to 580.5 µL of Premix G2, Pharmalyte pH 5-8 separation gradient. $(600 \times 1.3)/(40) = 19.5 \mu\text{L}$ and $600 - 19.5 = 580.5 \mu\text{L}$.
 - b. Add DMSO inhibitor to Bicine/CHAPS lysis buffer and sample diluent at 2:25 ratio to make the sample dilution reagent. Formula: $X(50X) = n(\mu\text{L})(4X)$. Example to make 150 µL master mix: add 12 µL DMSO inhibitor to 138 µL of sample diluent (B/C lysis buffer). $(150 \mu\text{L} \times 4)/(50) = 12 \mu\text{L}$ and $150 - 12 = 138 \mu\text{L}$.
 - c. Use sample dilution reagent to dilute sample lysates to 0.2 or 0.4 mg/mL by adding 1 part of diluted sample lysate to 3 parts of diluted pI standard/premix G2 (the final lysate concentration is 0.05 or 0.1 mg/mL), vortex the lysates; briefly centrifuge and store on ice until use.

We use a small table-top centrifuge that has no settings.

- 11.** Prepare the 384-well sample plate provided for the NanoPro™ 1000.
 - a.** Load 12 µL of the prepared sample lysates to wells in row A.
 - b.** Load 20 µL of the primary antibody dilutions to wells in row B.
 - c.** Load 20 µL of the secondary antibody to wells in row C.
 - d.** Load 20 µL of the tertiary reagent (streptavidin-HRP conjugate) to wells in row D.
 - e.** Load 20 µL of the luminol-peroxide XDR reagent to wells in row J.
 - f.** Centrifuge the sample plate at $1000 \times g$ for 5 minutes at 4°C.
- 12.** Run the NanoPro™ 1000 instrument using its Compass Software; define the assay protocol steps:
 - a.** 25 seconds for sample loading
 - b.** 21000 µW for 40 minutes for protein separation
 - c.** 3 seconds for exposure of pI standards
 - d.** 105 seconds for immobilization
 - e.** 2 washes with 20 seconds loading and 150 seconds wash soak
 - f.** Incubate 120 minutes for pan-AKT, pan-Erk1/2 and phospho-Erk1/2 antibodies, and 240 minutes for phospho-AKT antibody.
 - g.** 2 seconds primary antibody loading followed by 2 washes with 20 seconds loading and 150 seconds wash soak
 - h.** 60 minutes for each cycle of secondary antibody incubation
 - i.** 2 second secondary antibody loading followed by 2 washes with 20 seconds loading and 150 seconds wash soak
 - j.** 10 minutes for each cycle of HRP-conjugated tertiary reagent incubation

- k.** 2 seconds tertiary reagent loading followed by 2 washes with 20 seconds loading and 150 seconds wash soak
 - l.** 2 seconds wash load time followed by chemiluminescence exposures of 30, 60, 120, 240, 480 and 960 seconds
- 13.** Empty the waste reservoir and fill the water reservoir.
- 14.** Click on the resource tray icon to open/close the instrument resource tray.
- 15.** Fill the cups for wash buffer, anolyte and catholyte.
- 16.** Insert a capillary box and close the reservoir drawer.
- 17.** Click on the sample tray icon to open the sample tray and insert the lid-covered sample plate; click on the icon to close the sample tray. Save the assay protocol file and click the start button to initiate the run, which typically takes at least 8 hours depending on the number of cycles being run and the primary antibody incubation times.

We typically run the assay overnight.

- 18.** Analyze the data using data analysis module in Compass software.

Figure 13 illustrates the detection of modulation on (A) AKT and (B) Erk1/2 phosphorylation using antibody-based nanocapillary isoelectric focusing (NIA) on the NanoPro™ 1000 Simple Western system.

- (iv)** Activation and translocation of AKT and Erk1/2 signaling proteins, and downstream transcription factors, FOXO3a and CREB, by neuregulin-1 β (NRG) activation of ErbB pathway
 - 1.** Culture hiPSC-CMs for at least 14 days prior to testing according to methods described in Basic Protocol 1 for culturing hiPSC-CMs.
 - 2.** Prior to drug treatment, equilibrate the cells in 1.8 mL serum-free media for approximately 45 minutes in the incubator.
 - 3.** Prepare NRG in DPBS containing 0.1% BSA at 100 μ g/mL; aliquot the stock solutions and store NRG at -80°C for up to 3 months.
 - 4.** Dilute NRG stock in serum-free medium at 1 μ g/mL.
 - 5.** To treat the plates with NRG, add 200 μ L of 1 μ g/mL NRG to 2 wells for a final concentration of 100 ng/mL NRG; treat another 2 wells with serum-free medium only as a control; incubate the plates at $37^{\circ}\text{C}/5\%$ CO_2 for 30 minutes.

6. Collect cell lysates using the NE-PER® Nuclear and Cytoplasmic Extraction kit described above.
7. Load 0.2 µg/µL protein in each capillary.
8. Probe for AKT, Erk1/2, phospho-AKT and phospho-Erk1/2 using antibodies diluted 1:50.
9. Using a separate set of capillaries for the same lysates, probe for FOXO3a, CREB, phospho-FOXO3a and phospho-CREB using antibodies diluted 1:25 – 1:50.
10. Confirm protein expression using the Wes™ Simple Western system as described above.

Figure 14 illustrates phosphorylation and nuclear translocation of AKT and Erk1/2 after NRG activation of the ErbB2 pathway. Figures 15 and 16 illustrate activation and translocation of transcription factors, FOXO3a and CREB, respectively, in response to NRG activation of the ErbB2 pathway.

- (v) NRG-induced translocation of FOXO3a and phosphorylation of nuclear CREB detected by immunocytochemical stain
1. Plate hiPSC-CMs at 18,000 cells/well in a fibronectin-coated, 96-well acoustic uClear plate and culture for at least 14 days prior to testing.
 2. Replace the Maintenance Medium with serum-free DMEM (supplemented with 1 mM sodium pyruvate and 10 mM D-(+)-galactose); incubate cells for 4 hours in 37°C/5% CO₂ incubator.

Longer serum starvation, i.e. up to 24 hours, increases NRG-induced phosphorylated CREB signals.
 3. Dilute the NRG stock to 1 µg/mL in the serum-free DMEM; add 10 µL/well of the media containing NRG to wells containing 90 µL media; incubate the plate for 20 minutes in 37°C/5% CO₂ incubator.
 4. Dilute paraformaldehyde in PBS to make an 8% solution; pre-warm to 37°C and add 100 µL to each well; incubate for 15 minutes in 37°C/5% CO₂ incubator or at room temperature to fix cells.
 5. Remove paraformaldehyde; add 100 µL/well of 0.1% Triton X-100 in PBS and incubate 10 minutes at room temperature for membrane permeabilization.

6. Remove 0.1% Triton X-100 and wash cells with 200 μ L/well PBS twice at 5 minute intervals.
7. Add 100 μ L/well of Odyssey Blocking Buffer (OBB) containing 3% goat serum; incubate for 1 hour at room temperature to block non-specific binding.
8. Remove OBB; add 50 μ L/well of the rabbit anti-CREB, rabbit anti-phospho-CREB or rabbit anti-FOXO3a antibodies diluted 1:100 in OBB.
 - a. For anti-CREB or anti-phospho-CREB, incubate overnight at 4°C with gentle shaking.
 - b. For anti-FOXO3a, incubate for 1 hour at room temperature with gentle shaking.
9. Remove primary antibody solution and rinse cells quickly once with 200 μ L/well PBS followed by 3 times washes at 5 minute intervals.
10. Add 50 μ L/well of goat anti-rabbit antibody conjugated with Alexa Fluor 488 diluted in OBB at 1:1000 and mixed with Sytox red reagent at 50 nM; incubate for 1 hour at room temperature.
11. Remove the secondary antibody and Sytox red; rinse quickly once with 200 μ L/well PBS followed by 3 washes at 5 minute intervals; fill wells with 200 μ L/well PBS.
12. Analyze the stained image using IN Cell Analyzer 2000; refer to the section below entitled, “Assessment of mitochondrial membrane potential image analysis” for image acquisition.

It is critical to optimize the drug treatment and immuno-staining procedure to maximize the drug response and to improve the signal/noise (background, non-specific binding) ratio. For example, a longer serum starvation, i.e. up to 24 hours, increases NRG-induced phosphorylated CREB signals, but reduces the response of FOXO3a; the detected change in FOXO3a expression also decreases with longer antibody incubation, i.e. overnight at 4°C, likely due to increased non-specific binding.

Figures 15C–D and 16C further demonstrate activation and translocation of FOXO3a and CREB using standard immunocytochemical methods on fixed hiPSC-CMs in culture.

REAGENTS AND SOLUTIONS

Lysis buffer for denatured proteins

1. Dissolve 1 g of SDS in 10 mL distilled/deionized water (e.g., Milli-Q water) to make 10% SDS.
2. Dilute 1 mL 10X RIPA in 9 mL Milli-Q water to make 1X RIPA.
3. Transfer 9.2 mL 1X RIPA to a 15 mL centrifuge tube.
4. Add 0.5 mL 10% SDS, and 100 μ L each of protease inhibitor cocktail, phosphatase inhibitor cocktail and Halt protease inhibitor to make 10 mL complete RIPA lysis buffer containing 0.5% SDS.
5. Store the complete lysis buffer solution on ice before use.

Lysis buffer for native, non-denatured proteins

1. Add 4.85 mL of M-PER® reagent to a 15 mL centrifuge tube.
2. Add 50 μ L each of protease inhibitor cocktail, phosphatase inhibitor cocktail and Halt protease inhibitor to make 5 mL complete M-PER® lysis buffer.
3. Store the tube on ice for at least 30 minutes prior to use.

COMMENTARY

Background Information

Human heart is a major target organ of toxicity associated with cancer drug therapies (Albini et al., 2010; Curigliano et al., 2010; Mellor et al., 2011; Senkus and Jassem, 2011; Smith, 2014). Human cardiomyocytes differentiated from either human embryonic stem cells (hESCs) or human induced pluripotent stem cells (hiPSCs) provide a biologically relevant *in vitro* model and have been used increasingly in recent years to interrogate the potential cardiac safety liability of novel anticancer therapeutic agents during preclinical or clinical development (Force and Kolaja, 2011; Guo et al., 2011; Himmel, 2013; Khan et al., 2013; McGivern and Ebert, 2014; Scott et al., 2013; Sharma et al., 2013; Zeevi-Levin et al., 2012). Targeted therapeutics are associated with cardiotoxicity related to “on-target” effects when cardiomyocyte viability is dependent upon the targeted pathway for cell viability or survival (Force and Kolaja, 2011; Kao et al., 2012). *In vitro* cell systems employed to interrogate mechanisms of toxicity are suitable for generating experimental evidence supporting mechanistic hypotheses when the system has been adequately characterized. Since little is known about the functionality of cell signaling pathways in hiPSC-CMs, there continues to be an urgent need to develop more appropriate, relevant, and predictive tools and assays to inform a better understanding of the mechanistic basis of cardiotoxicity. Our lab has developed a strategy that employs multiple advanced technologies and analytic platforms to confirm the expression and functionality of key signaling proteins of interest, and to investigate the biological consequences of pathway modulation in hiPSC-CMs.

Critical Parameters

Cardiomyocyte quality—Differentiation of human pluripotent stem cells to cardiomyocytes is a fairly complex process and the purity of the resulting cardiomyocytes in cell culture is essential for characterization of the cardiomyocyte phenotype (Takahashi and Yamanaka, 2006; Yu et al., 2007). Human stem cell-derived cardiomyocytes manufactured from different suppliers often demonstrate differences in purity and phenotypes, e.g., different basal levels of beat rate and responses to the known tool drugs. Cardiomyocytes from the same supplier may also exhibit lot-to-lot variation in cell purity, plating efficiency, the time required to reach a stable beat rate and sensitivity to tool drugs. Therefore, a thorough characterization of each lot of cells may be necessary to ensure data consistency. When feasible, we recommend acquiring a large quantity of cells from a lot that has been pre-evaluated. Our lab uses hiPSC-CMs (iCell® Cardiomyocytes) from Cellular Dynamics International that are a mixture of spontaneously electrically active atrial, nodal, and ventricular-like myocytes that possess typical electrophysiological characteristics and exhibit expected electrophysiological and biochemical responses upon exposure to exogenous agents. Once plated, hiPSC-CMs undergo a continuous maturation process in culture (Babiarz et al., 2012; Ivashchenko et al., 2013; Lundy et al., 2013; Shinozawa et al., 2012); thus, it is recommended to perform test assays in the same time frame after plating to avoid potential impact of cell culture duration on the readout.

Use of serum-free medium—The serum in culture medium is generally required to achieve a high plating efficiency and maintain viability of cardiomyocytes, especially in long-term cultures or when cells are plated at a low plating density, e.g., less than 10,000 cells/well in a 96-well plate. However, the presence of serum in culture medium often confounds signaling protein analysis if the protein under investigation is activated by serum (likely due to multiple growth factors or other stimuli present in serum). For example, Erk1/2 is transiently activated by media change (Eldridge et al., 2014). Hence, activation of signaling pathways (such as ErbB2 by NRG) is typically performed in cells starved from serum for 30 minutes to 3 hours. A longer starvation may be required to bring activation down to basal levels for certain signaling proteins.

Erk1/2 was also activated when the media containing serum is added to neutralize trypsin activity during cell collection. The NE-PER® Nuclear and Cytoplasmic Extraction Reagents kit recommends using trypsin to collect adherent cells before lysing with CER I lysis buffer for maximal protein yield. Harvesting the cells with trypsin but without sequential addition of the serum-containing medium increases the protein yields of both cytoplasmic and nuclear fractions but may also cause an increased basal level activity of phospho-Erk1/2 in the cells (data not shown).

The serum in culture medium also interferes with the detection of cTnT by the MSD® platform (data not shown). Thus, assessment of doxorubicin-induced cTnT release has to be performed in cells cultured with serum-free medium. As removal of serum from media imposes stress and makes cells more vulnerable to external insults, the concentrations and duration of exposure to toxic drugs may need to be adjusted; i.e. doxorubicin exposure was reduced from 40 to 24 hours in studies in which cTnT was measured.

Monitoring of cardiomyocyte function—Synchronous beating of hiPSC-CMs may be detected as early as 24 hours after cell plating. However, when monitored with real-time impedance or field potential measurements, the amplitude of both contraction and Na⁺ spike is initially very small and the beat-to-beat interval is quite variable. With time in culture, these measurements improve. It generally takes about 10 days to reach stabilized beatings in all wells of an E-Plate. Typically, a well plated with iCell® cardiomyocytes is considered suitable for testing if the cell impedance is > 2 cell index units (CIU), the amplitude of impedance-measured contraction or Na⁺ spike is > 0.02 CIU or > 0.1 mV, respectively, and shows a stable, arrhythmia-free beat rate of around 30 beats/minute with a coefficient of variation (CV) < 10%.

Antibody quality—For immunocytochemistry (ICC) staining, it is recommended to select antibodies that are suitable for both western blot (WB) and ICC. Like all antibody-based proteomic analyses, it is critical to validate an antibody by demonstrating that it is specific, selective and reproducible in the context for which it is being used. The reproducibility of detection, signal/background noise ratio and the response to biological stimuli are major criteria in antibody selection. To confirm a lack or low expression of proteins of interest, antibodies raised against different epitopes/isoforms of the proteins of interest, and from multiple sources should be tested. Positive control specimen(s), cell lysates collected with different lysis buffers, i.e. denatured or non-denatured, and functional state, i.e. activated or deactivated, should also be tested. Different dilutions of both the cell lysates and primary antibodies are important variables to test. It is also important to rule out cross-reactivity and non-specific binding of antibodies by including appropriate negative controls [see (Bordeaux et al., 2010) for a comprehensive review of antibody validation].

Wes™ and NanoPro™ assay optimization—As equivalent to the conventional western blot (WB) assay, the size-based CE immunoassay is used primarily to quantify protein abundance in cell lysate samples, whereas the charge-based CE immunoassay is applied exclusively for detailed profiling of post-translational modification of signaling proteins. We employed both Wes™ (size-based) and NanoPro™ 1000 (charge-based) as the analytic platforms in this study based on 1) both instruments are fully automated systems; the conventional WB assay involves multiple steps of manual processing and takes about 24 hours to complete a run, while it takes about 3 hours for Wes™, or 11 hours for NanoPro™ 1000 to complete a run that automated and does not require manual attention; 2) both instruments have a higher throughput than conventional WB. Wes™ can test up to 25 samples in a single run or 50 samples (2 runs) in a day. NanoPro™ 1000 tests up to 96 samples in a run; 3) both instruments require smaller sample sizes than WB (approximately 0.5 – 2 µg protein/capillary, i.e. approximately 5 µL at 0.2 – 0.4 µg/µL for Wes™ and 10 – 12 µL at 0.05 – 0.1 µg/µL for NanoPro™, whereas approximately 30 µg protein/lane is needed for the conventional WB assay. This is a critical factor in conducting a time- and cost-effective experiment since the price to obtain a large quantity of hiPSC-CMs is relatively high.

A disadvantage of CE-based immunoassays is that the capillary is designed for only a single use, unlike the conventional WB assay in which the stains on the membrane may be stripped

off a couple of times, allowing the membrane to be re-probed with additional antibodies. Secondly, when using the streptavidin-HRP tertiary to amplify the target signal in the charge-based assay, two non-specific binding peaks (at pI 5.87–5.88 and pI 6.46–6.47) appear which is likely due to non-specific binding of streptavidin to the endogenous biotin of hiPSC-derived cardiomyocytes. This may cause misinterpretation when the target proteins have a weak signal that also located around pI 5.87 or 6.46.

To obtain high quality and reproducible data, optimization of primary antibody dilution, sample protein concentration, antibody incubation time, and exposure time for detection in capillaries must be performed. Typically for Wes™ Simple Western, protein concentration in capillaries starts at 0.4 µg/µL, primary antibodies are diluted 1:25 and incubation time in primary antibody is set for 60 minutes. For NanoPro™ Simple Western, protein concentration in capillaries starts at 0.1 µg/µL, primary antibodies are diluted 1:50 and incubation time in primary antibody is set for 240 minutes. A high intensity signal will “burn out” in the capillary, resulting in a dark gap in an otherwise continuous signal band. The saturated signal narrows the dynamic range of signal detection, reducing the sensitivity to identify any changes in signal. Thus, protein concentration, primary antibody dilution and incubation time need to be adjusted.

When using the streptavidin-HRP tertiary reagent is used to amplify the target signal in the charge-based NanoPro™ assay, two small peaks (at pI 5.87–5.88 and pI 6.46–6.47) are observed when no primary antibodies are used, suggesting that these peaks represent non-specific binding of streptavidin to endogenous biotin of hiPSC-CMs, and may interfere with detection of small target signals also located around the same pIs. Therefore, it is important to determine signal detection with or without using the streptavidin-HRP tertiary reagent.

siRNA knockdown—Because a potent and highly selective inhibitor of signaling proteins is not always available, down-regulation of a signaling protein of interest with targeted siRNA knockdown provides a valuable tool to analyze the role of a specific protein in the signaling cascade of interest. Reagents used in the siRNA transfection process are often toxic. Using the xCELLigence® impedance measurement, we found that the transfection reagent Lipofectamine® RNAiMAX is much less toxic than TransIT-TKO® or siLentFect™ when added at identical concentrations (data not shown). Though transfection for 72 hours continuously with 100 nM siRNAs results in approximately 70% knockdown of ErbB2 expression reproducibly, we found the same level of ErbB2 knockdown may be achieved with less siRNAs and/or shorter transfection time. In a recent study for siRNA-knockdown of a different signaling protein, we found transfection with 5 nM compared to 50 nM siRNA for 6 hours compared to 72 hours continuous transfection also resulted in approximately 70% knockdown of the target protein expression, similar to ErbB2 knockdown (data not shown). Moreover, reduced protein expression lasted for at least 2 weeks after washout of siRNAs.

Selection of methods for ErbB2 pathway modulation—To confirm functional activation of the ErbB2 pathway upon ligand binding by NRG, phosphorylation of AKT and Erk1/2 was confirmed. Inhibition of NRG-induced phosphorylation was further investigated using either an ErbB2 receptor antibody, trastuzumab, or an ErbB2 tyrosine kinase inhibitor,

lapatinib. Down-regulation of ErbB2 expression by siRNA knockdown, translocation of activated AKT, Erk1/2, FOXO3a and CREB in cytosol and nucleus were also examined. Taken together, these studies provide a comprehensive characterization of ErbB signaling in hiPSC-CMs.

Modulation of ErbB2 signaling on doxorubicin cardiotoxicity—Treatment of breast cancer patients overexpressing ErbB2 receptor with the ErbB2 monoclonal antibody, trastuzumab, is known to potentiate anthracycline chemotherapy-induced cardiotoxicity in the clinic (Bowles et al., 2012). In the protocols described here, inhibition of ErbB2 signaling by trastuzumab clearly potentiated cardiotoxic effects of doxorubicin in hiPSC-CMs *in vitro*. Furthermore, NRG attenuated doxorubicin-induced cardiotoxicity in this multiparameter model system. Our data confirm the role of the ErbB2 pathway in mediating mechanisms of cardiac toxicity and support the use of hiPSC-CMs to explore cardiac cell signaling pathways and mechanisms of cardiac toxicity for new uncharacterized early drug candidates.

Anticipated Results

ErbB signaling plays a critical role in maintaining the structural and functional integrity of normal myocardium (Fuller et al., 2008; Lemmens et al., 2007). HiPSC-CMs express receptors for EGFR/ErbB1, ErbB2, ErbB4, but not ErbB3 (Eldridge et al., 2014). This is not surprising since ErbB3 plays a role in cardiomyogenesis during heart development, but is not detectable in adult ventricular cardiomyocytes (De Keulenaer et al., 2010; Zhao et al., 1998). NRG is synthesized in endothelial cells near cardiomyocytes (in the endocardium and myocardial microvasculature). On release, NRG binds the ErbB4 receptor on cardiomyocytes that, after homodimerization with ErbB4 or heterodimerization with ErbB2, leads to activation of AKT and Erk1/2, with subsequent activation of downstream signaling proteins not only in the cytosol, but translocation to the nucleus with activation of nuclear transcription factors, such as FOXO3a and CREB (Brunet et al., 2001; Lemmens et al., 2007; Mebratu and Tesfaigzi, 2009; Takaishi et al., 1999). ErbB2 inhibitors, trastuzumab and lapatinib, were developed to treat the ErbB2-overexpressing breast cancer. Such treatment improves the cancer prognosis, but with an increased risk of cardiotoxicity and potentiates anthracycline-induced heart failure (Baselga, 2001; Bowles et al., 2012; Keefe, 2002). Upregulation of ErbB signaling by NRG may attenuate anthracycline-induced heart damage (Wadugu and Kuhn, 2012).

Consistent with our understanding of ErbB signaling in human myocardium, we show expression of EGFR/ErbB1, ErbB2 and ErbB4 receptors (but not the ErbB3 receptor), the presence of AKT and Erk1/2 in hiPSC-CMs and the phosphorylation of AKT and Erk1/2 upon activation with NRG (Figure 11). The expression of ErbB2 can be reduced effectively by siRNA knockdown (Figure 12) and the phosphorylation of both AKT and Erk1/2 can be blocked by the selective ErbB2 inhibitors, trastuzumab and lapatinib (Figure 13) as expected. NRG treatment increased the amount of phosphorylated AKT and Erk1/2 in the nucleus, which indicates nuclear translocation of phosphorylated AKT and Erk1/2 (Figure 14). Moreover, NRG treatment caused an increase in both total and phosphorylated FOXO3a in the cytosol and a decrease in the nucleus (Figure 15), suggesting a translocation

of FOXO3a from the nucleus to the cytosol. As expected, NRG treatment also increased the phosphorylated CREB in the nucleus as demonstrated by both Wes™ Simple Western and immunocytochemistry (ICC) (Figure 16). In agreement with both the preclinical and clinical observations, inhibition of ErbB2 signaling by trastuzumab or activation of ErbB signaling by NRG potentiated or attenuated doxorubicin-induced cardiotoxicity, respectively, as measured by cardiomyocyte cellular impedance and beating function (Figure 8), nuclear morphology (Figure 3), mitochondrial membrane potential (Figure 4), ATP, caspase 3/7 activity, LDH release (Figure 5). While NRG attenuated doxorubicin-induced cTnT release as expected, the potentiating effect of trastuzumab was not observed (Figure 6), which might be due to insufficient pretreatment with trastuzumab. Since removal of serum from the medium imposes stress to the cells, to minimize the impact of serum-withdrawal on cell responses, only a 3 hour pretreatment was performed, instead of 24 hour pretreatment when the experiments were conducted with the serum-containing medium.

Time Considerations

As it typically takes 10 to 14 days for cells to be ready for testing, the first step in the study is to plate cells, either on the optical-bottom plate 96-well plates for imaging, ATP, LDH, caspase 3/7 and cTnT measurements, or on the E-Plates for contraction, field potential, impedance cell injury, plus ATP, LDH, caspase 3/7 and cTnT measurements, or in 6- or 12-well plates for cell lysate collection. It is recommended to plate cells on a Monday and/or Wednesday morning, which allows switching Plating Medium to Maintenance Medium 2 days after plating, which would occur on a Wednesday and/or Friday. Once cells are ready for testing, all assays may be completed within a week.

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Figure 1.

Overview of the key transduction molecules of ErbB signaling pathway known to regulate cardiomyocyte viability and function. ErbB2, ErbB4, AKT, Erk1/2, FOXO3a and CREB were demonstrated as functional proteins in hiPSC-CMs in this unit. Scheme was prepared based on published literature (De Keulenaer et al., 2010; Fuller et al., 2008; Mebratu and Tesfagzi, 2009; Sussman et al., 2011; Yoon and Seger, 2006).

Figure 2.

Expression of cTnI and myomesin in hiPSC-CMs. Cells were plated at 5,000 or 10,000 cells/well in 96-well plates and cultured for 14 days, then fixed in 4% paraformaldehyde and stained for cTnI and/or myomesin by immunocytochemical methods. Images were captured using either a Nikon ECLIPSE Ti fluorescence microscope or IN Cell Analyzer 2000. Representative images of stained cells are presented. In the upper panels, hiPSC-CMs from one lot of cells are stained for cTnI (red cytoplasm) and F-actin (green cytoplasm). In the lower panels, cells from a different lot are stained for myomesin (green cytoplasm), F-actin (red cytoplasm) and Hoechst (blue nuclei). While all cells stained positive for F-actin, approximately 95% of the cells expressed cTnI, which represents the purity of cardiomyocytes in culture based on cTnI expression in this particular lot of hiPSC-CMs. Representative images of hiPSC-CMs from another lot are shown in the lower panel, with cells stained for myomesin (green cytoplasm), F-actin (red cytoplasm) and Hoechst (blue nuclei). Positive staining for myomesin showed a well-organized striated structure of sarcomere network in cardiomyocytes (in the lower middle panel), whereas non-cardiomyocytes stained only for F-actin. Based on the expression of myomesin in cells from this lot, the purity of cardiomyocytes was determined to be nearly 100%.

Figure 3.

ErbB signaling modulates doxorubicin (Dox)-induced alterations in nuclear morphology. Images acquired using the IN Cell Analyzer 2000 and analyzed using the IN Cell Workstation software. Cardiomyocytes were plated on uClear Flat-Bottom 96-well plates and cultured for a minimum of 14 days before testing. Cells were pre-treated with 1 μ M trastuzumab (Tras) or 100 ng/mL neuregulin 1 β (NRG) for 24 hours, then treated with 1 μ M Dox for 40 hours followed by fixation with 4% paraformaldehyde and stained with Hoechst 33258, or live-stained without fixation. (A) Representative images of cells exposed to 1 μ M Dox for 40 hours with or without pre-treatment with 1 μ M Tras or 100 ng/mL NRG; nuclei with a perimeter in red represents a nucleus with an area < 50 μ m². (B) Quantification of % nuclei with an area < 50 μ m². The average nuclear area of control cells was 110 $\pm$ 0.6 μ m², (mean $\pm$ SEM). (C) The total nucleus count of 6 fields/well and averaged from 3 wells in a 96-well plate. In this experiment, Dox by itself does not cause a significant loss of nucleus (cell) count; a small but statistically significant reduction in nucleus (cell) count is observed by Tras plus Dox, while a small but statistically significant increase in nuclei (cell count) is observed with NRG + Dox. Each bar represents “mean $\pm$ SEM” of 3 wells containing >16,000 cells; **P* < 0.05 vs. 0.1% DMSO control; #*P* < 0.05 vs. 1 μ M Dox group. Dox caused nuclear shrinkage which was potentiated by ErbB signaling inhibition with Tras and attenuated by ErbB signaling activation with NRG.

Figure 4.

ErbB signaling modulates doxorubicin (Dox)-induced alterations in the mitochondrial membrane potential (MMP). Cardiomyocytes were plated on the uClear Flat-Bottom 96-well plates and cultured for a minimum of 14 days before testing. Cells were pre-treated with 1 μ M trastuzumab (Tras) or 100 ng/mL neuregulin 1 β (NRG) for 24 hours, then treated with 1 μ M Dox for 40 hours before live-stained with JC-10 reagent. JC-10 enters the cell and distributes in the cytosol in a monomeric form that emits light in the green fluorescent range. Driven by the mitochondrial membrane potential, JC-10 enters the mitochondria and forms JC-10 aggregates inside the mitochondrial shifting the emission to reddish orange fluorescence; thus the ratio of orange/green fluorescence represents an indicator of the MMP. Dox caused a decrease in MMP, which was potentiated by Tras pre-treatment, and attenuated by NRG pre-treatment. As the positive control for disruption of MMP, CCCP induced a dose-dependent reduction in JC-10 aggregates and hence the intensity of orange fluorescence. CCCP at 1 and 10 μ M reduced the ratio of orange/green fluorescence by approximately 50% and 86%, respectively.

Figure 5.

ErbB signaling modulates doxorubicin (Dox)-induced alterations in (A) ATP, (B) LDH and (C) caspase 3/7. Cardiomyocytes were plated on the uClear Flat-Bottom 96-well plates and cultured for a minimum of 14 days before testing. Cells were pre-treated with 1 μ M trastuzumab (Tras) or 100 ng/mL neuregulin 1 β (NRG) for 24 hours, then treated with 1 μ M Dox for 40 hours. Upon completion of drug treatment, the culture medium was collected for LDH measurement, and cells were lysed for quantification of ATP content and caspase 3/7 activity. 1 μ M Tras caused a small but statistically significant reduction in ATP, and a small increase in LDH release and caspase 3/7 activation; 1 μ M doxorubicin induced a large reduction in ATP and a large increase in LDH release and caspase 3/7 activation. All of these alterations were potentiated by ErbB signaling inhibition with Tras and attenuated by ErbB signaling activation with NRG. Each bar represents the mean $\pm$ SEM of $n = 9 - 12$ wells of 5 replicate plates for ATP, and $n = 3 - 6$ wells from one of 4 replicate plates for LDH and caspase 3/7 measurement; * $P < 0.05$ vs. 0.1% DMSO control; # $P < 0.05$ vs. 1 μ M Dox group.

Figure 6.

ErbB signaling modulates doxorubicin (Dox)-induced release of cardiac troponin T (cTnT) as quantified using the MSD® platform. Cardiomyocytes were plated 96-well plates and cultured for a minimum of 14 days before testing. Cells were then cultured in serum-free DMEM medium for 4 hours, pre-treated with 1 μM trastuzumab (Tras) or 100 ng/mL neuregulin 1β (NRG) for 3 hours, then treated with 1 μM Dox for 24 hours. Upon completion of drug treatment, the culture media was collected for cTnT measurement. (A) The calibration curve shows the sensitivity and range of detection for cTnT. (B) Levels of cTnT released by hiPSC-CMs after drug treatment; the quantified data shows that 1 μM doxorubicin increased a cTnT release by 59%, which was mitigated by ErbB signaling activation with neuregulin. However, doxorubicin-induced cTnT release was not further aggravated by Tras treatment as expected. Each bar represents mean ± SEM, n = 3 wells from a representative plate; **P* < 0.05 vs. 0.1% DMSO control; #*P* < 0.05 vs. 1 μM Dox group.

Figure 7.

Example of data output for real-time monitoring of cardiomyocyte function by impedance-based contractility and electric field potential using xCELLigence® RTCA CardioECR system. Cardiomyocytes were cultured in a CardioECR eplate 48 for more than 14 days before testing. Drugs were dissolved in DMSO at 1 or 10 mM, then diluted to 10x target concentrations in fresh media and 20 μ L added to each well containing 180 μ L fresh medium. (A) Representative traces of field potentials (green lines) and impedance (red lines) of cells exposed to 30 nM E-4031 (a potent hERG channel blocker) for 30 minutes. The waveform of field potentials (green lines) precedes the impedance contractions in both regular and irregular (arrhythmic) beats. (B) Averaged impedance contraction and field potential waveforms from more than 30 beats at pre-dose (baseline) or 30 minutes after exposure to 1 μ M blebbistatin (a selective inhibitor of myosin II ATPase); blebbistatin reduced the amplitude of impedance contraction waveform by approximately 93% and had a negligible effect on the field potential.

Figure 8.

Representative results of an xCelligence® RCTA/ECR assay showing doxorubicin (Dox)-induced changes in both cellular impedance and field potential measurements that are attenuated by neuregulin-1 β (NRG) activated ErbB2 signaling, and potentiated by trastuzumab (Tras) inhibited ErbB2 signaling. Cardiomyocytes were cultured in a CardioECR Eplate-48 for more than 14 days before testing. Cells were pretreated with 1 μ M Tras, 100 ng/mL NRG or 0.1% DMSO for 24 hours before being exposed to 1 μ M Dox for 40 hours. (A) Screen shot of averaged global cell index (CI), normalized to the time when 1 μ M Dox or 0.1% DMSO was applied (marked by the solid vertical line and arrow head). CI in cells exposed to NRG or Tras did not differ from each other; 1 μ M Dox reduced the CI by 56%; the combination of 1 μ M Tras + 1 μ M Tras Dox reduced the CI further by 67%; NRG pretreatment attenuated the CI to 29% reduction by 1 μ M Tras Dox. Each trace represents the average of 3–6 wells $\times$ SD. (B) Screenshot of zoomed-in traces of contractions and field potentials taken after 40 hours Dox exposure. 1 μ M Dox reduced the amplitude of Na⁺ spike by approximately 72%, which was further reduced to approximately 83% by Tras pretreatment; NRG pretreatment attenuated the Na⁺ spike by approximately 23%.

Figure 9.

Standard curve for determining protein concentration in cell lysates. Protein standards (diamonds) show linearity between protein concentration and absorbance. Protein concentration of cell lysates (square and triangle) is derived from fitting two different dilutions (1:2 and 1:5) to the standard curve. Lysates were collected from 4 wells of 6-well plates, with cells plated at approximately 460,000 cells/well, cultured for 14 days and lysed with 150 μ L/well RIPA buffer for denatured proteins; the measured protein concentrations were 1.71, 1.66, 1.66 and 1.60 mg/mL, with a yield of 558, 541, 541 and 521 μ g/ 10^6 cells plated, respectively.

Figure 10.

Assessing the purity of separated cytoplasm and nuclear protein extracts using the Wes™ Simple Western system. Cells were cultured more than 14 days, lysates collected, and the fraction of cytoplasmic and nuclear proteins was separated using the NE-PER Nuclear and Cytoplasmic Extraction kit. Cytoplasmic and nuclear lysates were then loaded to each capillary at 0.2 $\mu\text{g}/\mu\text{L}$ protein concentration and probed with α -tubulin antibody (diluted 1:100) or with lamin B1 antibody (diluted 1:50) with 120 second exposure. A clean band at 55 KDa was detected by α -tubulin antibody only in cytoplasmic extracts, while a band at 72 KDa was detected by lamin B1 antibody only in nuclear extracts, indicating a distinct separation between cytoplasmic and nuclear protein extracts.

Figure 11.

Expression of EGFR/ErbB1, ErbB2 and ErbB4 receptors (A), and activation of AKT and Erk1/2 protein phosphorylation by neuregulin-1 β (NRG) (B) in lysates collected using the method for denatured proteins and detected using the WesTM Simple Western system. Cells were cultured for more than 14 days and treated with 100 ng/mL NRG or 0.1% DMSO for 30 minutes or untreated prior to lysate collection with RIPA buffer. (A) Lysates of untreated cells were loaded in each capillary at 0.4 μ g/ μ L for cardiomyocytes, and at 0.2 μ g/ μ L for NCI-H441, a human lung papillary adenocarcinoma cell line as the positive control for ErbB3 detection. The separated proteins were probed with EGFR/ErbB1, ErbB2, ErbB3 and ErbB4 antibodies diluted 1:25 (1:50 for H441), along with anti-GAPDH diluted 1:100 as the loading control. ErbB receptors were detected at 174 to 221 KDa; GAPDH was detected at 42 KDa. The detected ErbB signals were normalized to GAPDH and plotted in the bar graph; note ErbB3 was not detectable in cardiomyocytes. (B) Lysates of treated cardiomyocytes were loaded in each capillary at 0.2 μ g/ μ L and probed with anti-pan AKT and anti-pan-44/42 MAPK (Erk1/2) diluted 1:50, or with anti-phospho-AKT(Ser473) and anti-phospho-p44/42 MAPK (Erk1/2)(Thr202/Tyr204) diluted 1:25 together with anti-cofilin diluted 1:50 as the protein loading control. The bands for AKT and Erk1/2 detected by either anti-pan or anti-phosphorylated proteins were seen at 62 and 44/47 KDa, respectively. Cofilin was detected at 26 KDa. The AKT or Erk1/2 signals normalized to cofilin were plotted in the bar graph. While abundance of AKT and Erk1/2 was detected by anti-pan antibodies, the signal of both proteins were increased slightly (approximately 11%) by NRG treatment. An increase in both phosphorylated AKT and Erk1/2 was observed after NRG treatment.

Figure 12. Quantification of siRNA-mediated down-regulation of ErbB2 expression using the WesTM Simple Western system. After more than 14 days in culture, cells were exposed to Lipofectamine/Opti-MEM (transfection vehicle), 100 nM scrambled or targeted siRNAs (control or ErbB2 siRNAs) for 72 hours; cell lysates were then collected with the RIPA buffer, and loaded in each capillary at 0.4 $\mu\text{g}/\mu\text{L}$ protein concentration; the separated proteins were then probed with ErbB2 antibody (diluted 1:25) and GAPDH antibody (diluted 1:100), and resulted in one band at 173 KDa and another band at 43 KDa, representing ErbB2 and GAPDH, respectively (A). ErbB2 expression was normalized to GAPDH; efficiency of ErbB2 knockdown by siRNA was calculated as approximately 75% of the control siRNAs (B).

Figure 13.

Modulation of AKT (A) and Erk1/2 (B) as demonstrated using nanocapillary isoelectric focusing (NanoPro™ 1000 Simple Western system). Cells were cultured for more than 14 days, then pretreated with 1 μ M lapatinib, 1 μ M trastuzumab (Tras) or 0.01% DMSO for 30 minutes before addition of 20 ng/mL NRG or vehicle in fresh media for 30 minutes. Cell lysates were collected with M-PER lysis buffer and loaded in each capillary at 0.1 or 0.05 μ g/ μ L protein concentration, and probed with pAKT (Ser473) antibody (diluted 1:50) or pErk1/2 (Thr202/Tyr204) antibody (diluted 1:50). For data analysis using Compass software, chemiluminescence was detected using a CCD camera and converted into electropherogram form. In both (A) and (B), the left panel shows the image view for detection of specific isoforms separated, immobilized and exposed for 480 seconds; the center panel shows the lane view analogous to a western blot; and the right panel shows the graph plot of chemiluminescence for each peak detected. Isoelectric point (pI) is shown on x axis (or y axis in the center panel) and chemiluminescence on y axis. Phosphorylated AKT and Erk1/2 were not detectable in untreated cardiomyocytes, whereas multiple peaks of phosphorylated AKT and Erk1/2 occurred after treatment with NRG. Activation of AKT and Erk1/2 was blocked completely by lapatinib pretreatment and greatly reduced by trastuzumab pretreatment (approximately 84% and 72% for phosphorylated AKT and phosphorylated Erk1/2, respectively).

Figure 14.

Assessment of phosphorylation and nuclear translocation of AKT and Erk1/2 upon activation of ErbB signaling by neuregulin 1 β (NRG) using the WesTM Simple Western system. Cells were cultured more than 14 days prior to testing. Cells were equilibrated in serum-free media for about 45 minutes prior to exposure to 100 ng/mL NRG for 30 minutes. Cell lysates were collected; the fraction of cytoplasmic and nuclear proteins was separated using the NE-PER Nuclear and Cytoplasmic Extraction kit. (A) Lysates of cytoplasmic and nuclear fractions were loaded in each capillary at 0.2 μ g/ μ L protein, and probed with the mixture of anti-phospho-AKT and anti-phospho-Erk1/2 (diluted 1:50), or the mixture of anti-AKT and anti-Erk1/2 (diluted 1:50) with a 5 second exposure. AKT or phospho-AKT was detected as a 62 KDa band, while Erk1/2 or phospho-Erk1/2 was detected as a 44 KDa band. The total AKT or Erk1/2 was detected by anti-AKT or anti-Erk1/2 antibodies in both cytoplasmic and nuclear extracts, but was not altered by NRG treatment. NRG treatment increased both phospho-AKT and phospho-Erk1/2 in both cytoplasmic and nuclear extracts. (B) Quantified phospho-AKT and phospho-Erk1/2 normalized to total AKT or Erk1/2 detected in both cytoplasmic and nuclear extracts. Upon treatment with NRG, phospho-AKT was increased by 558% and 547% in cytoplasmic and nuclear extracts, respectively; similarly, phospho-Erk1/2 was increased by 533% and 584% in cytoplasmic and nuclear extracts, respectively.

Figure 15.

Assessment of cytoplasmic translocation of FOXO3a upon activation via ErbB signaling pathway using the Wes™ Simple Western system and IN Cell Analyzer 2000 microscopic cell imaging. Cells were cultured more than 14 days prior to testing. Cells were equilibrated in serum-free media for about 45 minutes prior to exposure to 100 ng/mL NRG for 30 minutes. Cell lysates were collected; the fraction of cytoplasmic and nuclear proteins was separated using the NE-PER Nuclear and Cytoplasmic Extraction kit. (A) Lane view of a Wes run. Lysates of cytoplasmic and nuclear fractions were loaded in each capillary at 0.2 $\mu\text{g}/\mu\text{L}$, and probed with the pan-FOXO3a antibody diluted 1:50 or phospho-FOXO3a (Ser253) antibody diluted 1:50. Both FOXO3a and phospho-FOXO3a were detected at 100 KDa (arrow). A band at 37 KDa detected only in nuclear extracts by the phospho-FOXO3a antibody is likely due to non-specific binding as it is not altered by NRG treatment. (B) Quantified total-FOXO3a and phospho-FOXO3a chemiluminescence values in both cytoplasmic and nuclear extracts. Compared to 0.1% DMSO, treatment with NRG increased total FOXO3a in the cytoplasm by approximately 51% and decreased it in the nucleus by approximately 60%. NRG treatment increased phosphorylated FOXO3a in the cytoplasm by approximately 106%, but decreased it in the nucleus by approximately 50%. (C) Detection of FOXO3a translocation with immune-staining. Cells were cultured in serum-free media for 4 hours prior to exposure to 100 ng/mL NRG for 20 minutes, and incubated with anti-FOXO3a antibody at 1:100 dilutions for 1 hour at room temperature. (D) Frequency distribution of nuclear FOXO3a staining intensity shows a remarkable leftward shift, indicating a significant loss of FOXO3a expression after NRG treatment. Assessment of at least 2500 cells/group, 94% of the cells exhibit > 250 RFU intensity in the 0.1% DMSO control group, and drops to 49% in cells treated with NRG.

Figure 16.

Nuclear specific expression of CREB and activation after neuregulin-1 β (NRG) treatment as shown by WesTM Simple Western (A) and IN Cell Analyzer 2000 microscopic cell imaging (B). Cells were cultured more than 14 days prior to testing, and equilibrated to serum-free media for about 45 minutes before being exposed to 100 ng/mL NRG for 30 minutes. Upon completion of NRG treatment, cells were lysed and the fraction of cytoplasmic or nuclear proteins was extracted using the NE-PER Nuclear and Cytoplasmic Extraction kit for WesTM Simple Western analysis (A). A band at 53 KDa was detected by both the pan- and phospho-CREB antibodies in nuclear, but not in cytoplasmic extracts (arrow). (B) Total CREB in the nucleus was not altered by treatment with 100 ng/mL NRG, whereas NRG treatment increased phosphorylated CREB by approximately 160% compared to the 0.1% DMSO group. Cells were fixed and stained with anti-phospho-CREB and Sytox Red (nuclear stain) for immunofluorescence using the IN Cell Analyzer 2000 system for imaging (C). Cells were treated with 0.1% DMSO or 100 ng/mL NRG as described for panel A. Images were acquired using the IN Cell Analyzer 2000 instrument and image analysis was performed using the IN Cell Workstation. Nuclear staining for phospho-CREB was clearly increased after treatment with 100 ng/mL NRG as seen by the increase in pCREB imaged in the green channel. Note the lack of phospho-CREB in many nuclei that stain only in the red (Sytox Red) channel in the DMSO control image. Based on analyzing more than 3,400 cells per group, treatment with NRG increased the average nuclear intensity of phospho-CREB by approximately 94% compared to the 0.1% DMSO treated cells, similar to the Wes data.