Application of Human Induced Pluripotent Stem Cell Technology for Cardiovascular Regenerative Pharmacology

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Springer Nature
2022

Majid , Q , Orsolits , B , Pohjolainen , L , Kovacs , Z , Földes , G & Talman , V 2022 , Application of Human Induced Pluripotent Stem Cell Technology for Cardiovascular Regenerative Pharmacology . in A Nagy & K Turksen (eds) , Methods in Molecular Biology . Methods in Molecular Biology , vol. 2454 , Springer Nature , pp. 163-196 . https://doi.org/10.1007/7651_2021_369

http://hdl.handle.net/10138/355657
https://doi.org/10.1007/7651_2021_369

acceptedVersion

Application of human induced pluripotent stem cell technology for cardiovascular regenerative pharmacology

Running title: hiPSC technology for cardiovascular regenerative pharmacology

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Summary/ Abstract:

Cardiovascular diseases are one of the leading causes of mortality in the western world. Myocardial infarction is amongst the most prevalent and results in significant cell loss within the myocardium. Similarly, numerous drugs have been identified as having cardiotoxic side effects. The adult human heart is however unable to instigate an effective repair mechanism and regenerate the myocardium in response to such damage. This is in large part due to the withdrawal of cardiomyocytes (CMs) from the cell cycle. Thus, Identifying,

screening and developing agents that could enhance the proliferative capacity of CMs holds great potential in cardiac regeneration. Human induced pluripotent stem cells (hiPSCs) and their cardiovascular derivatives are excellent tools in the search for such agents. This chapter outlines state-of-the art techniques for the 2- dimensional differentiation and attainment of hiPSC-derived CMs and endothelial cells (ECs). Bioreactor systems and 3-dimensional spheroids derived from hiPSC-cardiovascular derivatives are explored as platforms for drug discovery before focusing on relevant assays that can be employed to assess cell proliferation and viability.

Keywords:

Human induced pluripotent stem cells (hiPSCs), cardiomyocytes, endothelial cells, scalable manufacturing of hiPSC derivatives, 3D spheroid cell culture, regenerative pharmacology, cardiotoxicity

1. Introduction:

Given the high prevalence and poor prognosis of heart failure (HF), the emergence of regenerative medicine has rendered the heart an obvious target for therapeutic intervention. To this end, myocardial infarction (MI) or exposure to cardiotoxic drugs results in damage to the myocardium however, the adult human heart lacks the regenerative capacity to respond effectively to this damage. As such, progression towards HF ensues. Novel regenerative therapies including pharmacological interventions that repair the myocardium are sought after ***(1-3)***. In contrast to organisms that are capable of undergoing cardiac regeneration, the adult myocardium does not activate pro-regenerative neonatal-like gene regulatory networks in cardiomyocytes (CMs) and endothelial cells (ECs) ***(4)***. As a result, these two cell types have become promising target cells for regenerative therapies ***(5)***. The major hurdle in generating new myocardium in the adult mammalian hearts is cell cycle withdrawal of cardiomyocytes and

strategies that promote generation of new CMs are urgently sought after. Upon myocardial infarction in zebrafish and neonatal rodents, endogenous cardiac regeneration and production of new CMs takes place via dedifferentiation and proliferation of existing CMs in areas adjacent to the injury ***(6-9)***. Transient reactivation of the CM cell cycle is therefore considered a promising regenerative therapeutic strategy ***(2, 3)***. Another central process in cardiac regeneration is the revascularization of the injured area, which in zebrafish precedes and is necessary for CM cell cycle activation ***(10)***. Promoting a transient angiogenic response at the site of injury thus exhibits potential as cardio-regenerative therapeutic strategy.

As adult human CMs and ECs are not readily obtainable from human tissue samples, cardiovascular derivatives of human induced pluripotent stem cells (hiPSCs) hold great potential in the investigation of these cell types using human cells. However, these hiPSC derivatives do not fully resemble their adult human counterparts. To this end, whilst the hiPSC-derived CMs (hiPSC-CMs) express all major cardiac ion channels and intact intracellular signalling cascades, they have uncoordinated calcium kinetics resulting in spontaneous beating and the generation of a negative force-frequency relationship ***(11-13).*** As such, one should consider these discrepancies between hiPSC-derived cardiac cells and their bona-fide, native counterparts when interpreting results. Despite their limitations however, cardiovascular derivatives of hiPSCs exhibit several advantages relative to traditional in vitro models (i.e., primary culture). These include the elimination of species differences and, by deriving patient specific hiPSC lines, aid the creation of precision-medicine based therapeutic strategies. Therefore, hiPSC-based models have quickly become the state-of-the-art in vitro models employed in cardiac research. In this chapter, we describe techniques to produce and analyse hiPSC- derived CMs and ECs for the purpose of regenerative pharmacology.

1.1. hiPSC-derived cardiovascular cell culture platforms:

The composition of the adult human myocardium has been the subject of long-

standing debate. The observation that the non-CM populations play an integral role in both the regeneration of the neonatal myocardium and the homeostasis of the adult myocardium has led some to postulate that these cells types, rather than the CMs, are the most abundant within the myocardium. Indeed, lineage tracing experiments coupled to an in-depth analysis of cell-specific markers had determined ECs to be the most prevalent cell type within the myocardium ***(14, 15)***. Furthermore, other cells of the vasculature including the pericytes have similarly been shown to occur profusely throughout the myocardium ***(16)***. By contrast, recent single cell transcriptomic studies of human cardiac tissue refute this abundance of vascular cells and instead reaffirms the initial dogma of CMs existing as the predominant cell type within the myocardium ***(17)***. The question of cellular composition extends beyond purely academic interest. In fact, recapturing the correct ratio of the cell types contained within the myocardium is a pre-requisite to successful cardiac tissue engineering solutions, advanced drug discovery (including toxicology testing and drug safety assessment) as well as accurate disease modelling approaches. The advent of human pluripotent stem cell biology and the development of robust cardiogenic differentiation strategies has been essential to the advancement of these fields.

Although hiPSC-CMs and hiPSC-derived ECs (hiPSC-ECs) are attainable, efficient differentiation strategies that utilise either growth factors or definable small-molecules are required that mediate and mimic early cardiovascular development. Such processes can be scaled from a laboratory setting to a high-throughput industrial setting that aims to enhance the total cell yield by optimising the process parameters. Although the growth factors and small-molecules responsible for differentiation are expensive, the production of these hiPSC-derivates at scale helps to reduce the overall cost which in turn reduces the drug discovery and development costs incurred by pharmaceutical companies. Here we detail an appropriate protocol for the 2D differentiation of hiPSC-CMs and -ECs as well as a 3D-based EC differentiation protocol utilising a small-scale micro-Matrix bioreactor which could provide an in vitro diagnostic tool and drug screening platform in the future.

2. Materials:

2.1. Two-dimensional (2D) monolayer maintenance of hiPSCs:

1. Preparation of hiPSC culture media: Supplement 500ml Essential 8™ media (E8, Thermo Fisher) with 1 x 10 ml aliquot of E8 supplement. Alternatively, prepare mTeSR™1 media by supplementing 400 ml basal mTeSR1 media (STEMCELL TECHNOLOGIES) with the supplied 1 x 100 ml aliquot of 5X mTeSR1 supplement. Prepared media is stored at 4°C for up to 1 month.

2. Preparation of Matrigel: Prepare stock Matrigel (Mg) solution by diluting 1-part growth-factor reduced Mg (Corning) in 1-part KnockOut DMEM media (Gibco) (Note 1). 1 ml aliquots are prepared of this stock Mg solution and are stored at -20°C for up to 6 months. Thaw aliquots at 4°C and dilute 1:25 in 25 ml of KnockOut DMEM media. This 1:50 Mg-DMEM solution can be stored at 4°C for up to 1 month (Note 2).

3. hiPSC dissociation solution: Utilise stock Versene solution (Gibco) or dilute 0.5 M UltraPure™ EDTA (Thermo Fisher) in PBS w/o Ca^{2+}, Mg^{2+} to form a 0.05 mM solution.

4. Cell counting: Acquire the number of dissociated hiPSCs using acridine orange solution (Vitascientific), a LUNA-FL Dual Fluorescence cell Counter (Logos Biosystems) and Luna™ cell counting slides (Logos Biosystems).

5. Rho-associated protein kinase (ROCK) inhibitor: Reconstitute Y-27632 (herein ROCK inhibitor, Tocris) in EmbryoMax ultrapure H_2O (Sigma Aldrich) to obtain a 10 mM stock solution that is aliquoted and stored at -20 °C for up to 6 months.

2.2. CM differentiation:

1. Cell culture media for CM differentiation: CM differentiation requires three bespoke medias described below. Once prepared, the media can remain at 4°C for up to 1 month. Composition of the relevant media is summarised in Table 1.

A) 'RB$^-$media': 500 ml RPMI-1640 (Sigma Aldrich) containing 1 x 10 ml aliquot of B-27™ supplement minus insulin (Gibco)

B) 'RB$^+$ media': 500 ml RPMI-1640 containing 1 x 10 ml aliquot of fully supplemented B-27™ supplement (Gibco)

C) Metabolic selection media: 500 ml RPMI-1640 media without glucose (Gibco) containing 1 x 10 ml aliquot of B-27™ supplement.

[Insert Table 1 here]

2. Preparation of factors for CM differentiation: Reconstitute the GSK3 inhibitor CHIR99021 (STEMCELL TECHNOLOGIES) and the small molecule WNT inhibitor, Wnt-C59 (Tocris) in an appropriate volume of PBS containing 0.1% BSA to generate a 20 mM stock solution. Prepare aliquots and store at -20°C for up to 6 months.

3. Preparation of cell dissociation solution (CDS): Prepare CDS solution: 40% cell dissociation buffer (Gibco), 40% basal RPMI-1640 media w/o B-27™ supplement and 20% of 0.05% Trypsin-EDTA (herein TE, Gibco).

4. Preparation of hiPSC-CM neutralisation media: Prepare non-supplemented RPMI-1640 media containing 10% heat inactivated foetal bovine serum (herein FBS, Gibco)

5. Preparation of hiPSC-CM resuspension media: Supplement RB$^+$ media with 10% FBS and 10 μm ROCK inhibitor.

6. Preparation of fibronectin: Dilute bovine fibronectin (Sigma Aldrich) 1:100 in sterile PBS. Diluted fibronectin can be stored at 4°C for up to 1 week.

2.3. Two-dimensional (2D) monolayer endothelial differentiation of hiPSCs:

1. Cell culture media for EC differentiation: EC differentiation requires three bespoke media described below. Once prepared, the media can remain at 4^{o}C for up to 1 month.

A) Fully supplemented mTeSR1.

B) Stemline ® II Hematopoietic Stem Cell Expansion Medium (herein Stemline II, Sigma Aldrich)

C) EGM^{TM}-2 Endothelial Cell Growth Medium-2 fully supplemented with the supplied BulletKit^{TM} aliquots (herein EGM2, Lonza).

2. Growth factors for EC differentiation: Recombinant Human/Mouse/Rat Activin A Protein (R&D biosystems), Recombinant Human BMP-4 Protein (R&D systems), Recombinant Human bFGF (146 aa) Protein (R&D biosystems) and Recombinant Human $VEGF_{165}$ (PeproTech) are reconstituted to 10 μM in 0.1% BSA-containing PBS.

3. Cell dissociation reagent: 0.05% Trypsin-EDTA (herein TE, Gibco)

4. Preparation of Collagen: Reconstitute type IV Collagen from human placenta (Sigma Aldrich) in 22 ml 1x HBBS (Gibco) and 3 ml glacial acetic acid. Sterile water for injection (Sigma Aldrich) is also required for subsequent washing of collagen IV-coated TCP.

5. Preparation of FACS buffer: PBS w/o Ca^{2+}, Mg^{2+} (Merck Millipore) containing 1% FBS.

6. Preparation of samples for FACS: A 70 μm cell strainer (Corning®) and sterile polystyrene round- bottom tubes (herein FACS tubes, Falcon) are required.

7. FACS antibodies: AlexaFlour 488-conjugated human CD31 (BD

Biosciences) and APC-conjugated NRP1 (BD Biosciences).

8. FACS analyser: Aria™ Fusion (BD Biosciences).

9. FACS collection media: 40% fully supplemented EGM2, 40% supplemented Stemline II and 20% FBS (containing at least 1% Antibiotic-Antimycotic solution, Thermo Fisher).

2.4. Scalability of hiPSC derivatives in bioreactors:

1. Bioreactor system: The micro-Matrix platform (Applikon Biotechnology) and associated liquid handling platform (Applikon Biotechnology)

2. Bioreactor related consumables: Clear-bottom 24-well polystyrene cassettes with pre-calibrated optical sensors for pH and dissolved oxygen (DO) detection and disposable micro-Matrix filter bars (Applikon Biotechnology)

3. hiPSC-cardiovascular derivatives and their associated culture materials: hiPSC-CMs (section 2.2) and hiPSC-ECs (section 2.3).

4. Bürker cell counting chamber (Thermo Fisher)

5. Apoptosis assay: Molecular Probes™ Vybrant™ FAM Caspase-3 and -7 Assay Kit (Invitrogen) and a suitable immunofluorescent microscope.

6. Accutrend Plus Analyser (Roche) for the measurement and quantification of lactate production and glucose consumption

2.5. Generation of 3D spheroids from hiPSCs or their derivatives:

1. Undifferentiated hiPSCs or differentiated hiPSC-CMs/ -ECs and associated cell culture materials (sections 2.1, 2.2 and 2.3, respectively).

2. GravityPLUS™ Hanging Drop Plate (InSphero)

3. Bürker cell counting chamber

4. 8- or 12-channel pipette (Eppendorf) and medium reservoir.

5. Reagents for staining and imaging of the resultant 3D spheroids: 1X PBS (Sigma Aldrich), 4% PFA (Sigma Aldrich), Triton X-100 (Sigma Aldrich), Normal Donkey serum (Abcam), 0.05 M TBS (Sigma Aldrich), Borosilicate coverglass, round, #1.5 (VWR), SecureSeal Imaging Spacer (Sigma Aldrich)

6. Primary antibodies: CD31-Alexa Fluor 488 (BioLegend) and anti-Fibroblast-specific protein-1 (FSP1, EMDMillipore)

7. Secondary antibodies raised against the species of the primary antibody and conjugated with a fluorescent fluorophore (Jackson ImmunoResearch)

8. Confocal microscope (Nikon A1R Confocal Microscope)

2.6. Two-dimensional (2D) monolayer co-culture of hiPSC-CMs and -ECs:

1. hiPSC-cardiovascular derivatives and their associated culture materials: hiPSC-CMs (section 2.2) and hiPSC-ECs (section 2.3).

2. Preparation of co-culture media: Prepare a sterile media comprised of 50% RB^+ media and 50% fully supplemented EGM2. Co-culture media can be stored at 4^oC for up to 1 month.

2.7. Acute and chronic models of doxorubicin-induced cardiomyocyte toxicity:

1. 96-well TCP plates: Regular TCP plates such as Costar® multiple well cell culture plates (Corning) are recommended for viability assays or epifluorescence imaging with a 10X objective. Alternatively, CellCarrier ultra

96/ 384 well TCP plate (Perkin Elmer) are recommended for higher magnification imaging.

2. Coating of TCP: In addition to Mg (section 2.1. step 1) and bovine fibronectin (section 2.2. step 6), EmbryoMax® 0.1% gelatin solution (Sigma Aldrich) can also be utilised to adhere hiPSC-CMs.

3. hiPSC-CMs and their associated cell culture material (section 2.2)

4. Doxorubicin hydrochloride (Tocris bioscience) is reconstituted to the desired concentration in DMSO and aliquoted for storage at -70^{o}C for up to 6 months

2.8. Cell cycle/proliferation assays:

1. Preparation of a 10 mM BrdU stock solution: Dissolve 3 mg of BrdU (Abcam) in 1 mL purified water. Dissolution may take several minutes. Filter the 10 mM stock solution through a 0.2 μm filter under sterile conditions (Note 3). Aliquot and freeze at -20°C for up to 1 month. Avoid freeze- thawing of BrdU aliquots.

2. Preparation of 4% PFA: Dissolve 4 g of PFA in 100 ml of PBS. Add 2 drops of 5 M NaOH to the PFA- PBS solution and dissolve in a pre-heated water bath situated in a fume hood. The prepared 4% PFA can be used for up to 1 week when stored at 4^{o}C however storage at -20^{o}C is advised for longer periods.

3. Preparation of 0.1 M sodium borate buffer: Dissolve 3.8 g sodium borate (MW = 381.4 g/mol) in 100 ml distilled water. Adjust pH to 8.5 with NaOH and store at RT.

4. Immunostaining reagents: Phosphate buffered saline (PBS), 0.1% Triton X-100 in PBS, 2 M HCl, 4% FBS in PBS, anti-BrdU antibody (Abcam), primary antibodies (e.g., anti-Ki67, anti-phospho-histone H3) and 4′,6-diamidino-2-

phenylindole (DAPI).

5. 96-well TCP plate, such as CellCarrier ultra 96-well TCP plate (Perkin Elmer) or Costar® 96-well plate (Corning).

6. High content imaging and analysis system (e.g., ImageXpress® Nano or ImageXpress® Micro Confocal and MetaXpress® analysis software, Molecular Devices).

2.9. Methyltiazoletetrazolium (MTT) cell viability assay:

1. Preparation of MTT stock solution: Dissolve 5.5 mg of MTT in 1 ml of Hank's Balanced Salt Solution (HBSS). Larger stocks can be prepared, aliquoted and stored at -20°C for several months. Do not freeze-thaw aliquots.

2. DMSO

3. 96-well flat-bottom plates

4. Plate reader with filters for 550 nm and 650 nm

3. Methods:

3.1. hiPSC culture:

1. Coating of tissue culture plastic for hiPSC culture: Add 100 µl/cm^2 1:50 Mg-DMEM solution per well of a sterile tissue culture plastic (TCP) plate (Nunc) and incubate for 30 min at 37^{o}C in a cell culture incubator.

2. Thawing of hiPSC vials: Remove hiPSC vial(s) from liquid nitrogen and place within a pre-heated (37^{o}C) water bath for 1 min (Note 4). Upon thawing,

the content of the vial is transferred into a falcon tube containing pre-heated E8/mTeSR1 media containing 10 μm ROCK inhibitor.

3. Centrifuge the resultant cell resuspension at 200 x g/ 4 min. Resuspend the pellet in 1 ml fully supplemented E8/mTeSR1 containing 10 μm ROCK inhibitor.

4. Counting thawed hiPSCs: Prepare a 9:1 solution of thawed hiPSC resuspension and acridine orange solution. Pipette the solution into the chamber of a LUNA™ Cell counting slide and place into the LUNA-FL Dual Fluorescence Cell Counter. The number of viable cells per 1 ml is attained and used to calculate the volume of hiPSC resuspension that is to be added per well (Note 5).

5. Plating of thawed hiPSCs onto Mg-coated TCP: Plate the thawed hiPSCs onto Mg-coated TCP at a density of 50,000 hiPSCs/cm^2 and incubate in E8/mTeSR1 media containing 10 μm ROCK inhibitor for 24 h

6. Maintenance of thawed hiPSCs: Following 24 h, replace the cell culture media with fresh E8/mTeSR1 media devoid of ROCK inhibitor. Cell culture media is replaced once every 24 h until a confluency of 60-70% is reached (Note 6).

7. Dissociation of hiPSCs: Wash the hiPSC maintenance wells with 250 μl/cm^2 sterile PBS and incubate with 250 μl/cm^2 Versene solution for 5 min at 37°C. Aspirate the Versene solution and allow the well to dry under the sterile environment of the laminar flow hood (Note 7). Add 1 ml of E8/mTeSR1 media to each maintenance well undergoing dissociation. Gently pipette the hiPSC resuspension to further facilitate dissociation of the hiPSCs (Note 8).

8. Viability and counting of dissociated hiPSCs: Refer to step 4.

9. Plating of dissociated hiPSCs: Replate the dissociated hiPSCs onto Mg-coated TCP at a density of 25,000 hiPSCs/cm^2 and incubate in E8/mTeSR1 media containing 10 μm ROCK inhibitor for 24 h at 37°C.

10. Culture and maintenance of subcultured hiPSCs: Refer to steps 7-9.

3.2. Cardiomyocyte differentiation of hiPSCs:

CMs are derived from the mesodermal germ layer during embryonic development. The earliest reports of beating CM-like cells obtained from pluripotent cells date back to the 1970s where embryoid bodies (EBs) derived from mouse embryonic carcinoma cells (mECCs) were allowed to spontaneously differentiate ***(19)***. CM differentiation protocols have since undergone iterative cycles of refinement including the transition towards 2D monolayers supported by feeder layers, growth factor-directed differentiations, and subsequently small molecule-defined differentiations of 2D monolayers. More recently, transdifferentiation approaches that avoid a pluripotent state and may therefore have a lower risk of tumorigenesis have been developed. The advances in CM differentiation are summarised in Table 2.

[Insert table 2 here]

Although directed differentiation strategies utilising EBs can be adapted to attain 70% pure hiPSC- CM populations ***(21)***, the generation of homogenous EBs is laborious and not readily reproducible. As such, alternative differentiation strategies have been sought. Indeed, the temporal modulation of the canonical WNT/ β-catenin signalling pathway has been identified as an essential regulator of cardiogenesis. To this end, WNT activation prior to gastrulation is necessary for mesodermal specification ***(36)*** however prolonged activity is detrimental to cardiogenesis in both zebrafish and mESCs ***(37)***. An understanding of this signalling cascade has allowed for the attainment of chemically defined 2D monolayer CM differentiation protocols ***(25, 26, 28)***. Whilst newer strategies are emerging, including transdifferentiation based approaches, these do not yet offer comparative robustness concerning CM yield and adaptability to several hPSC lines. The protocol outlined below has been utilised across several hiPSCs lines including those derived from diseased patients. Furthermore, the incorporation of a metabolic selection step, whereby a

substrate that is discriminately utilised by the CMs and non-CM populations is administered to the differentiating culture to expunge it of non-CMs, is readily scalable and conducive to GMP. This contrasts with other purification techniques, including fluorescence activated cell sorting (FACS) that is both laborious and not scalable.

1. Seeding dissociated hiPSCs onto Mg coated TCP: Seed 25,000 hPSCs/cm^2 onto Mg-coated TCP in E8 media containing 10 μm ROCK inhibitor for 24 hours (Notes 9-11).

2. Growth of hiPSCs on Mg coated TCP: Replace media every 24 h with fresh E8 until hiPSCs reach optimal confluency at which differentiation can be commenced (Note 12).

3. Mesodermal specification of the hiPSC monolayer: Dilute 20 mM CHIR99021 to 6 μM in RB^- media and apply to cells for 48 h (day 0 – day 2, Note 13). Replace media with fresh RB^- for a further 24 h (day 2 – day 3).

4. Cardiogenic specification of mesodermal germ layer: Dilute 20 mM Wnt-C59 to 2.5 μM in RB^- media and apply to cells for 48 h (day 3 – day 5).

5. Continuation of hiPSC-CM differentiation: Replace media every 48 hours with fresh RB^- media until day 11 of the differentiation protocol.

6. Metabolic selection of hiPSC-CMs: On day 11 of the differentiation protocol, replace RB^- media with 'metabolic selection media'. Supply fresh media once every 48 hours until day 15 at which point the media is replaced with RB^+ (Note 14).

7. Coating of tissue culture plastic for longer-term hiPSC-CM culture: Add 250 μl/cm^2 of 1:100 bovine fibronectin solution to sterile TCP and incubate for 1 h at 37^{o}C (Note 15)

8. Dissociation of purified hiPSC-CMs: On day 15 of the differentiation

protocol, wash the hiPSC-CM differentiation wells with sterile PBS. Add 250 µl/cm^2 pre-heated (37^{o}C) CDS solution to the differentiation wells and incubate for 5-8 min at 37^{o}C. Gently pipette the CDS solution within the well to further detach hiPSC-CMs from their culture wells. Dilute the CDS containing hiPSC-CMs in hiPSC-CM neutralisation media at a 1:4 ratio. Subsequently centrifuge the hiPSC-CM suspension at 200 x g/4 min. Discard the supernatant and resuspend the pellet in 1 ml hiPSC-CM resuspension media.

9. Viability and counting of dissociated hiPSC-CMs: Refer to section 3.1. step 4.

10. Replating of purified hiPSC-CMs: Replate the dissociated hiPSC-CMs onto bovine fibronectin- coated TCP at a density of 125,000 hiPSC-CM/cm^2 and incubate in hiPSC-CM resuspension media for 24 h at 37^{o}C

11. Longer-term maintenance of hiPSC-CMs: Replace media every 3 – 4 days with fresh RB$^+$ media (Note 16).

The hiPSC-CM differentiation protocol is schematically illustrated in Figure 1.

[Insert Figure 1 here]

3.3. hiPSC-EC differentiation:

1. This protocol is based on a previously established protocol for endothelial differentiation ***(38).***

2. Seeding dissociated hiPSCs onto Mg-coated TCP: Seed 6,500 hiPSC/cm^2 onto Mg-coated TCP in 260 µl/cm^2 mTeSR1 media containing 10 µm ROCK inhibitor for 24 h (day -2) (Note 17). Media is replaced with fresh mTeSR1 on day -1.

3. Commencement of EC differentiation: On day 0, replace media with 520

µl/cm^2 mTeSR1 containing 10 ng/ml of the four growth factors: Activin A, BMP4, bFGF, and $VEGF_{165}$.

4. Continuation of EC differentiation: On day 1, replace media with Stemline II media supplemented with 10 ng/ml BMP4, bFGF, and $VEGF_{165}$. This media composition is utilised on days 3, 5, 7, 9 and 11 of the differentiation protocol.

5. Fluorescence activated cell sorting (FACS): On day 12 of the EC differentiation protocol, select for the $CD31^+/NRP1^+$ (Neuropilin 1) double-positive population of hiPSC-ECs via FACS.

6. Collagen IV coating of TCP: Add 130 µl/cm^2 of collagen IV solution to TCP and incubate for 30 min at 37^{o}C (Note 18). Remove collagen IV solution and wash the TCP with sterile water for 1 hour at 37^{o}C (Notes 19, 20).

7. Replating of FACS sorted hiPSC-ECs: Replate the day 12 FACS sorted hiPSC-ECs onto collagen IV coated-TCP at a density of 2500 hiPSC-ECs/cm^2. Culture cells in media comprised 50% fully supplemented EGM2, and 50% supplemented Stemline II Medium for 48 h (day 12 – day 14).

8. Weaning of hiPSC-ECs from Stemline II. After 48 h (day 14), replace media with 75% fully supplemented EGM2 and 25% supplemented Stemline II media for a further 48 h (day 14 – day 16). On day 16, replace media with 100% fully supplemented EGM2 that is thereafter replaced every 48h.

The hiPSC-EC differentiation protocol is schematically illustrated in figure 2.

[Insert Figure 2 here]

3.3.1. FACS of day 12 hiPSC-ECs:

1. Remove media from each differentiation well and wash with 250 µl/cm^2 PBS (without Ca^{2+}, Mg^{2+}).

2. Dissociation of EC differentiation cultures: Add 60 µl/cm^2 of pre-heated

(37ºC) TE to each well and incubated for 5 min at 37°C (Note 21). Gently pipette TE-cell resuspension to further dissociate hiPSC-EC networks.

3. Dilute the TE-cellular resuspension 1:5 in Stemline II media containing 10% FBS in order to neutralise the TE.

4. Centrifuge the TE-cellular resuspension at 250 x g/5 min.

5. Resuspend the resultant pellet in 130 µl of FACS buffer solution and pass through a 70 µm strainer to eliminate clumps and debris.

6. Preparation of FACS samples:

(A). Unstained control: 10 µl cell suspension in 500 µl FACS buffer solution

(B). CD31 single positive control: 10 µl cell suspension in 100 µl FACS buffer solution

(C). NRP1 single positive control: 10 µl cell suspension in 100 µl FACS buffer solution

(D). CD31/NRP1 double-positive sample: 100 µl cell suspension.

7. Incubation with FACS antibodies: Incubate CD31 samples with Alexa Fluor 488-conjugated anti- human CD31 at a 1:20 dilution. NRP1 samples are incubated with APC-conjugated antihuman NRP1 at a 1:11 dilution. Vortex samples and incubate in the dark for 30 min at 4°C.

8. Wash samples with 5 ml FACS buffer and centrifuge at 250 x g/5 min to wash away excess antibodies that did not conjugate.

9. Resuspend the pellet in 0.5 ml FACS buffer and transfer the cell suspensions to sterile FACS tubes. Samples are stored in the dark at 4°C until sorting (Note 22).

10. Isolate the CD31/NRP1 double-positive population by passing the samples through a Aria^{TM} Fusion FACS analyser. The unstained control sample is used

as a negative control to eliminate sample auto-fluorescence. The single stained controls samples are employed to exclude non-specific antibody binding on the cell surface thus allowing for stringent gates to be set.

11. Collect the sorted CD31/NRP1 double-positive cells in a FACS tube containing 500 µl collection media.

12. Replate the FAC sorted day 12 CD31/NRP1 double-positive hiPSC-ECs onto Collagen IV-coated TCP at a density of 2500 hiPSC-ECs/cm^2 and culture in 50% EGM2 and 50% Stemline II for 48 h.

3.3.2. Passage of hiPSC-ECs

1. Prepare collagen IV-coated TCP (section 3.3 step 6).

2. Remove media from each EC maintenance well and wash with 250 µl/cm^2 PBS

3. Add 60 µl/cm^2 of pre-heated (37^{o}C) TE to each well and incubate for 2-3 min at 37°C.

4. Dilute the TE-cellular resuspension 1:5 in EGM2 media to neutralise the TE.

5. Centrifuge the TE-cellular resuspension at 250 x g/5 min.

6. Resuspend the resultant pellet in an appropriate volume of EGM2 media. hiPSC-ECs are subcultured at a 1:3 ratio with media replaced every 48 h until an 80% confluency is reached.

3.4. Scalability of hiPSC derivatives in bioreactors:

Drug discovery and development programmes require a large number of cells for investigation. Given the ability mentioned above to generate patient- and

disease-specific lines, hiPSCs and their derivates have become a mainstay in the pharmaceutical industry. Mini- and micro-bioreactors offer the possibility to culture hiPSCs and their cardiovascular derivates at scale in high-density cultures. Furthermore, the closed-system nature of a bioreactor is favourable to GMP as it allows for real-time detection and monitoring of culture parameters. This allows for deviations in culture conditions to be corrected immediately, thereby enhancing the reproducibility and robustness of the differentiation protocol. Optimisation of bioreactor conditions including the seeding density, volume of media, frequency of media exchange, RPM of the cell agitator/pendulum should be considered and optimised in order to establish an efficient and reproducible culture strategy that yields the high number of cells required for drug discovery and development programmes.

To this end, the scale-up of hiPSCs in large volume bioreactors needs to be automated for closed-loop continuous manufacturing and scale-up of cells ***(39)***. Non-invasive automated bioanalyser technologies can be utilised to continuously monitor critical parameters of hiPSC quality, such as lactate levels. The variables that influence the quality and quantity of cells produced in bioreactors include cell aggregate formation, inoculation methods by single cells or cell clumps, agitation rate, pH, average and deviation size of cell clumps, means of harvest and total expansion time ***(40, 41)***.

3.4.1. Preparation of hiPSC-ECs:

1. Dissociate hiPSC-ECs from their maintenance (section 3.3.1. step 2).

2. Count the number of dissociated hiPSC-ECs using a Bürker chamber

3. Dilute the cell suspension to 2.5×10^5 cells/ml

4. Transfer the 1 ml cell suspension into each well of the micro-matrix bioreactor cassette

3.4.2. The micro-Matrix bioreactor:

1. Calibrate the 24 pH sensors and the 24 DO sensors by scanning the QR codes.

2. Define setpoints for each individual well.

3. Define the process parameters for pH, DO and temperature sensors via individual PID controllers (Note 23). This provides a total of 72 PID control loops to be visualised. Values can be defined for each parameter in each well individually or as a group of wells.

4. Control loop parameters are subsequently defined.

5. Seed counted hiPSC-ECs into the micro-matrix bioreactor cassette (Note 24)

6. Connect the gas supplies (O_2, CO_2 and N_2) to the bioreactor

7. Fill the liquid addition feed bottle (LAFB) with EGM2 media.

8. Connect the LAFB to the compressed gas outlet (Note 25)

9. Confirm all parameters in the micro-Matrix software (Figure 3)

10. Place the assembled cassette into the micro-Matrix and connect the gas bars to the filter bars

11. Commence measurement and cell expansion activity of the bioreactor system.

[Insert Figure 3 here]

3.4.3. Drug treatment:

1. Unclamp the plate from the micro-Matrix bioreactor and transfer it to a laminar flow cabinet at which point the top plate can be removed from the

cassette.

2. Add the relevant drugs to the required wells at the desired concentrations

3. Reassemble the cassette and return to the bioreactor.

4. Reconvene measurements.

3.4.4. Completion of the drug treatment regime:

1. Select the 'stop experiment' function on the micro-Matrix software package.

2. Export data of all parameters as an excel file.

3. Unclamp the plate from the micro-Matrix bioreactor and transfer it to a laminar flow cabinet at which point the top plate can be removed from the cassette.

4. Transfer cells from the cassette into centrifuge tubes.

5. Centrifuge the collected hiPSC-ECs at 250 x g/ 5 min.

6. Resuspend the resultant pellet in EGM2 media.

3.4.5. Viability assessment of collected hiPSC-ECs:

1. Resuspend the harvested hiPSC-ECs to a concentration of 1 x 10^6 cells/ml in EGM2 media.

2. Add 30 µL of 30x FLICA working solution and 5 µL of 1 mM Hoechst 33342 dye directly to the 1 ml cell suspension and pipette gently.

3. Incubate samples for 60 min at 37^{o}C and 5 CO_2 and ensure samples are protected from light.

4. Centrifuge the samples at 250 x g/5 min.

5. Resuspend the pellet in 1 ml of wash buffer and re-centrifuge the sample at 250 x g/5 min.

6. Resuspend the pellet in EGM2 and transfer into a glass-bottom TCP plate suitable for immunofluorescent microscopy analysis (Note 26).

7. Use 350 nm excitation and blue wavelength emission for Hoechst 33342 visualisation and 488 nm excitation and green wavelength emission for FAM Caspase-3/7 visualisation.

3.5. Generation of 3D spheroids from hiPSCs or their derivatives:

3D cell culture circumvents many limitations associated with 2D cell culture including the laborious nature of TCP based media exchange. Furthermore, 3D cell culture systems can, to an extent, recapture the complex, multi-cellular in vivo environment. As such, this architecturally relevant model can better mimic physiological and pathophysiological conditions. The hanging drop system outlined below is an example of a versatile platform that can generate and observe 3D spheroids.

1. Prepare a medium reservoir containing 20 ml of 1X PBS

2. Place the humidifier pad (provided as part of the GravityPLUS^{TM} plate) into the PBS containing medium reservoir for ~5 min.

3. Transfer the soaked humidifier pad to the bottom of the GravityPLUS^{TM} plate

4. Dissociate the relevant cell type (section 3.1. step 7, section 3.2. step 8 and section 3.3.1. step 2) and count viable cells via the Bürker cell counting chamber

5. Prepare a cell suspension with 5000-10,000 cells/ 40 µl

6. Pipette this 40 µl cell suspension into each well of the GravityPLUS™ Plate

7. Transfer the plate into a humidified cell culture incubator (37°C and 5% CO_2)

8. After three days, the cells will have re-aggregated to form compact spheroids

9. Harvest the spheroids into either a 96 well plate for immunostaining or centrifuge them into the GravityTRAP™ plate for live cell imaging

3.5.1. Immunostaining of 3D spheroids:

1. Fix the spheroids in 4% PFA for 15 min at RT.

2. Wash the spheroid three times, each for 5 min, with 100 µl/well PBS

3. Simultaneously block the non-specific binding of the antibodies and permeabilise the cells by using 0.1 % Triton X-100, and 2% normal donkey serum in 0.05 M TBS for 60 min

4. Dilute the primary antibodies in 0,1 % Triton X-100 in 0.05 M TBS

5. Incubate samples with primary antibodies overnight at 4°C

6. Wash the spheroid three times, each for 5 min, with 0.05 M TBS

7. Dilute the secondary antibodies in 0.1% Triton X-100 in 0.05 M TBS

8. Incubate samples with secondary antibodies for 90 min at RT

9. Wash the spheroid three times, each for 5 min, with 0.05 M TBS

10. Wash the spheroid two times, each for 5 min, with 100 µl/well PBS

11. Cover the samples with Fluoromount G (with DAPI) and mount samples

#1.5 thick borosilicate coverslips with the use of SecureSeal Imaging Spacer

12. Image the spheroids with a confocal microscope and analyse z-stacks using ImageJ software (Figure 4)

[Insert figure 4 here]

3.6. Two-dimensional (2D) monolayer co-culture of hPSC-CMs and -ECs:

Given the relative abundance of ECs within the myocardium in addition to the complex role they play in CM function and homeostasis, drug development platforms should aim to utilise co-culture systems derived from these two cell types.

1. Coating of tissue culture plastic for hiPSC-CM and -EC co-culture: Add 250 µl/cm^2 of 1:100 bovine fibronectin solution per well of a sterile TCP plate and incubate for 1 hour at 37^{o}C.

2. Dissociation of hiPSC-CMs and hiPSC-ECs: Dissociate purified day 30 hiPSC-CMs and day 19 hiPSC-ECs (section 3.2. step 8 and section 3.3.1. step 2, respectively) (Note 27).

3. Seeding dissociated hiPSC-derived cells onto fibronectin-coated TCP: Seed hiPSC-CMs and hiPSC- ECs onto bovine fibronectin-coated TCP at a ratio of 3:1 in co-culture media containing 10 µm ROCK inhibitor for 24 h (Note 28).

4. Maintenance of hiPSC-CM and hiPSC-EC co-culture wells: Replace media with fresh co-culture media and replenish every 48 h.

3.7. Acute and chronic models of doxorubicin-induced cardiomyocyte toxicity:

Doxorubicin is a widely used chemotherapeutic agent with dose-dependent

cardiotoxic effects that limit its clinical use. It has been known to cause CM cell death through various mechanisms, including apoptosis, autophagy and necrosis ***(42)***. Most in vitro studies have evaluated the effects of short-term, high-dose doxorubicin treatments ***(43)***. Additionally, in vitro and in vivo animal models are confounded by interspecies differences, and therefore do not accurately predict toxicity in humans. To better mimic long-term doxorubicin dosing and delayed cardiotoxicity observed in clinical practice, we have developed a model for chronic low-concentration doxorubicin-induced toxicity in hiPSC-CMs ***(44)***. This model provides a platform for screening and characterisation of potential cardioprotective or cardio-regenerative agents. In this section, we present a protocol for the induction of both acute and chronic cardiotoxicity in hiPSC-CMs using doxorubicin (Figure 5).

[Insert Figure 5 here]

1. Preparation of doxorubicin stock solution: Weigh doxorubicin and reconstitute to the desired concentration in DMSO (Note 29).

2. Dissociate hiPSC-CMs (section 3.2. step 8) and seed either 15,000 - 20,000 cells/well on gelatin-coated 96-well TCP plates, or 10,000 - 15,000 cells/well on Mg-coated 96-well TCP plates in 100 µl/well of hiPSC-CM resuspension media (Day -7).

3. Replace media with fresh RB^+ media 48 h post-seeding (day -5).

4. Allow hiPSC-CMs a further 5 days to adhere and settle in the gelatin- or Mg-coated TCP. Replace media with fresh RB^+ media on day -2.

5. Commence doxorubicin treatment on day 0. Prepare doxorubicin-containing medium by diluting the thawed doxorubicin stock solution into RB^+ media at the desired concentration. Based on our concentration-response experiments, we use the following concentrations for hiPSC-CMs derived from the iPS(IMR90)-4 cell line (Wicell).

a. **Acute toxicity:** 1 μM doxorubicin in RB+ medium (Note 30).

b. **Chronic toxicity:** 100 nM doxorubicin in RB+ medium (Note 30).

6. Acute toxicity: End the treatment and commence the end-point assay of choice after 4, 8, 24 or 48 h, depending upon the desired readout.

7. Chronic toxicity: Replace the media with freshly prepared doxorubicin-containing RB^+ media every 3-4 days. End the experiment after 21 days of doxorubicin treatment and carry out the end-point assay of choice.

3.8. Cell cycle/proliferation assays:

Cell cycle activity and cell proliferation can be studied by assessing DNA synthesis and DNA content, cellular metabolism or proliferation-associated proteins. DNA synthesis is a strong and reliable sign of proliferating cells. Newly synthesised DNA can be detected by staining for nucleoside analogues (including 5-bromo-2'-deoxyuridine, BrdU or 5-Ethynyl-2′-deoxyuridine, EdU) that are incorporated into replicated DNA ***(45)***. Alternatively, whole DNA content can be investigated via high-content analysis of DAPI or Hoechst-stained nuclei to assess the different phases of the cell cycle ***(46)*** whilst co-staining with phospho-histone H3, Aurora kinase B or Ki-67 reaffirms the hit compound is enhancing cell proliferation rather than simply upregulating DNA synthesis (as is the case when CMs become polyploid or multi-nuclear).

3.8.1. 5-bromo-2'-deoxyuridine (BrdU) cell proliferation assay:

BrdU is a thymidine analogue that is incorporated into replicating DNA to detect proliferating cells. Prior to fixation, BrdU is added to the cell culture media allowing for its incorporation into the DNA of proliferating cells. Upon fixation, immunofluorescence for cell-specific markers is conducted alongside DNA hydrolysis, an essential step that allows anti-BrdU antibodies to access

DNA. The following BrdU labelling protocol is based on a protocol established by Abcam ***(47)***. In addition, specific proteins present at different phases of the cell cycle can be detected via immunofluorescence. To this end, Ki-67, a protein synonymous with cell proliferation, is present during the active phases of the cell cycle (G1, S, G2, and M). At the same time, phosphorylation at Ser10, Ser28, and Thr11 residues of histone H3 is tightly associated with chromosome condensation during mitosis ***(48. 49)***. These markers can be detected by immunofluorescence staining and can be combined with BrdU assay. Furthermore, this protocol can be combined with the assessment of DNA content. The following protocol is described for cells cultured on a 96-well TCP plate however this can be rescaled for other multi-well plate formats.

3.8.1.1. BrdU labelling:

1. Remove the 10 mM BrdU stock solution aliquot from -70°C storage and allow it to equilibrate to RT

for at least for one hour.

2. Dilute the 10 mM BrdU stock solution in the relevant cell culture medium to obtain a 10 μM BrdU labelling solution.

3. Remove the existing culture medium from the cells and replace with 10 μM labelling solution (Note 31).

4. Incubate the cells in the BrdU labelling solution for 1–24 h at 37°C in a CO_2 incubator (Note 32).

5. Remove the BrdU labelling solution and wash the cells twice quickly with PBS

6. Perform a secondary PBS washing step of three washes, each for 2 min.

3.8.1.2. Fixing and Immunostaining of BrdU labelled samples:

Perform steps 1-12 at RT.

1. Fix the cells with 75 µl/well of 4% paraformaldehyde (PFA) for 15 min.

2. Wash the cells three times, each for 5 min, with 100 µl/well PBS (Note 33)

3. Permeabilise the cells with 0.1% Triton X-100 in PBS for 10 min, 75 µl/well.

4. Wash the cells two times, each for 5 min, with 100 µl/well PBS

5. Hydrolyse DNA by incubating the cells with 50 µl/well 2 M HCl for 30 min (Note 34).

6. Remove HCl and neutralise with 50 µl/well 0.1 M sodium borate buffer pH 8.5 for 30 min

7. Wash the cells a further three times, each for 5 min, with 100 µl/well PBS

8. Block non-specific binding sites with 4% FBS in PBS for 45 - 60 min at RT, 50 µl/well.

9. Dilute the primary antibody in 4% FBS-containing PBS (Note 35).

10. Remove the blocking solution and add the primary antibodies (30 µl/well). Incubate for 60 min on a shaker (300 rpm). A secondary antibody control well is prepared by omitting the primary antibody solution and instead only administer 4% FBS-containing PBS.

11. Wash the cells three times, each for 5 min, with 100 µl/well PBS

12. Dilute the secondary antibody in 4% FBS-containing PBS. Add DAPI (or Hoechst) into the same dilution and ensure protection from light (Note 36)

13. Remove PBS and add 30 µl/well secondary antibody solution. Cover the plate with foil to protect from light and incubate for 45 min on a shaker (300 rpm) at RT.

14. Wash the cells three times, each for 5 min, with 100 µl/well PBS ensuring 100–200 µl of PBS remains within the well following the final wash.

15. Seal the plate with Parafilm and cover with foil to protect from light. The plate can be stored at 4°C until imaged.

3.8.1.3. Automated high-content image analysis of BrdU/ immunofluorescent stained plates:

The image analysis is to some extent dependent upon the high content imaging and analysis system utilised. However, similar analysis pipelines can be created also with other image analysis software. In this section, a general pipeline is described. Pipelines traditionally commence through the identification of either the cells or their nuclei (steps 1 and 2). The following steps may vary across different systems.

1. Generate a nuclei mask: Identify nuclei based on their DAPI intensity, round shape and size.

2. Generate a cytoplasmic mask: Detect the cytoplasm by measuring the intensity of cytoplasmic markers (e.g., a cytoskeletal protein) (Note 37).

3. Filter out non-cardiomyocytes: As the non-myocyte contaminants present in hiPSC-derived CM cultures at very low numbers are often more proliferative than CMs, it is essential to filter out non-CMs. This can be done by staining a CM-specific marker (e.g., cardiac troponin T or α-actinin) and excluding cells that are negative for this staining (Note 38).

4. Quantification of BrdU positive cells: Measure the average intensity of BrdU within the nuclei mask (or within the CM marker positive nuclei mask). Classify nuclei as BrdU positive and negative by setting a threshold with the help of the secondary antibody control (Figure 6). Calculate the percentage of BrdU positive present in the well relative to the other cells or the non-BrdU positive CMs.

5. Cell cycle analysis by quantification of DNA content: Measure the integrated/total intensity of DAPI within each nuclei mask (or within the CM marker positive nuclei mask). Create a histogram, where the cell count is plotted against their integrated intensity. Provided a sufficient number of cells can be analysed, two peaks indicative of different cell cycle phases should be visible (figure 7). The first peak represents cells at the G1 (growth) phase, whilst the second peak represents cells at G2/M (further growth/mitosis) phase. The difference between these two peaks is representative of the cells in S (DNA synthesis) phase. Set an intensity threshold between these two peaks, positioning at the start of the second peak. Classify cells above the threshold as G2/M cells. If this threshold is established using a control sample, it can be re-used to classify cells in all samples of that particular experiment.

[Insert figure 6 here] [Insert figure 7 here]

3.8.2. Methyltiazoletetrazolium (MTT) cell viability assay:

Increased cellular metabolism indicates increased proliferation of cells, while decreased metabolism is a sign of cell toxicity ***(51)***. Metabolic activity can be measured via the colorimetric MTT assay.

The Methyltiazoletetrazolium (MTT) assay is an indirect colourimetric assay that measures the enzymatic activity of mitochondria. NAD(P)H, produced by viable mitochondria engaged in oxidative phosphorylation, can reduce MTT into purple insoluble formazan crystals. Thus, increased purple colouring indicates an increased number of viable cells.

1. Add 10 µl of 5.5 mg/ml MTT stock solution into the 100 µl of cell culture media present in the wells, resulting in a final concentration of 0.5 mg/ml.

2. Incubate the plate for 2 - 4 h at 37 °C in a CO2 incubator (Note 39)

3. Aspirate the culture medium from the wells by pipetting carefully (Note 40)

4. Add 200 µl of DMSO to each well and carefully pipette carefully up and down to dissolve the generated crystals.

5. Measure the absorbance at wavelength 550 nm and subtract the absorbance at 650 nm as background.

6. Calculate the cell viability by direct comparison of the absorbance values of treated cells to those of control (untreated or vehicle-treated) cells.

4. Notes:

1. When working with Mg, it is essential that the manufacturer's instructions on cold pipette tips, tubes and KnockOut DMEM are followed, as Mg will rapidly form a viscous gel at room temperature (RT).
2. Alternative culture substrates, including recombinantly derived Laminin ***(50)*** or synthetic matrices like synthemax (synthetic peptide-acrylate) can be used and have shown promise in maintaining the pluripotency of hiPSCs ***(51)***.
3. Alternatively, this filtering step can occur once stock BrdU solution has been added to cell culture media to form the labelling solution.
4. To facilitate homogenous thawing of hiPSC vials, continually move the vial in a ‘figure of 8’ pattern once placed within the water bath.
5. Alternatively, cells may be counted in a Bürker chamber following Trypan blue staining
6. Overconfluent hiPSC cultures have been known to undergo spontaneous differentiation, thus impacting the efficiency of any future directed differentiation strategy. As such, care should be taken to ensure hiPSCs are passaged in a routine and timely manner (often once every 4 days however this is dependent mainly upon the hiPSC line) ensuring confluency does not exceed 80%.
7. To ensure efficient dissociation of hiPSCs, maintenance wells should be allowed to dry for approximately 1 min following aspiration of the Versene or EDTA solutions. Mechanical dissociation of the wells should be commenced immediately after adding the media to the well as short-term

culture with E8/mTeSR1 at RT results in the cells re-attaching to the TCP plate.

8. To prevent hiPSC colonies from completely disintegrating during dissociation, a process not entirely favourable to hiPSC culture, a wide orifice 1000 µl tip can be employed during the gentle pipetting of the hiPSC resuspension.
9. A lower concentration of Mg (1:800) can be utilised as a cost-saving measure, although this requires a longer incubation period (overnight at 37^{o}C).
10. The initial seeding density of different hiPSC lines should be tested if differentiation proves inefficient.
11. The passage of the hiPSCs should be recorded as differentiation efficiency has been noted to decrease as hiPSCs undergo continual passage. For the iPS(IMR90)-4 line this occurs between passages 50-60 and is exacerbated if hiPSCs become too confluent and commence spontaneous differentiation at the expense of pluripotency.
12. The duration between seeding and commencement of CM differentiation should be optimised for different hiPSCs lines as this affects the confluency at day 0 which can influence the CM differentiation.
13. Variable concentrations of CHIR99021 (6 - 10 µM) and the length of CHIR exposure (24 h – 48 h) should be tested if the CM differentiation is proving ineffective.
14. hiPSC-CMs typically commence beating between days 7 - 11 of the differentiation protocol however it can take up to day 15 for differentiations that are otherwise equally efficient to begin beating.
15. Gelatin and Mg coated TCP can also be used to seed hiPSC-CMs
16. hiPSC-CMs can be maintained in TCP wells for >1 year. During this period, it is advisable to dissociate and replate hiPSC-CMs onto freshly coated fibronectin TCP upon evidence of hiPSC-CMs detaching. Furthermore, the metabolic selection regime (section 3.2. step 6) can be repeated during this 1-year period if non-CM populations begin to emerge.
17. The initial seeding density of hiPSCs may need to be optimised for different hiPSC lines.

18. Collagen-IV should be protected from light.

19. Alternatively, 0.2 mg/ml collagen type I from rat tail solution (Merck) can also be utilised.

20. Sterile water should only be removed from TCP prior to seeding cells to prevent the collagen coating from drying out.

21. By day 12 of the EC differentiation protocol, hiPSC-ECs form dense networks that are not readily dissociated into single cells, a pre-requisite for FACs. As such, the incubation with TE (section 3.3.1. step 2) can be repeated several times to facilitate this.

22. As the processes of cell dissociation, antibody incubation and passage through the FACS analyser are relatively harsh a degree of cell death is to be expected. DNase I (from bovine pancreas, Sigma Aldrich) may be added to the FACS samples to degrade released DNA from apoptotic cells thus preventing cells from amalgamating with one another.

23. pH and DO can be controlled per well via the influx of four gasses and the addition of one liquid. Thus, experiments may be conducted under hypoxic conditions that mimic ischemia.

24. The square shape of the cassette wells allows for adequate mixing of the different factors. This can be enhanced by using a 25 mm orbital shaker operating at a 400 rpm to ensure the shear forces remain low

25. At the start of the experiment, utilise 90-100% DO and a continuous fixed perfusion rate of 20% media exchange per day. On the second day of the experiment, the rotation speed may be increased to 100 RPM.

26. Following a drug treatment samples may be fixed for later analysis by adding 40 µL of fixative (i.e., 4% paraformaldehyde, herein PFA). The Caspase-3/7 assay allows for the identification of live, dead and apoptotic cell populations.

27. As hiPSC-ECs are notoriously unstable and prone to transdifferentiation towards a fibroblast-like cell type, utilisation of early passage hiPSC-ECs is advised.

28. Unlike hiPSC-CMs, hiPSC-ECs have a strong proliferative capacity at day 19 thus are seeded at a lower initial density than the hiPSC-CMs.

29. To maintain the DMSO concentration at or below 0.1%, ensure the stock solution has a concentration at least 1,000X that of the final concentration. (A final doxorubicin concentration of 100 nM would thus require a >100 μM stock solution. In the chronic doxorubicin model, we routinely prepare a stock solution that is 10,000X the final concentration).

30. The sensitivity of hiPSC-CMs to doxorubicin may vary depending upon the hiPSC cell line from which the CMs are derived. It is therefore recommended to optimise the doxorubicin concentration in preliminary experiments. For acute toxicity, we recommend testing concentrations from between 300 nM to 3 μM, and for chronic toxicity concentrations from between 30 nM to 300 nM.

31. If the cell culture media of the cells does not need to be changed (ie. no pre-exposure or co-exposure to treatments), an appropriate volume of stock BrdU solution can be added directly into the existing cell culture medium ensuring a final BrdU concentration of 10 μM.

32. BrdU incubation times are dependent upon how quickly the cells proliferate. hiPSC-CMs may need up to 24 h whilst other, more rapidly proliferating cells (including hiPSCs) may require a shorter incubation period.

33. If the cells are not to be stained immediately following fixation then following this washing step, plates can be stored at 4ºC for several months provided a sufficient volume of PBS is added to each well, and the plate is sealed with parafilm and covered with foil.

34. The optimal HCl concentration may vary between 1 – 2.5 M and the optimal incubation time may vary between 10 min - 1 h. If using a shorter incubation time, incubating at 37°C may be more effective than at RT.

35. Dilution of primary antibodies is dependent upon the specific antibody used. In our hands, a 1:250 dilution of rat monoclonal anti-BrdU antibody (ab6326, Abcam) robustly identifies proliferating cells. To identify proliferating hiPSC-CMs, we suggest using anti-BrdU antibody alongside a cardiac-specific marker (e.g., cardiac troponin T, α-actinin). Robustness of BrdU staining can be assessed by co-staining for other proliferation markers (e.g., anti-Ki67, anti-phospho-histone H3) using antibodies produced in

different hosts. Make at least 300 µl of extra antibody-4% FBS PBS solution per 96-well plate to enable multi-channel pipetting.

36. Dilution of secondary antibodies depends on which fluorophore-conjugated antibodies are used. For example, Alexa Fluor-conjugated antibodies are usually used at a dilution of 1:200. A final concentration of 1 µg/ml DAPI is used to identify the nuclei. Alternative markers, including Hoechst may be used.

37. The detection of cytoplasm is challenging in confluent hiPSC-CM cultures. However, multiple platforms can expand or grow nuclei to create a mask covering both the nuclei and the perinuclear area. Such an approach can be used as an alternative means of identifying the cytoplasm provided all markers are expressed in that perinuclear area and no measurements requiring the whole cell, e.g., cell surface area or fibre analysis, is required. If only the nuclear area is of interest, the CM marker can be measured within the nuclei mask and non-myocytes can be filtered out based on the absence of the marker in within the nuclear mask.

38. The analysis pipeline used to quantify to BrdU can also be applied to detect other proliferation markers (e.g., Ki67, phospho-histone H3).

39. When performing this assay with a new cell line, follow the formation of formazan crystals by periodic observation of the plate under a light microscope. Once blue crystals begin to emerge, step 3 can be enacted.

40. Ensure care is taken to not pipette and discard the formazan crystals from the wells as this will impact the measurements.

Acknowledgements:

The authors thank Ms S. Tuuli Karhu for the expert advice on the doxorubicin model. The research was funded by the Hungarian National Research, Development and Innovation Fund (NKFIA; NVKP-16-1- 2016-0017, ‘National Heart Program’ and 128444), the Medical Research Council (MR/R025002/1), Academy of Finland (grants 321564, 328909), the Finnish Foundation for Cardiovascular Research, Sigrid Jusélius Foundation, University of Helsinki and Drug Research Doctoral Programme.

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Figure Legends:

Figure 1. A schematic illustration of the multiple steps involved in the cardiomyocyte differentiations of hiPSCs.

Figure 2. A schematic illustration of the multiple steps involved in the endothelial differentiation of hiPSCs.

Figure 3. A representative screen capture of the micro-Matrix software running with hiPSC-ECs. Measured data is displayed in tables and line graphs. One well

is selected at a time and the parameters of this well are displayed.

Figure 4. Representative 3D spheroid culture derived from hiPSC-ECs. Green (CD31), Blue (Hoechst).

Figure 5. Human induced pluripotent stem cell-derived cardiomyocyte (hiPSC-CM) based models of acute and chronic doxorubicin-induced cardiotoxicity. A, Timeline of acute and chronic doxorubicin (DOX) toxicity experiment. B, Concentration-dependent toxicity of doxorubicin in hiPSC-CMs. Toxicity of DOX was determined using the MTT assay after 2, 7, 14 and 21 days of DOX treatment. The data in B have been published previously (Karhu et al. 2020) and are expressed as percentage of control (mean ± SEM; n = 4). ***P < 0.001 vs. DMSO; **P < 0.01 vs. DMSO; *P < 0.05 vs. DMSO.

Figure 6. Identification of proliferating cardiomyocytes using BrdU staining. Nuclei are identified based on DAPI staining. Cardiomyocyte marker, cardiac troponin T (cTnT), staining is used to filter out nuclei of non-cardiomyocytes. Of cTnT-positive nuclei BrdU staining intensity is measured and nuclei are classified as BrdU positive or negative. In the example, identification of BrdU positive nuclei is done with MetaXpress software (Molecular Devices).

Figure 7. Cell cycle analysis based on DNA content. Cardiomyocytes are stained with DAPI and intensity of the staining is measured in each nucleus. In the histogram, integrated DAPI intensity is plotted against cell count. Thresholds for the different phases of the cell cycle are set manually. In this example, MetaXpress software (Molecular Devices) is used for DNA content classification.

Table 1. Summary of the different media compositions required for the cardiomyocyte differentiation of hiPSCs.

Table 2. A summary of the various differentiation strategies that have thus far been employed in the generation of CMs from pluripotent cells.

Media	Component	Supplier
RB minus (RB^-)	RPMI-1640 media	Sigma Aldrich
	B-27™ supplement, minus insulin	Gibco
RB plus (RB^+)	RPMI-1640 media	Sigma Aldrich
	B-27™ supplement	Gibco
Metabolic selection media	RPMI-1640 media, No Glucose	Gibco
	B-27™ supplement	Gibco

Table 1

Differentiation strategy	Cell type	Culture conditions	Factors	Comments		Reference
Spontaneous EB	Mouse embryonic carcinoma cells (mECCs)	EBs	N/A	Inefficient differentiation.	The process of attaining EBs is laborious and requires specialised culture vessels to aid the attainment of homogenous EBs.	***(18)***
	Mouse embryonic stem cells (mESCs)	EBs	N/A			***(19)***
	Human embryonic stem cells (hESCs)	EBs	N/A			***(20)***
Directed EB	hESC	EBs	DNA-hypomethylating agent **5-aza-2′-deoxycytidine**	70% pure populations of hPSC-CMs attained following Percoll density centrifugation		***(21)***
2D monolayer driven by co-culture	hESC	2D monolayer + visceral endodermal cell feeder layer	N/A	35 ± 10% of the well contained areas of contracting CM-like cells		***(22)***
Directed 2D monolayer	hESC (H7)	2D monolayer plated on Matrigel-coated TCP	Sequential administration of 100 ng/ml **Activin A** (24 h) followed by 10 ng/ml **BMP4** for 4 days in RPMI media supplemented with B-27™ (RB)	Beating CM-like cells appeared on day 12 of differentiation. Could attain 71–95% pure populations of hESC-CMs following Percoll gradient centrifugation Protocol used to obtain $1x10^9$ hPSC-CMs	Initial culture of hESCs relied on mouse embryonic fibroblast conditioned medium (MEF-CM) containing bFGF Protocol does not readily work on other hPSC lines	***(23)***
	hESC (H1 and H7)	2D monolayer plated on Matrigel-coated TCP	Sequential administration of 100ng/ml **Activin A**, 100 ng/ml WNT ligand (**Wnt3a**) followed by 10	Modulation of the Wnt/β-catenin signalling pathway through the addition of Wnt3a enhanced CM differentiation to a	hESC cultures were maintained in MEF-CM supplemented with bFGF	***(24)***

			ng/ml **BMP4** for 4 days in RB	pre-purification yield of 27%	The protocol was not always reproducible with some differentiation yielding <10% hPSC-CMs	
	hESCs (H9, H13 & H14) and hiPSCs (6-9-9, 19-9-11, IMR90-4)	2D monolayer plated on Matrigel or Synthemax coated TCP (Also conducted in EB format)	Sequential administration of 100ng/ml **Activin A**, 12 μM GSK3β inhibitor **CHIR99021** (CHIR) followed by 5 ng/ml **BMP4** in RPMI supplemented with B-27 without insulin	Generate highly pure populations of hPSC-CMs (up to 90%) without the need for purification		***(25, 26)***
			12 μM **CHIR** administration followed by downregulation of β-catenin either via targeted **shRNA** or **IWPs** (inhibitors of WNT ligand production)	Produces a high yield (0.8–1.3 million hPSC-CMs/ cm^2) hPSC-CMs are 80-98% without purification	Used to generate $5x10^8$ hiPSC-CMs for the recellularisation of decellularised human hearts ***(27)***	
	hESCs (H7 and H9) and 11 'homemade' hiPSCs	2D monolayer plated on Matrigel or Synthemax II-SC coated TCP	6 μM **CHIR** in chemically defined media (CDM3) followed by 2 μM **Wnt-C59** (WNT inhibitor)	First chemically defined CM differentiation protocol. Of the factors contained in B-27, identified 500 μg/mL **human albumin** and 213 μg/mL **L-ascorbic acid 2-phosphate** dissolved in basal **RPMI-1640** to be sufficient to drive CM differentiation	Generates a heterogenous population of ventricular, atrial and sinoatrial-like hPSC-CMs 10-day (d10-d20) purification via metabolic selection using RPMI w/o glucose yields ~95% pure hPSC-CM populations	***(28)***

Transdifferentiation of a 2D monolayer	Mouse fibroblasts	2D monolayer	Delivery of three transcription factors: **Gata4**, **Mef2c**, and **Tbx5**	By circumventing the pluripotent state, this transdifferentiation approach instead proceeds via a progenitor cell stage that may be less tumorigenic		***(29)***
Chemically induced transdifferentiation of a 2D monolayer	Human fibroblasts	2D monolayer	9 compounds including CHIR in media containing VEGF, Activin A and BMP4	Chemically defined PSCs (ciCMs)		***(30)***
Chemically defined transdifferentiation of a 2D monolayer	Mouse fibroblasts	2D monolayer	7 compounds: **CHIR, RepSox, Forskolin, VPA, Parnate, TTNPB** and **Rolipramd**	In situ generation of ciCMs from mouse fibroblasts post-MI albeit at a low yield of ~1%	Resulted in a reduction in scar size and improvement in cardiac function	***(31)***
Chemically defined co- and trans-differentiation of cardiogenic cells	hPSCs	2D monolayer		Co- and tri-cultures yielding hPSC-derived CMs, ECs and smooth muscle cells (SMCs)		***(32, 33)***
Differentiation strategies that improve the quality of attained hPSC-CMs	hiPSCs derived from cardiac fibroblasts Seeded on Matrigel coated TCP	2D monolayer	Administration of 100ng/ml Activin A in RB- media for 24 h followed by 10 ng/mL BMP4 and 7.5 ng/mL bFGF up to day 5 of the differentiation protocol.	Deriving hiPSCs from cardiac fibroblasts rather than human umbilical cord blood mononuclear cells or from human dermal fibroblasts yielded hiPSC-CMs that displayed superior calcium handling kinetics		***(34)***
Directed EB differentiation for chamber specific CMs	hPSCs	EB	Activin A, BMP4 and variable concentrations of retinoic acid	Retinoic acid was required for the attainment of atrial hPSC-CMs		***(35)***

Table 2 (above)

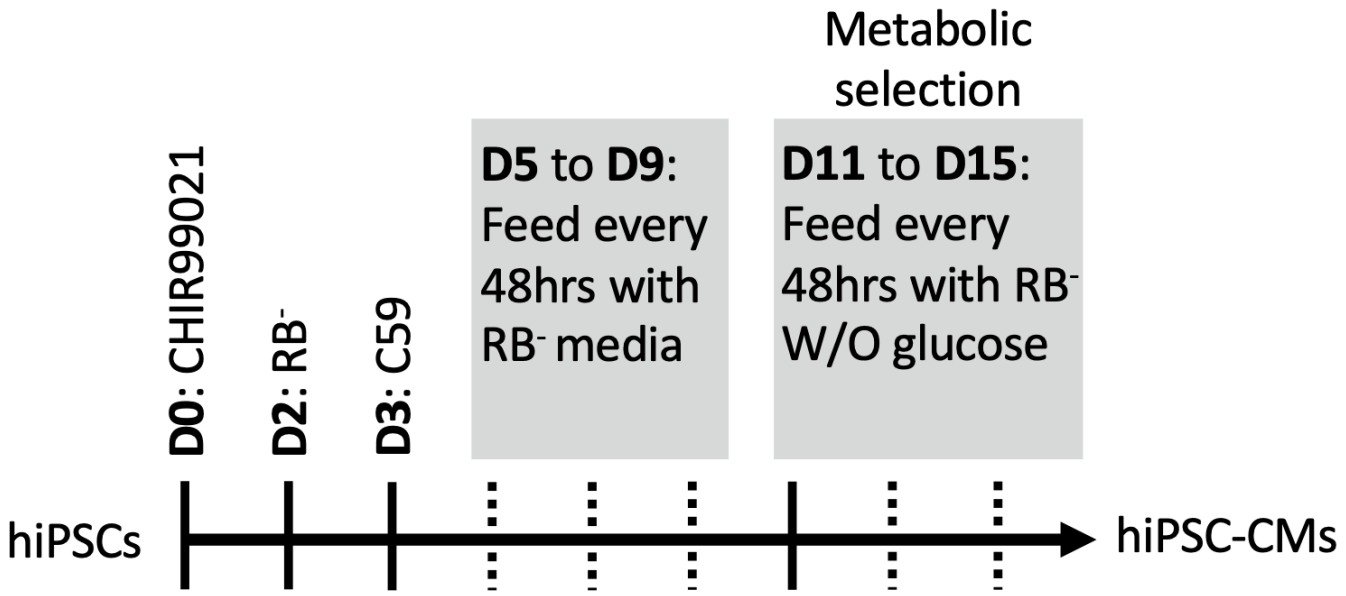

Figure 1

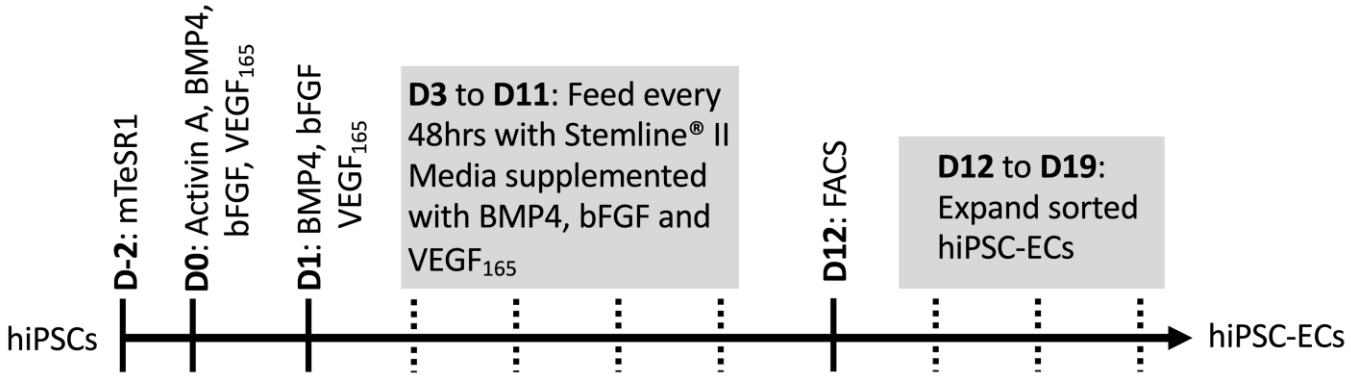

Figure 2

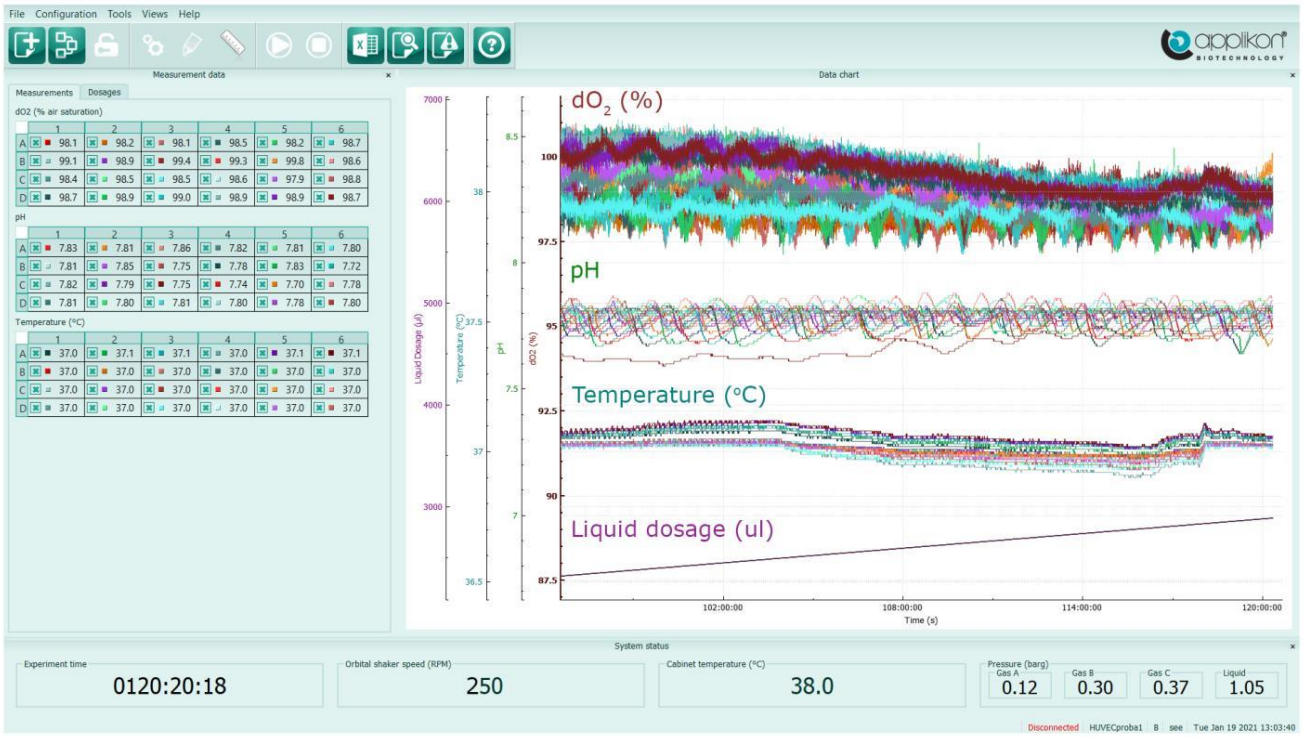

Figure 3

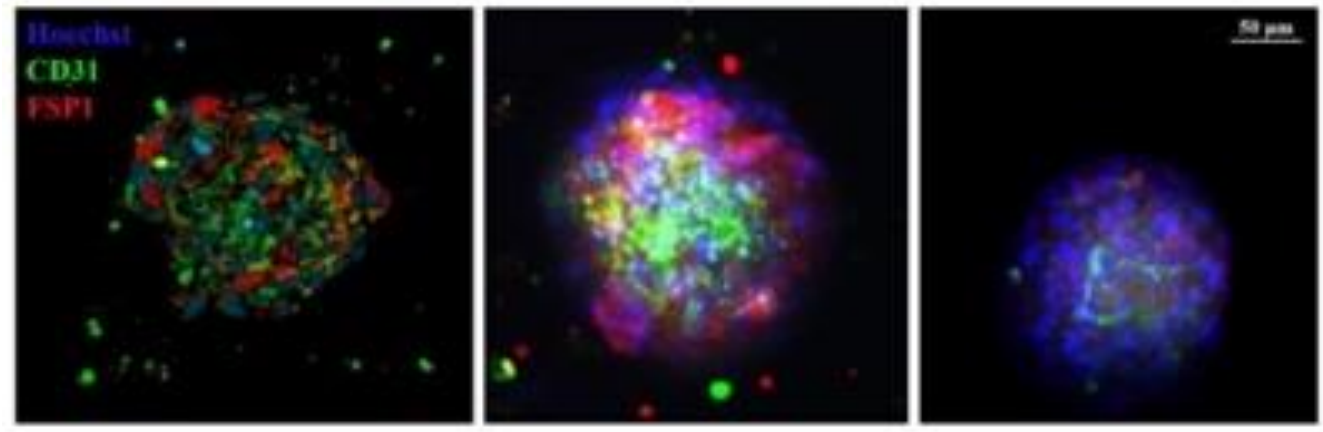

Figure 4

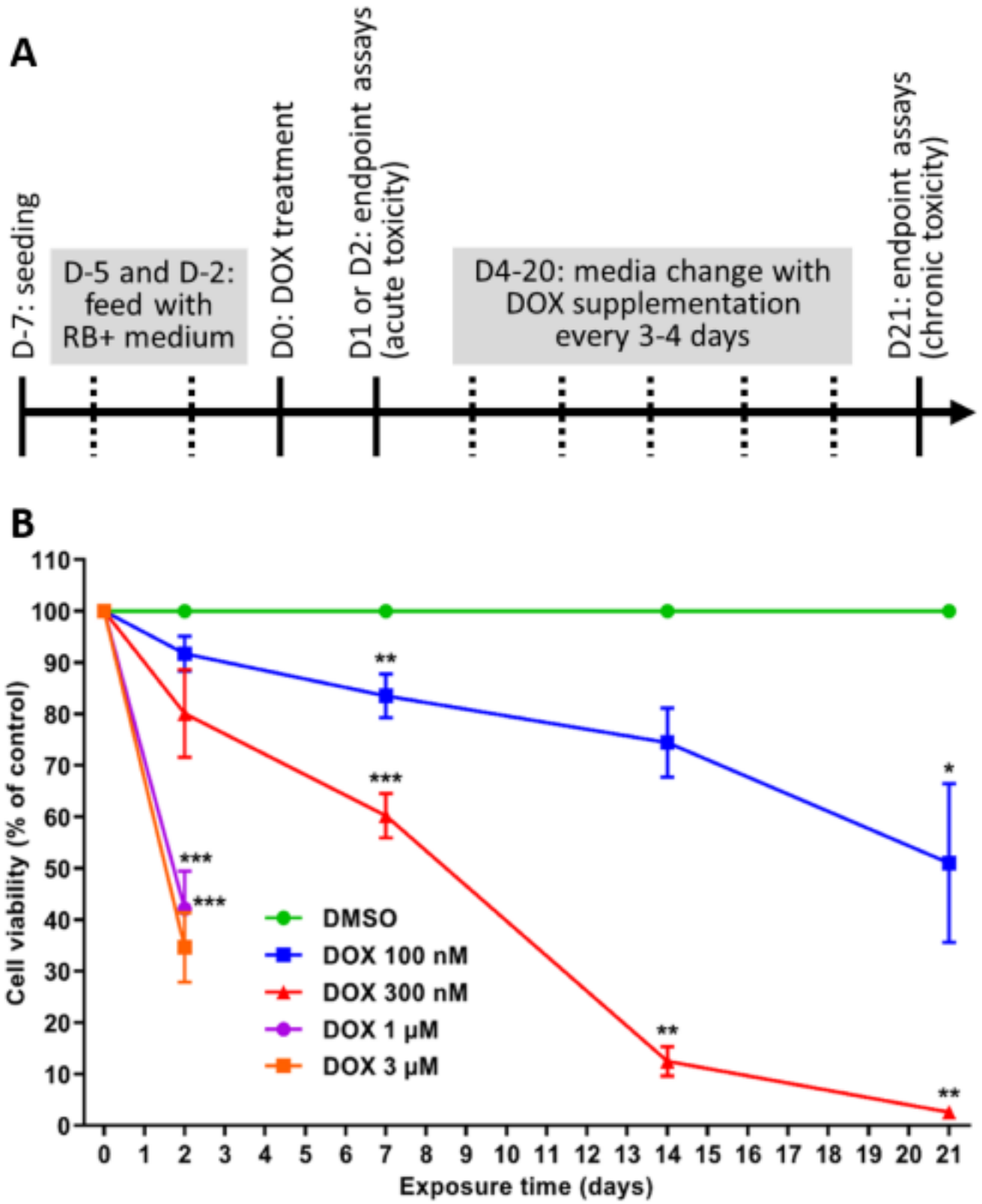
A
D-7: seeding
D-5 and D-2: feed with RB+ medium
D0: DOX treatment
D1 or D2: endpoint assays (acute toxicity)
D4-20: media change with DOX supplementation every 3-4 days
D21: endpoint assays (chronic toxicity)
B
Cell viability (% of control)
Exposure time (days)
DMSO
DOX 100 nM
DOX 300 nM
DOX 1 µM
DOX 3 µM

Figure 5

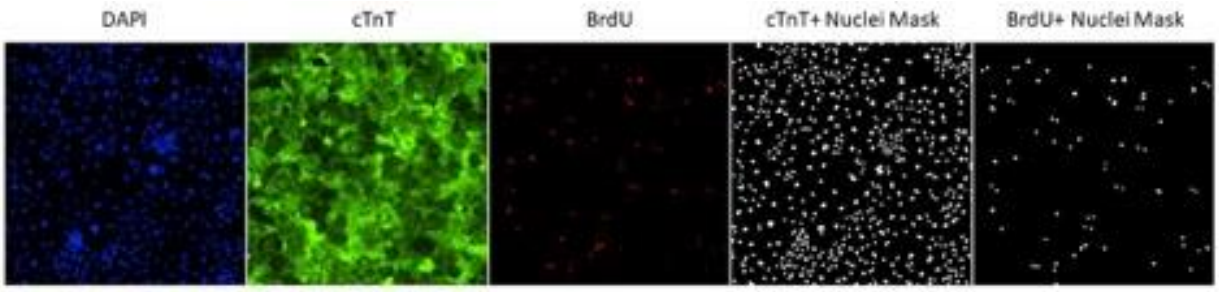

Figure 6

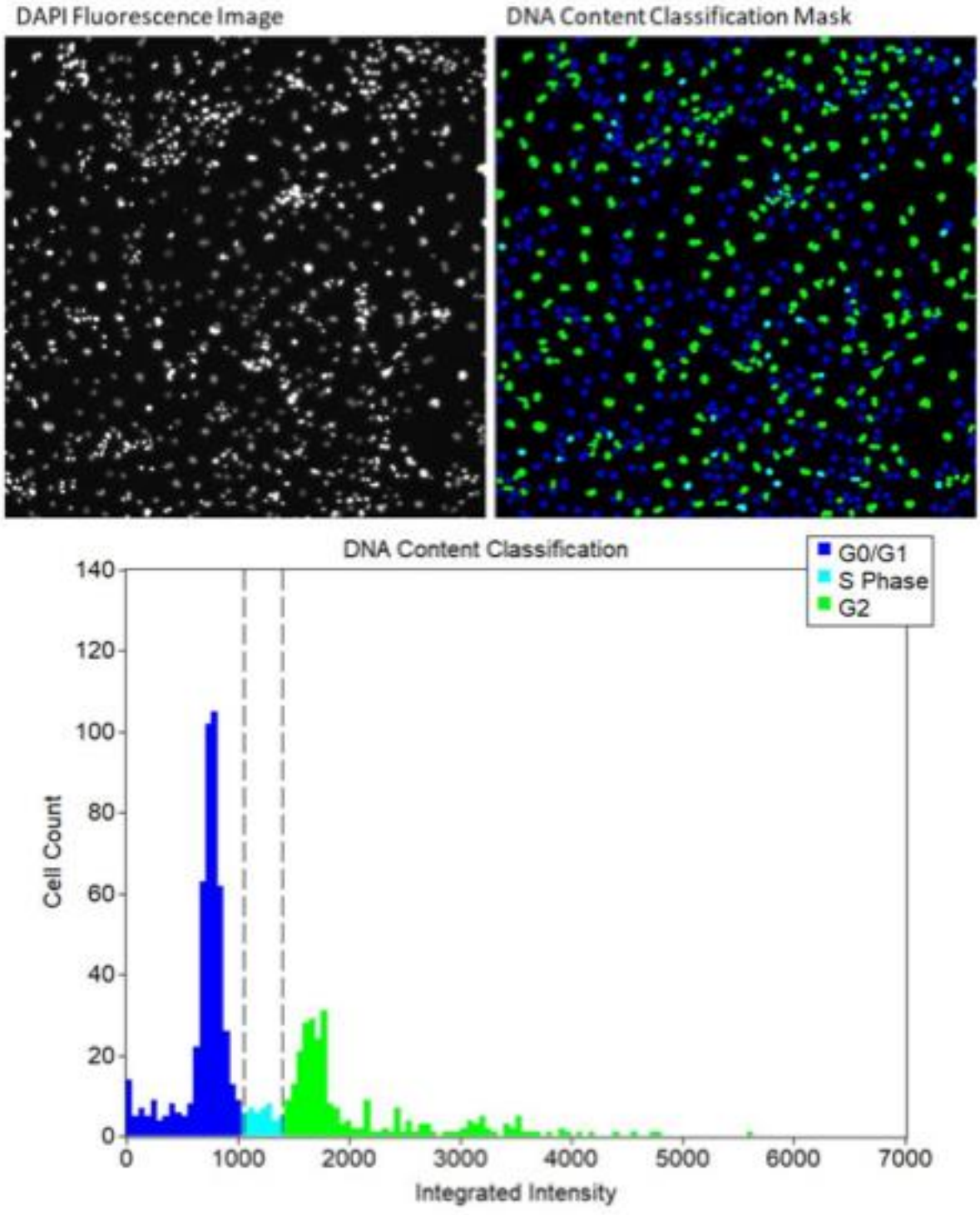
DAPI Fluorescence Image
DNA Content Classification Mask
DNA Content Classification
G0/G1
S Phase
G2
Cell Count
0
20
40
60
80
100
120
140
0
1000
2000
3000
4000
5000
6000
7000
Integrated Intensity

Figure 7