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Generation, High-Throughput Screening, and Biobanking of Human-Induced Pluripotent Stem Cell-Derived Cardiac Spheroids

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Abstract

Human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) are of paramount importance for human cardiac disease modeling and therapeutics. We recently published a cost-effective strategy for the massive expansion of hiPSC-CMs in two dimensions (2D). Two major limitations are cell immaturity and a lack of three-dimensional (3D) arrangement and scalability in high-throughput screening (HTS) platforms. To overcome these limitations, the expanded cardiomyocytes form an ideal cell source for the generation of 3D cardiac cell culture and tissue engineering techniques. The latter holds great potential in the cardiovascular field, providing more advanced and physiologically relevant HTS. Here, we describe an HTS-compatible workflow with easy scalability for the generation, maintenance, and optical analysis of cardiac spheroids (CSs) in a 96-well-format. These small CSs are essential to fill the gap present in current *in vitro* disease models and/or generation for 3D tissue engineering platforms. The CSs present a highly structured morphology, size, and cellular composition. Furthermore, hiPSC-CMs cultured as CSs display increased maturation and several functional features of the human heart, such as spontaneous calcium handling and contractile activity. By automatization of the complete workflow, from the generation of CSs to functional analysis, we increase intra- and inter-batch reproducibility as demonstrated by high-throughput (HT) imaging and calcium handling analysis. The described protocol allows modeling of cardiac diseases and assessing drug/therapeutic effects at the single-cell level within a complex 3D cell environment in a fully automated HTS workflow. In addition, the study describes a straightforward procedure for long-term preservation and biobanking of whole-spheroids, thereby providing researchers the opportunity to create next-generation functional tissue storage. HTS combined with long-term storage will substantially

contribute to translational research in a wide range of areas, including drug discovery and testing, regenerative medicine, and the development of personalized therapies.

Introduction

The discovery of human-induced pluripotent stem cells (hiPSCs) offered unprecedented opportunities to study human development and disease at the cellular level. Over the past decade, using developmental lessons, various protocols have been established to ensure the efficient differentiation of hiPSCs into cardiomyocytes (CMs)[1,2,3,4]. hiPSC-derived cardiomyocytes (hiPSC-CMs) can serve as a resource for modeling genetically inheritable cardiovascular diseases (CVDs), testing cardiac safety for new drugs, and cardiac regenerative strategies[5,6,7,8]. Despite the directed cardiac differentiation of hiPSCs, indefinite CM numbers remain a challenge in the cardiac field, since matured hiPSC-CMs generally are non-proliferative, and primary human cells are not available in high quantities.

Recently, we described that concomitant Wnt signaling activation with low cell-density culture resulted in a massive proliferative response (up to 250-fold) of hiPSC-CMs[9,10]. This cost-effective strategy for the massive expansion of hiPSC-CMs *via* serial passaging in culture flask format facilitates the standardization and quality control of large numbers of functional hiPSC-CMs. Additionally, to keep up with the demand for large batches of hiPSC-CMs from various donors, the biobanking of hiPSC-CMs has been described[10]. However, cardiomyocyte monolayers seeded in these standard culture dishes are not representative of the complex 3D structure present in the heart. Moreover, the immaturity of hiPSC-CMs has remained an obstacle, thus falling short in mimicking the biological and physiological phenotype of the *in vivo* cardiovascular environment.

Novel 3D *in vitro* models have been developed where hiPSC-CMs show closer physiological behavior such as self-organization[11,12], extracellular matrix (ECM) remodeling[13], enhanced maturation[14,15,16], and synchronized contraction[17,18,19]. 3D models have been utilized for drug discovery, drug cardiotoxicity testing, disease modeling, regenerative therapies, and even the first clinical trials[20,21,22,23,24]. One of the most used models is the fibrin-based engineered heart tissue (EHT), which exhibits a tissue-like arrangement and cardiac contractility[13,17,25]. Previously, we showed that EHTs generated from expanded hiPSC-CMs displayed comparable contractility to those from unexpanded hiPSC-CMs, demonstrating uncompromised cellular functionality after expansion[9]. Nevertheless, even though the generation of EHTs from hiPSC-CMs has been well established, further developments are anticipated regarding the establishment of an HT assessment platform. Here, the rapid generation of large numbers of self-aggregating cardiac spheroids (CSs) in 96-well format allows an improvement in 3D conditions for high-throughput screening (HTS) purposes.

Overall, the advantage of CSs as 3D cell culture is their high reproducibility and scalability. In particular, CSs combined with robotic sample handling can standardize and automate CS culture, drug treatment, and high-content analysis[20]. Here, we describe optimized protocols to generate high-purity and high-quality CSs, which can be efficiently cryopreserved and screened for cardiac function by performing Ca^{2+} transient measurements using an optical

calcium acquisition and analysis system. This model provides a simple yet powerful tool to perform high-throughput screens on hundreds to thousands of spheroids[17,18].

Protocol

NOTE: hiPSC-CMs used in this study were generated according to previously described hiPSC culturing and CM differentiation protocols[26,27]. Optionally, the hiPSC-CMs can be expanded and cryopreserved as recently published before starting the CS protocol (section 4)[10].

1. Preparation of cell culture media, solutions, and aliquots

1. Prepare basal medium
    1. Equilibrate penicillin-streptomycin and the medium (RPMI 1640) to room temperature (RT) and ensure it has thawed completely. Mix 500 mL of the medium and 5 mL of pen/strep. Store at 4 °C for up to 8 weeks; equilibrate to 37 °C before use.
2. Prepare RPMI + B27
    1. Equilibrate the B27 supplement and the basal medium to RT. Ensure to thaw the supplement completely. Mix 490 mL of the basal medium and 10 mL of the 50x B27 supplement. Store at 4 °C for up to 2 weeks; equilibrate to 37 °C before use.
3. Prepare hiPSC-CM re-plating media
    1. Add Rho-associated, coiled-coil containing protein kinase (ROCK) inhibitor (2 µM final concentration) and 10% knockout serum replacement (KSR) to RPMI + B27 media. Add ROCK inhibitor directly to the RM media as needed. Do not store culture media once supplemented.
4. Prepare CM thawing media
    1. Add a 1:100 concentration of cell survival supplement (e.g., Revitacell) and 20% KSR to RPMI + B27 media and equilibrate to 37 °C before use.
5. Prepare maturation supplement
    1. The previously described maturation medium formula[28] consists of: 3 mM glucose, 10 mM L-lactate, 5 mg/mL vitamin B12, 0.82 mM biotin, 5 mM creatine monohydrate, 2 mM taurine, 2 mM L-carnitine, 0.5 mM ascorbic acid, 1x NEAA, 0.5% (w/v) albumax, 1x B27, and 1% KOSR. To prepare one full bottle (500 mL) of maturation supplement, remove 65 mL from a bottle of DMEM without glucose and supplement with 2.7 g of glucose, 5.6 g of L-lactate, 0.025 mg of vitamin B12, 1 mg of Biotin, 3.73 g of creatine monohydrate, 1.25 g of taurine, 1.975 g of L-carnitine, 0.7125 g of ascorbic acid, 50 mL of NEAA, 12.5 g of albumax, and 5 mL of penicillin-streptomycin.
    2. Filter through a sterile disposable filter unit with a 0.22 µm pore Polyethersulfone (PES) membrane.
    3. Aliquot into 45 mL (to prepare 500 mL of maturation medium) or 4.5 mL (to prepare 50 mL of maturation medium). Store at 20 °C for up to 6 months.
6. Prepare maturation media
    1. Equilibrate the B27 supplement, knockout SR, penicillin-streptomycin, the maturation supplement[28], and the DMEM no-glucose medium at RT. Ensure to thaw the supplement completely. Mix 435 mL of the DMEM no glucose medium with 10 mL of the 50x B27 supplement, 5 mL of penicillin-streptomycin, 5 mL of knockout SR, and 45 mL of

maturation supplement. Store at 4 °C for up to 2 weeks; equilibrate at 37 °C before use.

7. Prepare fluor bright medium

    1. Equilibrate penicillin-streptomycin and DMEM fluorobrite medium at RT. Ensure that the supplement is completely thawed. Mix 500 mL of the DMEM fluorbrite medium with 5 mL of penicillin-streptomycin. Store at 4 °C for up to 1 month; equilibrate at 37 °C before use.

8. Prepare the non-ionic detergent solution

    1. Mix 20% w/v non-ionic detergent powder (e.g., F-127) with PBS. Filter using a 0.22 µm filter and store at 4 °C for up to 6 months; equilibrate at RT before use.

9. Prepare calcium dye medium

    1. Mix the non-ionic detergent solution (final concentration of 0.04% v/v) and 0.1x of the calcium dye (e.g., Cal520 AM) in fluor bright medium. In a 50 mL conical tube, add 10 µL of Cal520 and 20 µL of the non-ionic detergent solution. Mix until fully dissolved. Keep the solution in the dark before adding to the cells.

2. Preparation of buffers

1. Prepare permeabilization and blocking buffer: This buffer contains 10 mL of PBS, 5% wt/v BSA, and 0.3% v/v Triton-X-100.
2. Prepare the flow cytometry buffer: This buffer contains 50 mL of PBS, 1% wt/v BSA, and 0.3% v/v Triton-X-100.
3. Flow cytometry washing buffer: This buffer contains 50 mL of PBS and 1% wt/v BSA.
4. Spheroid washing buffer (SWB): This buffer contains 1 mL of Triton-X-100, 2 mL of 10% (w/v in DPBS) SDS, and 2 g of BSA in 1 L of PBS.

    NOTE: SWB can be stored at 4 °C for up to 2 weeks.

5. Prepare the embedding solution (ES): To prepare 100 mL of the embedding solution, mix 50 mL of glycerol with 9.09 mL of dH_2O, 1 mL of Tris buffer (1 M, pH 8.0), and 200 µL of EDTA (0.5 M). Add 22.7 g of fructose and mix at RT in the dark until dissolved. When clear, add 22.2 g of fructose and mix until dissolved. Then add 15 g of urea and mix until dissolved (store at 4 °C in the dark).
6. Prepare PBT (PBS with Tween-20) buffer. This buffer contains PBS/Tween-20 (0.1% v/v). For 1 L of PBS, add 1 mL of Tween-20.

3. Preparation of small molecules

1. Reconstitute thiazovivin (ROCK inhibitor) powder in 10 mM aliquots of 50 µL in DMSO and store at -20 °C for up to 6 months. Protect from light.
2. Prepare 2.5 mM aliquots of 10 µL each of Cal-520 AM in DMSO and store them at -20 °C for up to 6 months. Protect from light.

4. Cardiac spheroid generation

NOTE: For larger amounts of CSs, seed up to 1 million CMs in a 6-well ultra-low attachment plate with 2 mL of hiPSC-CM re-plating media. This study used a minimum of 2,500 (2.5k CS) up to 20,000 (20k CS) hiPSC-CMs per well of a 96-well plate.

1. For one 96-well plate, prepare a cell culture containing a minimum of 2 x 10^6 human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs)[10].

2. When the cultured hiPSC-CMs reach confluence, add 0.1 mL/cm^2 of sterile cardiac detachment solution (e.g., tryple) to each well. Incubate the plate at 37 °C for 15 min.
3. Using a 5 mL pipet, mechanically dissociate the cells by flushing with 2 mL of warm basal medium to make a single cell suspension. Confirm the detachment with a bright field microscope (4x magnification); cells will look white and have a round shape.
4. Transfer the cell suspension to a 15 mL conical tube and centrifuge for 3 min at 300 x *g*.
5. Aspirate the supernatant and resuspend the cells in 1 mL of hiPSC-CM re-plating media.
6. Using a 1,000 µL pipet tip, mechanically dissociate the cell pellet. The solution appears homogeneous after three or four mixes. Count the cells. Transfer the appropriate amount of cells in 100 µL of the re-plating medium to each ultra-low attachment round-bottom 96-well well.
7. Place the plate of CSs on an orbital shaker at 70 rpm in the incubator for 24 h. Set the incubator conditions to 37 °C, 5% CO_2, 21% O_2, and 90% humidity.
8. Aspirate 50 µL of medium from each well and add 100 µL of RPMI + B27 medium per well for the first 48 h.
   NOTE: Always keep 50 µL of the medium in the 96-well plate to avoid accidental aspiration and spheroid rupture.
9. Aspirate 100 µL of the medium from each well and add 100 µL of the maturation medium per well. Maintain the cells in the maturation medium and refresh the medium every 2-3 days.

5. Cryopreservation of CSs

NOTE: CSs can be cryopreserved for long-term storage. Cryopreservation can be performed from day 3 after the generation of CSs. CSs can be cryopreserved directly in the wells of a 96-well plate or as a CS suspension in cryovials.

1. Pre-cool the plate by placing the plate on ice for 10 min.
2. Centrifuge the spheroid plate for 3 min at 70 x *g*.
3. Remove the supernatant till 50 µL remains and add 200 µL of ice-cold hiPSC freezing medium per well.
   NOTE: Keep the CS suspension on ice for the entire duration of the procedure. In the case of a 6-well plate with spheroids, freeze one well in a 500 µL freezing medium cryovial.
4. Seal the plate with plate sealing film.
   NOTE: The 96-well plate needs to be stored in a polystyrene box or, when not available, a silicon mold can be made as described in step 5.5.1.
5. To ensure uniform heat exchange between the well plate and the freezer, place the plate carefully into a polystyrene box or in a silicon mold.
   1. To prepare the silicon mold: Vigorously mix two components of the silicon elastomer kit in a 10:1 ratio. De-bubble the solution using a vacuum pump for 15-20 min. Subsequently, cast the solution inside the bottom part of the well plate and de-bubble using a vacuum pump for 10 min. Place the mold into an oven and cure at 60 °C for 8 h to obtain a semi-flexible elastomer that is peeled off the plate.
6. Freeze the plate at -80 °C for a minimum of 4 h in the polystyrene box or the prepared silicon mold.

7. Transfer the plate to a liquid nitrogen tank or a -150 °C freezer for long-term storage.

6. Thawing of cardiac spheroids

NOTE: Do not thaw more than one plate at a time to ensure a quick thawing process.

1. Prepare 20 mL of 37 °C preheated basal medium in a 50 mL conical tube.
2. Collect the cell plate with CSs from the liquid nitrogen and place it in the incubator for 15 min. Set the incubator conditions to 37 °C, 5% CO_2, 21% O_2, and 90% humidity.
3. Remove the supernatant and the cell pellet remains, and resuspend each well in a warm basal medium. Use 200 µL of medium per well.
4. Centrifuge for 3 min at 70 x *g*.
5. Repeat steps 6.3 and 6.4.
6. Remove the supernatant until the cell pellet remains and add 200 µL of CM thawing medium in each well.
7. Place the plate of CSs on an orbital shaker at 70 rpm in an incubator for 24 h. Set the incubator conditions to 37 °C, 5% CO_2, 21% O_2, and 90% humidity.
8. Aspirate 50 µL of the medium from each well and add 100 µL of RPMI + B27 medium per well for the first 48 h.
9. Aspirate 100 µL of the medium from each well and add 100 µL of maturation medium per well. Maintain the cells in the maturation media and refresh the medium every 2-3 days.

7. Assessment of intracellular Ca^{2+} transients

NOTE: CSs are in culture for a total of 3 weeks; 2 weeks before freezing, and 1 week after thawing. The 'fresh' controls are age-matched.

1. After 1 week of culture, the thawed CSs are optimal for calcium handling optical imaging. Use a calcium dye (e.g., Cal520AM) to assess the uptake and release of Ca^{2+} from the cells.
2. Treat them with 100 µL of calcium dye medium per well and incubate for 60 min in the incubator. Set the incubator conditions to 37 °C, 5% CO_2, 21% O_2, and 90% humidity.
   NOTE: Cal520AM is light-sensitive. Perform all loading procedures and experiments in the dark.
3. Prepare the calcium acquisition and analysis system.
   1. Power the microscope, ensuring the environmental control option is on.
   2. Adjust the camera and framing aperture dimensions to minimize the background area.
      NOTE: Here, the Leica Thunder DMi8 microscope was used; other microscope systems are applicable as well until they allow a sampling rate above 30 frames/second (fps).
4. Record a video with a consistent stream of 2−10 peaks within 10 s and scan across the 96-well plate, initially moving to the left, then downward in a zig-zag fashion to cover the whole plate. Measure the calcium signal using a 488 nm laser; set the contrast to a black background with a bright green signal during calcium release.
5. After acquiring the Ca^{2+} transients, analyze the data with the fluorescence traces analysis software

(e.g., CyteSeer, Vala Sciences) according to the manufacturer's instructions.

8. Flow cytometry analyses of dissociated cardiac spheroids

NOTE: In this study, flow cytometry was used to determine the viability of the CSs before and after the thawing process.

1. Collect the CSs in a 15 mL conical tube using a 5 mL pipet to avoid spheroid damage and centrifuge for 3 min at 70 x *g*. Aspirate the supernatant and add 1 mL of PBS.
2. Centrifuge for 3 min at 200 x *g*. Aspirate the supernatant and dissociate the CSs by adding 1 mL of cardiac detachment solution (e.g., tryple). Incubate the tube at 37 °C for 15 min.
3. Using a 5 mL pipet, mechanically dissociate the cells by flushing with 2 mL of basal medium until single cells can be seen when observed under the microscope.
4. Centrifuge for 3 min at 200 x *g*.
5. Aspirate the supernatant and fix the CMs with 200 µL of 4% paraformaldehyde (PFA) solution in 1x PBS. Incubate for 10 min at RT.
6. Centrifuge for 3 min at 200 x *g*. Aspirate the supernatant and add 1 mL of PBS.
   NOTE: Pause point: The fixed hiPSC-CMs can be stored at 4 °C for up to 4 weeks.
7. Transfer the cell suspension to a FACS tube and centrifuge for 3 min at 200 x *g*. Aspirate the supernatant and resuspend 1 x 10^5 cells in 50 µL of the permeabilization buffer.
8. Incubate the cells for 30 min at 4 °C.
9. For immunofluorescence flow cytometry analysis, perform steps 8.9.1-8.9.4.
   1. Resuspend the cells in the flow cytometry buffer (50 µL) containing the α-actinin antibody at a dilution of 1:300. In another FACS tube, resuspend 1 x 10^5 cells in the flow cytometry buffer (50 µL) with the respective isotype control (e.g., FITC mouse IgM, κ isotype [1:200 dilution]). Similarly, resuspend 1 x 10^5 cells in 50 µL of flow cytometry buffer for negative control.
   2. Incubate the cells for 30 min at 4 °C.
   3. Add 2.5 mL of flow cytometry buffer and centrifuge the cells at 200 x *g* for 3 min at 4 °C; discard the supernatant and repeat this washing step twice.
   4. Resuspend the cells in 50 µL of flow cytometry buffer with the secondary-antibody goat-anti-mouse (1:300 dilution).
      NOTE: Place the tube in the dark since the secondary-antibody solution is light-sensitive.
10. For viability check with propidium iodide (PI), add 150 µL of PI per sample (1:1,000) and incubate for 15 min.
    NOTE: Place the tube in the dark since the PI solution is light-sensitive.
11. Adjust the gates according to the standard gating strategy as shown in **Supplementary Figure 1** and analyze the cells with a flow cytometer.

9. Immunofluorescence staining of whole 3D spheroids

NOTE: This protocol is based on the protocol for high-resolution 3D imaging of whole organoids upon immunofluorescent labeling, which was previously published[29] and adjusted for cardiac spheroids. During the procedure, all pipet tips and conical tubes can be coated with 1% wt/v BSA-PBS in order to prevent the spheroids from

sticking to plastics. To coat the materials, dip into the 1% BSA-PBS. Be careful not to damage the spheroids by using the 5 mL pipet, avoiding mechanical disruption.

1. Collect the CSs in a 15 mL coated tube with a 5 mL pipet. The spheroids are visible to the eye. Collect ~20-50 spheroids per antibody combination. Centrifuge for 3 min at 70 x *g* and aspirate the supernatant.
2. Carefully resuspend the spheroids in 1 mL of ice-cold 4% paraformaldehyde (PFA) solution in 1x PBS using a coated 1 mL tip.
3. Fix at 4 °C for 45 min. After 20 min, gently resuspend the spheroids using a coated 1 mL tip. This evens fixation among all spheroids.
4. Add 10 mL of ice-cold PBS to the tube and gently mix by inverting the tube. Incubate for 10 min at 4 °C and spin down at 70 x *g* for 3 min.
   NOTE: From this step onward, coating of tips and conical tubes is generally not needed as CSs do not stick to the tip after fixation.
5. Block the CSs by resuspending the pellet in ice-cold SWB (200 µL of SWB per well) and transfer the spheroids to a 24-well suspension culture plate.
   NOTE: CSs from one large pellet can be split over multiple wells to perform different stainings. Use ~20-50 CSs per antibody combination.
6. Incubate at 4 °C for at least 15 min.
7. Add 200 µL of the SWB in an empty well to serve as a reference well.
   NOTE: For the immunofluorescent staining, 48- or 96-well plates can also be used to reduce antibody usage. However, the staining and washing results can be reduced due to the smaller volume per well.
8. Allow the spheroids to settle at the bottom of the plate, by leaving the plate tilted at a 45° angle for 5 min.
9. Remove the SWB, leaving the CSs in 200 µL of the SWB (use the reference well to estimate the minimal volume of 200 µL).
10. Add 200 µL of the SWB with the primary antibodies 2x concentrated (e.g., α-actinin [1:200] and Troponin T [1:200]) and incubate overnight at 4 °C while rocking/shaking (40 rpm on a horizontal shaker).
11. The next day, add 1 mL of the SWB to each well.
12. Allow the spheroids to settle at the bottom of the plate by leaving the plate at a 45° angle for 5 min.
13. Remove the SWB, leaving 200 µL in the plate. Add 1 mL of SWB and wash for 2 h with slow rocking/shaking.
14. Repeat steps 9.12 and 9.13 twice more.
15. Allow the CSs to settle at the bottom of the plate by leaving the plate tilted at 45° for 5 min. Remove the SWB, leaving 200 µL in each well
16. Add 200 µL of the SWB with secondary antibodies, conjugated antibodies, and dyes 2x concentrated (e.g., DAPI [1 µg/mL], mouse-AF488 [1:500], rabbit-AF568 [1:500]), and incubate overnight at 4 °C in the dark, while slowly rocking/shaking.
17. The next day, repeat steps 9.12 and 9.13 twice more.
18. Carefully transfer the CSs to a 1.5 mL tube and spin down at 70 x *g* for 3 min.
19. Remove as much of the SWB as possible by pipetting without disrupting the CSs.
20. Add the embedding solution (ES; at least 50 µL, at RT) using a 200 µL tip with the end cut off and resuspend

gently to prevent bubble formation and incubate at RT for 20 min.

21. In the meantime, create a square container on a glass slide with either nail polish or silicone sealant.
22. Cut off the end of a 200 µL tip and transfer the CSs in ES to the middle of the square container.
23. Place a square coverslip on top. To reduce air bubbles, place one side of the coverslip first, then slowly lower the coverslip from one side to the other until there is no trapped air under the surface, and then release the coverslip.
24. Gently push on all edges of the coverslip to seal it to the nail polish or silicone sealant.
25. Leave the slide overnight at RT. The next day, the slide is ready for imaging.

    NOTE: Optical clearing by the ES can cause minor tissue shrinkage. However, this cannot affect the overall morphology of the CSs. The staining procedure can be paused here by storing the slides at 4 °C (for at least 1 week) or at -20 °C (for at least 6 months).

Representative Results

The protocol shown in **Figure 1A** describes the generation of CSs from previously expanded hiPSC-CMs. The CSs acquire a 3D structure by day 1 post-seeding in ultra-low attachment round-bottom plates and can be cultured for up to 6 weeks (**Figure 1B**). As assessed by immunofluorescence staining, the majority of the cells in 3-week-old CSs expressed sarcomeric proteins such as α-actinin and troponin T and displayed regular sarcomere organization (**Figure 1C**). For the quantification of α-actinin positive cells, flow cytometry analysis was performed. In accordance with the immunofluorescence results, the flow cytometry data demonstrated comparable high levels of α-actinin in both day 0 (76.9% ± 16.6%) and 3-week-old CSs (71.1% ± 22.7%) (**Figure 1D**), indicating a constant and highly pure cellular composition during culturing. There was an increased expression of the cardiac genes for junctions (GJA1, JPH2, and PKP2), desmosomes (DES), and mitochondria (ATP5A) in hiPSC-CM derived spheroids (day 42) versus hiPSC-CMs cultured in 2D for 90 days (**Figure 1E**). The expression of these genes is a hallmark of cell-cell interaction and maturation[30].

Subsequently, the functional properties of CSs, namely beating rate and Ca^{2+} handling, were assessed at different time points (**Figure 2**). Calcium transient parameters such as rise time, peak time, decay time, and calcium transient duration (CTD90) were evaluated as indicated in **Figure 2A**,**B**. The percentage of beating CSs is similar in the first 3 weeks post-generation but significantly dropped in week 6 (Wk6) CSs (**Figure 2C**). The beating rate was significantly reduced at Wk3 compared to Wk1 and, similar to the percentage of beating CSs, dramatically dropped at Wk6 (**Figure 2D**). At Wk6, CS deterioration was observed, which can explain the drop in both the beating rate and the number of beating CSs. Measurement of calcium transient parameters indicated a significantly higher peak value at Wk2 (**Figure 2E**), while the rise time, decay time, and CTD90 were significantly increased at Wk3 compared to Wk1 (**Figure 2F-H**). Taken together, these results show that hiPSC-CM-derived spheroids are functionally optimal at around weeks 2 and 3 post-generation.

Figure 3 shows the effect of spheroid size on the beating rate and calcium handling. CSs were generated by seeding 2.5 x 10^4, 5 x 10^4, 10 x 10^4, and 20 x 10^4 hiPSC-CMs in a well of a 96-well plate for a total of 24 CSs/wells per condition (**Figure**

3A). As expected, the spheroid size increased as the number of cells used increased, ranging from 178 ± 36 µm to 351 ± 65 µm (**Figure 3A**, right panel). Ca^{2+} transients were measured in 3-week-old CSs at the four different seeding densities (**Figure 3B**). Measurements of beating CSs indicated that only about 50% of the smaller size-CSs (2.5K- and 5K-CSs) were beating, while the percentage of bigger size-beating CSs (10K- and 20K-CSs) was significantly higher (about 85%) (**Figure 3C**). A similar beating rate (approximately 28 bpm) was shown by 5K-, 10K-, and 20K-CSs, which was significantly higher compared to 2.5K-CSs (**Figure 3D**). The peak values of calcium images were similar in all tested conditions (**Figure 3E**), however, rise time (**Figure 3F**), decay time (**Figure 3G**), and CTD90 (**Figure 3H**) were significantly increased in larger size-CSs (10K- and 20K-CSs) compared to the smaller ones (2.5K- and 5K-CSs). Taken together, these results show that hiPSC-CM-derived spheroids are optimal for calcium handling screening when a seeding density between 10K- and 20K hiPSC-CMs/well is used.

Next, we evaluated the impact of cryopreservation on CS's viability and function. Before analysis, thawed CSs were maintained in culture for 1 week (**Figure 4A**). As shown by both flow cytometry (**Figure 4B**) and Calcein-AM (**Figure 4C**) cell viability tests, cryopreservation did not affect cell viability within the CSs. Additionally, thawed CSs showed similar expression levels of sarcomeric proteins as compared to the fresh age-matched CSs (**Figure 4D**). These data indicate that CSs can be efficiently cryopreserved for subsequent cardiac function analysis and high-throughput screening.

Finally, the beating activity and Ca^{2+} handling were measured in both fresh and cryopreserved CSs (**Figure 5**). The percentage of beating CSs was measured at different time points after thawing, respectively, at 2, 5, and 7 days. While most of the fresh CSs showed beating activity over time, clearly the cryopreserved CSs needed up to 1 week of culturing in order to recover their beating activity (**Figure 5B**). There was no significant change in the beating rate of thawed CSs versus fresh; however, no spontaneous beating activity was observed in some frozen CSs (**Figure 5C**). Although peak values were significantly reduced in frozen/thawed CSs compared to fresh (**Figure 5D**), no significant changes were observed in rise time, decay time and the CTD90 of frozen/thawed CSs compared to fresh (**Figure 5E-G**). These data indicate that, after thawing, it is important to let the CSs recover in the incubator for at least 1 week before measuring beating activity and Ca^{2+} transient.

Taken together, these results show that cryopreservation of hiPSC-CM-derived spheroids preserves cardiomyocyte viability, the sarcomeric structure, and their functional characteristics such as spontaneous beating activity and calcium handling. Thus, hiPSC-CM-derived spheroids represent a suitable model to accurately recapitulate cardiac electrophysiology *in vitro*.

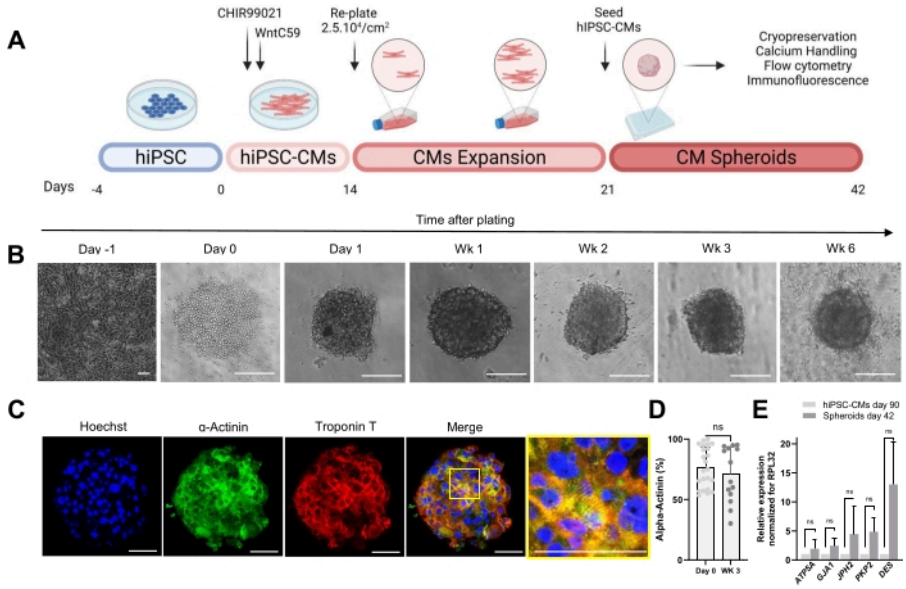

Figure 1: Generation of cardiac spheroids. (**A**) Schematic representation of Wnt-based directed cardiac differentiation, the subsequent expansion of hiPSC-CMs, and the generation of CSs. Created with biorender.com. (**B**) Bright-field images at different time points of CS culturing. Scale bar, 200 µm. Wk represents week. (**C**) Representative immunofluorescence images for cardiac sarcomeric proteins α-actinin and troponin T in 3-week-old CSs. Immunofluorescence: Hoechst (blue), α-actinin (green), and troponin T (red). Scale bar, 200 µm. The zoomed-in merged picture on the right displays the sarcomere organization. Scale bar, 50 µm. (**D**) Flow cytometry quantification of α-actinin positive cells before (day 0) and 3 weeks after the formation of CSs. (n = 14-23 per condition. (**E**) RT-qPCR performed on hiPSC-CMs cultured for 90 days (2D) and spheroid samples cultured for 42 days to establish expression levels of different cardiac genes related to cell junctions, intermediate filaments, and mitochondria. (n = 1-3 batches). Data are represented as mean ± SD. NS (non-significant) as calculated by an unpaired t-test. Please click here to view a larger version of this figure.

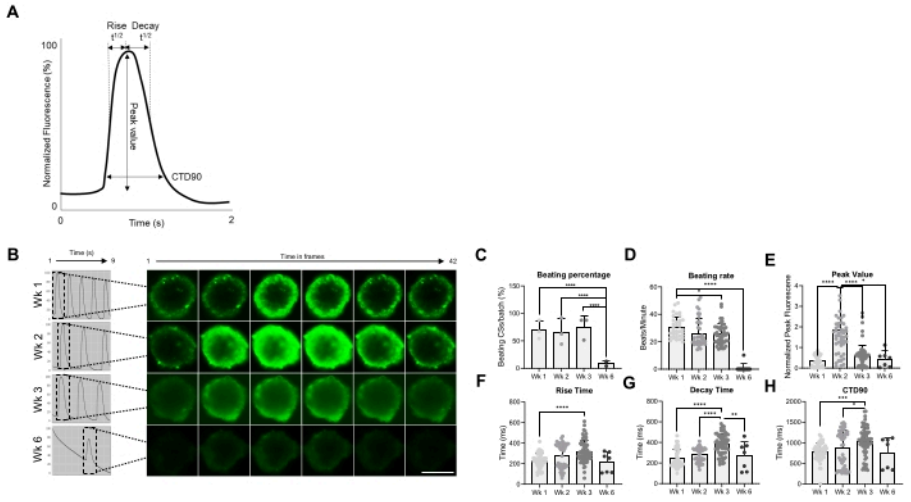

Figure 2: Beating rate and Calcium handling in CSs at different weeks post generation. (**A**) Examples of calcium transient parameters calculated by the Vala sciences analysis algorithm in Cyteseer Software. (**B**) Representative calcium transient traces and time-lapse images of the CSs at different time points (weeks) post-generation. Scale bar, 200 µm. (**C**) Time course quantification of spontaneous beating activity is expressed as the percentage of beating CSs. (**D**) Beating rate of CSs during culturing time. (**E-H**) Quantification of the calcium transients showing peak value, rise time, decay time, and CTD90. Data shown are mean ± SD. Biological replicates = three, technical replicates = 38, 50, 66, and 7, respectively. *p < 0.05, ****p < 0.001; one-way ANOVA followed by Tukey's post hoc multiple-comparisons test. Abbreviations; CTD = calcium transient duration, Wk = week, CSs = human cardiac spheroids. Please click here to view a larger version of this figure.

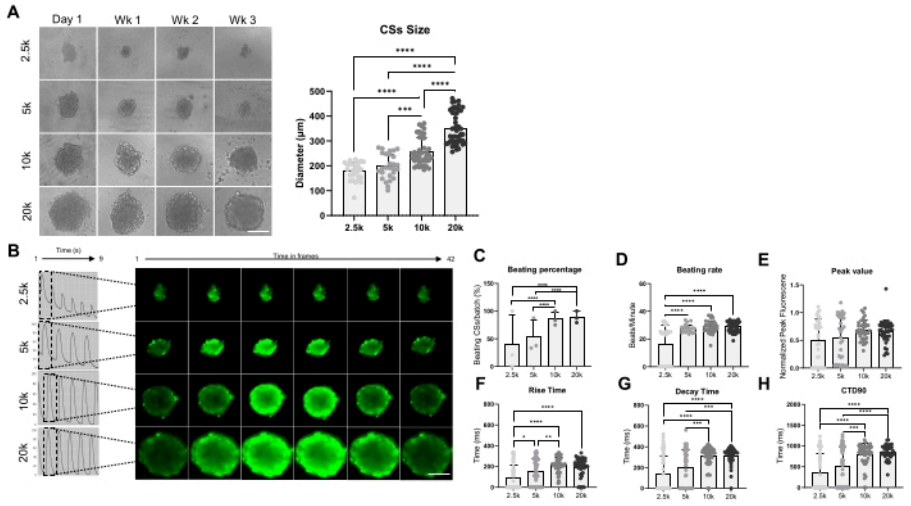

Figure 3: Beating rate and calcium handling in CSs generated using different cell seeding densities. (**A**) Bright-field imaging (left) and size measurements (right) of CSs generated using different numbers of hiPSC-CMs. Scale bar, 200 µm. (**B**) Representative calcium transient traces and time-lapse images of the 2.5K-20K-CSs. (**C,D**) Beating percentage and beating rate of 2.5K-20K-CSs. (**E-H**) Peak value, rise time, decay time, and CTD90 in 2.5K-20K-CSs. Data are mean ± SD. Biological replicates = three, technical replicates = 28-39. $*p < 0.05$, $****p < 0.001$; one-way ANOVA followed by Tukey's post hoc multiple-comparisons test. Abbreviations: CTD = calcium transient duration, Wk = week, k = x 1,000 cells, CSs = cardiac spheroids. Please click here to view a larger version of this figure.

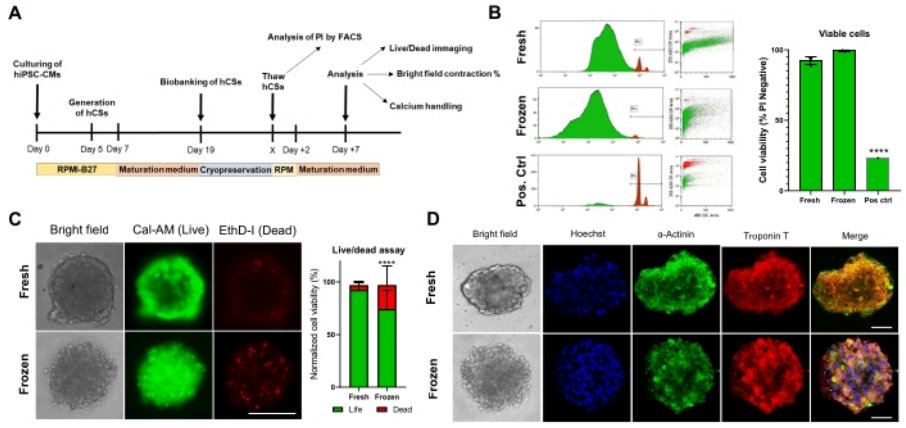

Figure 4. Effect of cryopreservation on cardiac spheroids' viability and structure. (**A**) Schematic representation of CS generation, subsequent biobanking, and thawing. (**B**) Flow cytometry cell viability test in both fresh and cryopreserved CSs. As a positive control, a treatment with 10% Triton-X solution for 5 min was used. (n = 4 per condition). Data are represented as mean ± SD. ****p < 0.001; one-way ANOVA followed by Tukey's post hoc multiple-comparisons test. (**C**) Calcein-AM cell viability test in fresh versus thawed CSs after 7 days of culturing (n = 15-17 per condition, ****p < 0.001, by paired t-test; scale bar, 200 µm). (**D**) Representative bright-field (left) and immunofluorescence staining for α-actinin and troponin T expression in fresh and thawed CSs. Immunofluorescence: Hoechst (blue), α-actinin (green), and troponin T (red). The merged pictures on the right display sarcomere striations in the CSs. Scale bar, 50 µm. Abbreviations: X = thawing day of choice, PI = propidium iodide, Cal-AM = calcein-AM, EthD-I = Ethidium Homodimer I. Please click here to view a larger version of this figure.

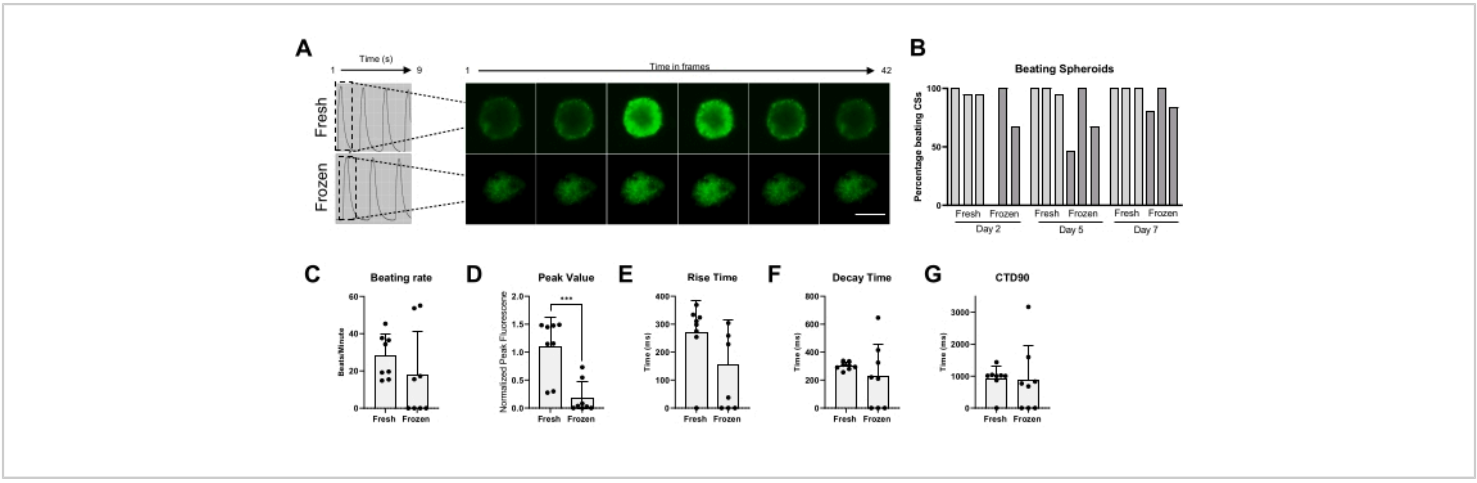

Figure 5: Calcium transients in fresh versus thawed CSs. (**A**) Representative calcium transient traces and time-lapse images of the CSs before cryopreservation and 1 week after thawing. (**B**) Beating percentage of fresh and frozen/thawed cardiac spheroids. Bars represent individual experiments. (**C**) Beating rate of fresh and frozen/thawed cardiac spheroids. (**D-G**) Quantification of calcium transient parameters: peak value, rise time, decay time, and CTD90. Data are mean ± SD. *p < 0.05, ****p < 0.001; one-way ANOVA followed by Tukey's post hoc multiple-comparisons test. Abbreviations; CTD = calcium transient duration, CSs = cardiac spheroids. Please click here to view a larger version of this figure.

Supplementary Figure 1: Representative gating strategies for flow cytometry analysis. (**A**) Representative gating strategy for α-actinin positive hiPSC-CMs in a pure population versus negative control and isotype control. The number of α-actinin positive analyzed cells is 25 x 10^5. Abbreviations; SSC = side scatter, PI+ = propidium iodide positive. (**B**) Representative gating strategy for the viability analysis in both fresh, thawed, the positive control (Triton-X), and the negative control (unstained). Please click here to download this File.

Discussion

Cardiac drug discovery is hampered by a reliance on non-human animal and cellular models with inadequate throughput and physiological fidelity to accurately perform readouts. hiPSC-CM biology coupled with HT instrumentation and physiological probes has the potential to re-introduce human models into the earliest stages of cardiac disease modeling and drug discovery. We developed a 3D cardiac tissue generation method that produces high-quality and functional CSs for an optimal cardiac disease modeling and drug screening platform. Additionally, combining the spheroid technology in 3D bioreactor systems for industrial EV production allows a necessary step toward the clinical translation of EV-based therapy. The method described here relies on several crucial factors and is a variant of existing protocols[9,10,28,29]. These methods include: 1) the generation of 3D tissue constructs, 2) the optimal cell number and timing before the screening, 3) improving sensitivity and high-throughput capacity of instruments, and 4) being able to freeze the spheroids before any functional analysis. Unlike previously described protocols, the proposed protocol describes the generation of up to 1,500 spheroids per day and the suitability for HTS. Conventional analysis of a

hundred compounds over 6 x 0.5 log doses for 10 replicates using existing 96-well calcium imaging systems or 24-well multiplexed engineered heart tissues require approximately 500 million to 3 billion hiPSC-CMs[31,32]. The proposed application makes cardiac screenings less costly and time effective compared to the conventional systems since the 96-well plates required only 10% of the seeding density compared to the described method. Moreover, compared to previous protocols, such as the hanging-drop method, the generation of spheroids by self-aggregation in ultra-low attachment plates enables high-quality automated imaging of single microtissues[33].

This small 3D model mimics the biological and physiological phenotype of the *in vivo* cardiovascular environment. As previously demonstrated, calcium transients dramatically increase in 3D cardiac tissue constructs as compared to 2D monolayer cell cultures[34].

Next, we found that the seeding density and proper culturing time are also critical factors for a successful CS screening. The densities of 10K-20K hiPSC-CMs per spheroid and screening between weeks 2-3 after generation were optimal, whereas too small or too old spheroids show disturbed calcium handling (**Figure 2** and **Figure 3**). Therefore, it is of importance to maintain seeding densities as consistent as possible, since size influences the functional parameters. Also, although this optical method provides good results for live 3D cultures as a whole tissue, obtaining data within larger spheroids at (sub-)cellular level is challenging without relying on time-consuming histology methods. Recently, several approaches have been published that used "optically clearing", which enables the acquisition of whole 3D spheroids with the opportunity for single-cell quantification of markers. Here, we adapted a 3-day protocol from CS harvesting to image analysis, which is optimized for 3D imaging using confocal microscopy[29] (**Figure 1C** and **Figure 4D**).

Lastly, with the increase in 3D cardiac tissue applications and commercial applications, the demand for long-term storage and patient-specific biobanking from various donors is rising. Cryopreservation is an effective strategy to generate HTS-plates from multiple batches over time. The freezing of hiPSC-CMs has been described previously and is not different compared to other cultured cell types[10,35,36]. Recently, approaches for freezing plates with 2D cells have been described[37]. Here, we found the PSC cryopreservation kit is the most optimal condition as compared to three others (data not shown) and used this medium for the efficient freezing of spheroids. After cryopreservation, viability remains high (**Figure 4B**,**C**), but CSs' electrophysiological properties are affected and a period of incubation after thawing is required. Indeed, 1 week after thawing, CSs displayed spontaneous beating activity and calcium handling. However, it has been described that fresh and recovered hiPSC-CMs do not always show identical molecular and physiological properties[38]. This limitation needs to be considered when cryopreserved hiPSC-CMs are used for assessing drug-induced cardiac read-outs. Moreover, although we effectively modulate the number of cells per spheroid and the optimal timing of the calcium transient imaging, the cardiac spheroids could be improved by mixing hiPSC-derived cardiomyocyte cells with endothelial cells, fibroblasts, cell-cell junctions, and extracellular matrices, such as chitosan, collagen IV, fibronectin, matrigel, or laminin, mimicking the *in vivo* cardiac environment[39,40]. Overall, we propose a step-by-step protocol to efficiently generate CSs which are suitable

for downstream applications such as disease modeling and HT drug screening.

Disclosures

The authors have nothing to disclose.

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