Cardiac casting protocol

Protocol for:

axoCells™ hiPSC-derived ventricular cardiomyocytes and human cardiac fibroblasts

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AXOL

OPTICS life

SUPPORT AND CONTACT DETAILS

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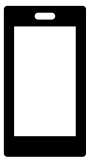

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BEFORE WE START

Introduction

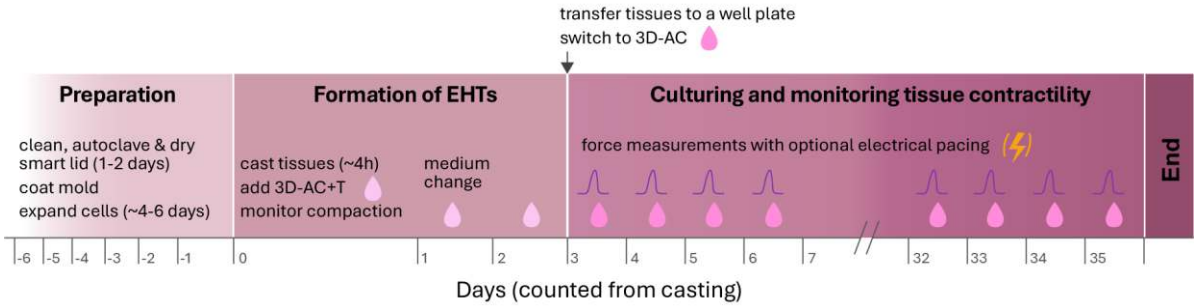
This protocol focuses on the procedure of generating 3D engineered heart tissues (EHTs) composed of cardiomyocytes and fibroblasts in the Cuore system. Techniques for safely handling and operating Cuore are described in detail in the *Cuore Manual* and *Cuore Handling video*. Please familiarize yourself with them before starting an experiment.

The protocol described here, based on Tiburcy et al. 2020, has been developed in collaboration with the Zoccarato group at King's College London and validated by Optics11 Life using axoCells™ human iPSC-derived ventricular cardiomyocytes in combination with human primary cardiac fibroblasts.

To adapt the protocol to a different cell line, optimization of hydrogel and media composition may be required.

Procedure overview

A typical experiment in Cuore consists of several phases:



Approximate experimental timeline. Note that the preparation phase may take shorter or longer, depending on the cell expansion needed. Timing of force measurements, electrical pacing, as well as total duration may also be adjusted to the specific experimental setup and scientific question.

Preparation	• Acquire the required reagents and components, including a sufficient amount of axoCells™ hiPSC-derived ventricular cardiomyocytes (this protocol requires 3.5×10^5 cardiomyocytes per tissue). Prepare stock solutions. • Grow a sufficient amount of fibroblasts (this protocol requires 1.5×10^5 fibroblasts per tissue). • Clean and sterilize the Cuore smart lid components, pre-coat the mold.
Formation of EHTs	• Mix the two cell types and the hydrogel, cast them into the Cuore smart lid. • Cell-hydrogel mixture polymerizes and compacts around the cantilevers. • Tissue formation can be monitored using an inverted microscope. • Formed tissues are transferred to a well plate.
Culturing and monitoring tissue contractility	• The force and dynamics of the spontaneous contractions of maturing EHTs can be measured using Cuore system. Daily medium refreshments are needed to maintain tissues in a healthy state. • Optionally, electrical pacing can be applied to synchronize contraction or if otherwise required by the specific experimental design. • Tissue morphology can be monitored using an inverted microscope.

End of experiment	• Remove tissues from the cantilevers. Optionally, the tissues can be collected and used for downstream biochemical analysis or imaging. • Clean the Cuore smart lid before storage or further experiments.

References

Tiburcy, M., Meyer, T., Liaw, N. Y., & Zimmermann, W. H. (2020). Generation of Engineered Human Myocardium in a Multi-well Format. *STAR protocols*, *1*(1), 100032. doi:10.1016/j.xpro.2020.100032.

axoCells™ hiPSC-derived ventricular cardiomyocytes User Guide

Innoprot human cardiac fibroblasts Culture Protocol

STEP BY STEP GUIDE

To prepare beforehand

The list of required materials and reagents, as well as instructions on preparing and aliquoting the stock solutions, can be found in the Appendices at the end of this document.

Expanding the cells

The axoCells™ human iPSC-derived ventricular cardiomyocytes (hereafter: CM) employed in this protocol are thawed directly at the casting step and they do not therefore require pre-culturing. The casting of a full smart lid (24 tissues), considering the preparation of 10% excess solution, requires a minimum of 8.4 million viable CM. However, to account for possible cell loss during preparation, and pipetting imprecisions we recommend acquiring 10 million cells.

Conversely, the supporting cardiac fibroblasts need to be expanded beforehand. In this protocol, we employed human cardiac fibroblasts (hereafter: CFb) sourced from Innoprot and cultured according to the supplier's instructions. A recap of the protocols for thawing and passaging Innoprot's CFb can be found in the Appendices at the end of this document. Casting 24 tissues requires a minimum of 3.6 million of CFb and therefore a fully confluent T-75 flask is typically sufficient to provide enough cells. However, to be able to prepare excess cell resuspension, compensate for possible variations in proliferation or loss of cells during preparation, and pipetting imprecisions, we advise seeding 3 T-75 flasks before the experiment. For better results, we recommend using CFb at passage 3 to 6 at the moment of casting.

Cleaning and autoclaving smart lid components

Before each experiment, ensure that the components of the Cuore smart lid are cleaned, autoclaved, dried and back to room temperature. For the appropriate cleaning and sterilization procedure, please refer to the *Cleaning Protocol*.

The full cleaning and sterilization procedure, excluding the pre-wash of the stimulation plate, requires several (~3-6) hours, plan well in advance to avoid delaying your experiments.

The full cleaning of the stimulation plate is significantly more time-consuming and might take up to 3 days. It is, however, worth noting that the stimulation plate is not assembled in the system on the casting day, therefore affording more time for preparation.

All components should be kept in autoclave bags until use and only unpacked and handled in sterile conditions.

Pre-cooling of required equipment

Maintaining all reagents cold during the casting procedure prevents premature gelation of the collagen hydrogel, that can compromise homogeneity of the 3D tissues. To ensure the temperature remains low during handling, we recommend pre-chilling the following material:

- Cooling block fitting a standard well plate
- A few sterile 15 ml conical test tubes
- Sterile micropipette tips (for 200 µl and 1000 µl pipettes)

Note: *Storage between -20°C and 4°C for multiple hours and preferably overnight is required to evenly reach a sufficiently low temperature.*

Coating the mold

To minimize adhesion of the hydrogel/cells mix to the casting mold, and to favor effective compaction of the 3D tissues, it is crucial to pre-coat the casting chambers as follows:

- Unpack the sterile mold from the autoclave bag in a biological safety cabinet.
- Dispense 100-150 µl of sterile-filtered 2.5% Pluronic solution directly into each casting chamber. Note that the casting chamber volume is ~50 µl, the liquid will therefore overflow into the mold well.
- Cover the mold with a sterile well plate lid. For extra safety, the lid can be secured to the casting mold with parafilm or tape to ensure that sterility is maintained.
- Incubate undisturbed for at least 4 h or preferably overnight at room temperature.

Casting mixture composition

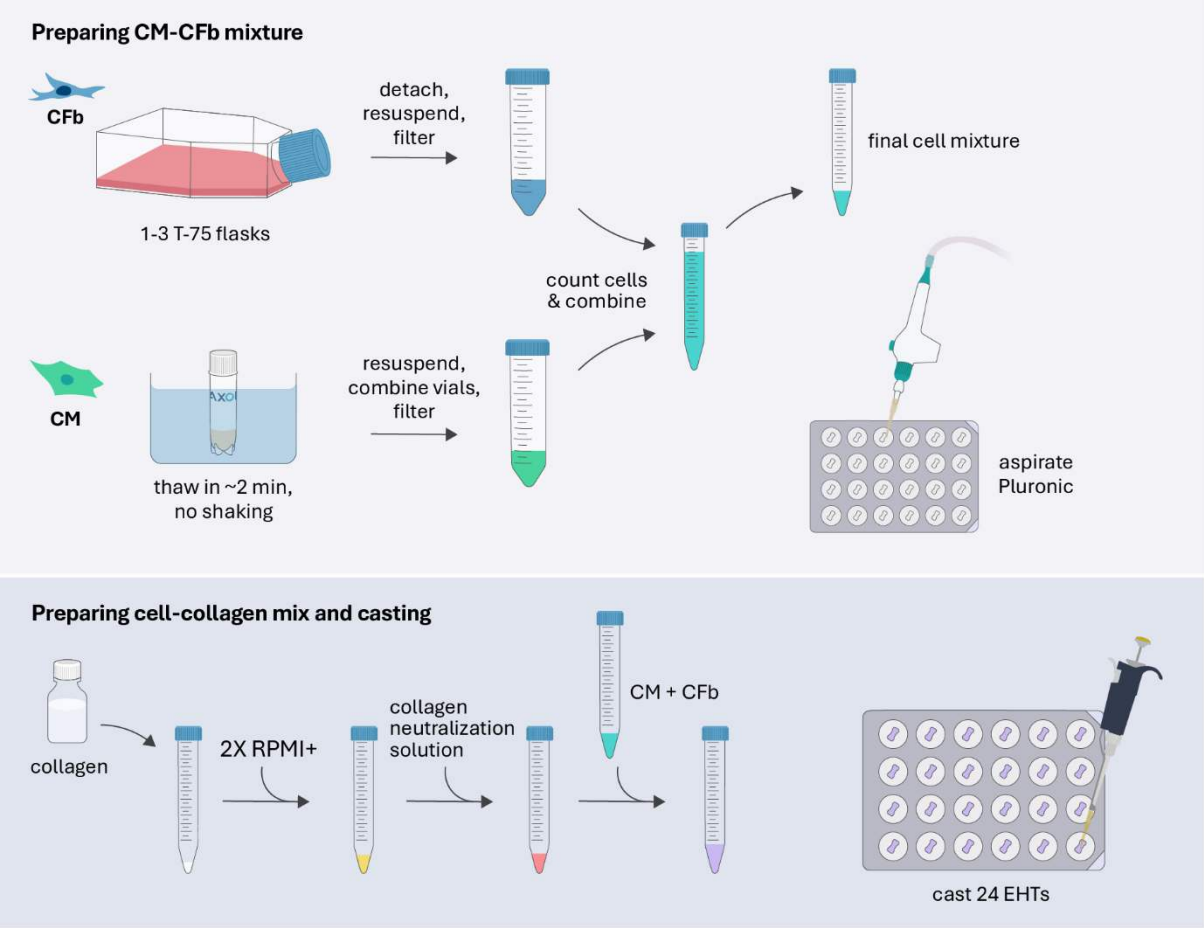
The volumes and amounts mentioned below are calculated for the casting of a full Cuore smart lid (24 wells, 50 µl per tissue) and already include a 10% excess to minimize the influence of volume inaccuracies. Because of the viscous nature of collagen, and the resulting volume loss during pipetting steps, we recommend further increasing the excess volume (for example using the counts for 28 EHTs instead of 24 for a full plate) for the first experimental rounds. We advise keeping large excesses if scaling up or down, according to your experimental design.

Reagent	Stock concentration	Final concentration	Volume per tissue (including 10% excess)	Volume for 24 tissues (including 10% excess)	Volume for 28 tissues (including 10% excess)
Collagen type I	~6 mg/ml	~1.34 mg/ml	12.24 µl	293.76 µl	342.72 µl
2X RPMI+	-	-	12.24 µl	293.76 µl	342.72 µl
Collagen neutralization solution	-	-	1.67 µl	40.08 µl	46.76 µl
Cell mixture‡ (containing both CFb and CM) in AC-3D+T medium	5.20 M/ml CFb and 12.13 M/ml CM	2.72 M/ml CFb and 6.36 M/ml CM	28.85 µl (0.15 M CFb and 0.35 M CM)	692.4 µl (3.6 M CFb and 8.4 M CM)	807.8 µl (4.2 M CFb and 9.8 M CM)
Total			55* µl	1320 µl	1540 µl

* This volume includes a 10% excess, 50 µl of the mixture will be dispensed in each casting chamber.

‡The cell mixture referenced here is obtained by counting the two different cell types, mixing at the correct proportion, centrifuging and resuspending at the given concentration, as indicated in the step-by-step procedure. For an example on how to calculate the amounts of cells to pre-mix see Appendix 5.

Casting procedure



Schematic illustration of the EHTs casting procedure.

In preparation for casting the 3D tissues:

- Before casting, place the coated mold to 4°C, preferably on a pre-chilled cooling block on ice.
- Warm up to room temperature at least 10 ml of fibroblast medium-2 (FM-2), and, for each flask of CFb to resuspend, at least 5 ml of HI-FBS, 2 ml of trypsin/EDTA (T/E) and 10 ml of trypsin neutralization solution (TNS).
- Prepare an ice box sufficiently big to contain the cooling block and a few (up to 4) 15 ml falcon tubes.
- Thaw 2X RPMI+ on ice. Move collagen, collagen neutralization solution and an empty 15 ml conical tube to ice as well.
- Prepare the necessary material for cell counting.

The steps described hereafter should be performed in a biological safety cabinet and in accordance with aseptic technique practices.

- Prepare 13.5 ml of fresh Axol cardiomyocyte plating medium (AC-PM) for each CM vial to thaw. Warm up to room temperature.
- Prepare fresh Axol cardiomyocyte 3D medium with TGF-β (AC-3D+T). For a full smart lid (24 EHTs) 40 ml will be required. Set aside 1 ml (for a full smart lid) of AC-3D+T and keep at hand for cell resuspension. The remaining volume can be stored at 4°C and will be used later.
- Unpack the smart lid components in the biological safety cabinet. If space allows, leave the components disassembled as this will facilitate the assembling steps.

Casting the 3D tissues

CFb detachment

Note: *Multiple flasks might be required for a full lid casting, we recommend growing 3 T-75 flasks to 70-90% confluency before the experiment. The following steps should be then performed in parallel for each flask.*

- Detach the CFb as per supplier's protocol. For each flask:

1. Aspirate culture medium.
2. Rinse cells with 10 ml DPBS, remove by aspiration.
3. Add 8 ml DPBS and 2 ml T/E to the flask. Rock gently to ensure complete coverage.
4. Move flask to the incubator for 1-3 minutes, monitoring at an inverted microscope from time to time.
5. During incubation, add 5 ml of FBS to a 50 ml conical centrifuge tube.
6. Once most cells are rounded up and before a high percentage of them is detached, transfer the T/E solution from the flask to the tube.
7. Put back the flask without liquid into the incubator for an additional minute.
8. Remove the flask from incubator and gently tap the sides to promote cell detachment, confirm detachment at the microscope and repeat if needed.
9. Add 5 ml of TNS to the flask, pipette gently up and down to ensure rinsing the entire growth surface to collect all cells and transfer to the 50 ml centrifuge tube containing FBS prepared in step 5.
10. Add an extra 5 ml of TNS to the flask, pipette gently up and down to ensure rinsing the entire growth surface once more and add to the same 50 ml centrifuge tube.

- Centrifuge the tube(s) at 210 rcf for 5 minutes. Aspirate and discard the supernatant, taking care not to disturb the cell pellet.
- Gently resuspend the cell pellet in 10 ml of FM-2, combining the cells from multiple tubes if necessary, and move the cell suspension to ice.

Thawing CM

Note: *The* Cuore cardiac tissue kit *includes 2 vials of CM, each containing 5M of cells. Casting a full lid requires the thawing of both vials. To minimize the duration of the procedure we recommend starting the thawing of the second vial while the content of the first one is being resuspended. That would mean, for example, that while the main operator is performing steps 4-7 for the first thawed vial, a colleague is proceeding with step 2 with the second vial. Extra care should be taken in ensuring that thawing times are not exceeded.*

➔ Thaw the CM as per Axol Bioscience's protocol. For each vial:

1. Collect the cell vials from liquid nitrogen storage and bury them in dry ice. Transfer the vial quickly to a 37°C water bath, taking care not to immerse it for more than two thirds.
2. Thaw the vial of cells without shaking for ~2 minutes. Remove the vial before the last bit of ice has melted.
3. Spray the thawed vial thoroughly with 70% ethanol, wipe it with a clean paper towel and move it to the biological safety cabinet.
4. Using a 1000 µl micropipette, immediately transfer the cells to an empty 15 ml sterile conical tube.
5. Wash the now empty cryovial with 1 ml of room temperature AC-PM.
6. Slowly add the AC-PM used for rinsing the vial to the 15 ml conical tube now containing the CM.

Note: *To prevent osmotic shock, the medium should be added dropwise to the conical tube containing the cells, whilst gently swirling the receiving tube. It should take ~60 seconds to dispense 1 ml of medium*.

7. Using a serological pipette, slowly add 8 ml of room temperature AC-PM to the conical tube containing the cells.

Note: *To prevent osmotic shock, the medium should be added dropwise to the conical tube containing the cells, whilst gently swirling the receiving tube. It should take ~60 seconds to dispense 8 ml of medium.*

8. Repeat steps 2 to 7 until all vials have been resuspended.

➔ Once all vials have been thawed, centrifuge all the tubes containing the CM at 200 rcf for 5 minutes.

➔ Aspirate and discard the supernatant, taking care not to disturb the cell pellet. Resuspend the cell pellet in each tube in 1-3.5 ml of AC-PM, so that the expected cell concentration sits between 1 and 2 M cells/ml. Combine the resulting volume of cell suspension into the same tube, mixing well.

Preparing final cell mixture

Once the two cell types have been resuspended separately, the necessary cell amounts should be combined and resuspended in a single, highly concentrated, final cell mixture, according to the following steps.

- Using 70 µm cell strainers, filter both CFb and CM separately into fresh 50 ml tubes. Dispense the cell suspension on the strainers at an angle applying sufficient pressure to facilitate passage through the mesh.
- Proceed to count both cell types. Store cells on ice during counting. Depending on your cell counting method of choice, diluting a small volume of the cell suspensions in their corresponding medium may be required.

Note: *We recommend using a cell counting method that discriminates viable cells (e.g. using trypan blue and a hemocytometer). Consider only the number of viable cells for the following step.*

- Calculate the volumes of each cell type resuspension required to obtain the correct cell concentration in the final cell mixture. Use the example in the cell-mixture calculations section as a reference.
- Mix the volumes of CFb and CM cell resuspension containing the required amounts of cells in a conical falcon tube.
- Centrifuge the cell mixture at 200 rcf for 5 minutes.
- During the centrifugation time, if not previously done, move the casting mold to the biological safety cabinet and place it on top of the cooling block on ice. Using a vacuum aspirator, remove the Pluronic coating solution and any condensation from the mold, ensuring no liquid is left before proceeding with the next steps.

Note: *During all the following steps and until assembly into the smart lid, the casting mold should be kept cold, preferably on a pre-chilled cooling block. If a cooling block is not available, the mold can be placed directly over ice instead.*

- Aspirate and discard the supernatant from the centrifuged cell suspension, taking care not to disturb the pellet.
- Resuspend the cell mix in AC-3D+T, using the volume indicated in the Casting mixture composition section, and place the tube containing the cell suspension on ice.

Casting the EHTs

- Using cold tips, pipette the required volume of collagen into the empty cold 15 ml conical tube previously placed on ice. As the solution is quite viscous, slow pipetting is recommended.
- With cold tips, add an equal volume of 2X RPMI+ to the collagen and mix well by gentle pipetting. The RPMI in the solution will turn yellow due to the acidic pH of the collagen. The color can assist in monitoring the homogeneity of the mix.

Note*: Pipette gently to avoid introducing bubbles.*

- Still employing cold tips, add the required amount of collagen neutralization solution to adjust the pH. The mixture will turn pink. Mix well by pipetting up and down until reaching a homogenous mix.
- With cold tips, add the cell suspension to the collagen solution and mix slowly 10 times, or until mixture is homogenous.
- Gently but quickly pipette 50 µl of casting mixture into each casting mold chamber, paying attention not to introduce air bubbles.
- Once all your tissues have been cast, quickly proceed to assemble the smart lid by placing the mold into the base frame, followed by fiber plate, cantilever plate, locking screws (if using Cuore V2.1), smart lid cover and a sterile well plate lid (you may reuse the one that was covering the mold before if sterility has been maintained). Please refer to *Cuore Manual* and *Handling video* for the correct assembling technique.

Note: *Please note that at this stage the stimulation plate should be left out of the smart lid assembly, as it is not compatible with the casting mold.*

- Move the assembled smart lid to the incubator (5% CO_2, 37°C), for 1 hour to allow polymerization of the EHTs.
- During this time, warm up the previously prepared AC-3D+T to 37° C.
- After the incubation, move the smart lid back to the biological safety cabinet (on the laptop stand if available) and gently add 1.5 ml of AC-3D+T to each mold well using a 1000 µl micropipette. Make sure to follow pipetting recommendations as described in *Cuore Manual* and *Handling video* and to proceed with care to avoid disturbing the forming 3D tissues.

Note: *We recommend only removing the well plate lid, keeping the smart lid cover on, when adding or changing the medium. This minimizes exposure to contaminants.*

- Place the smart lid back in the incubator, on top of a rocker and incubate in gentle oscillation (5-10 rpm).

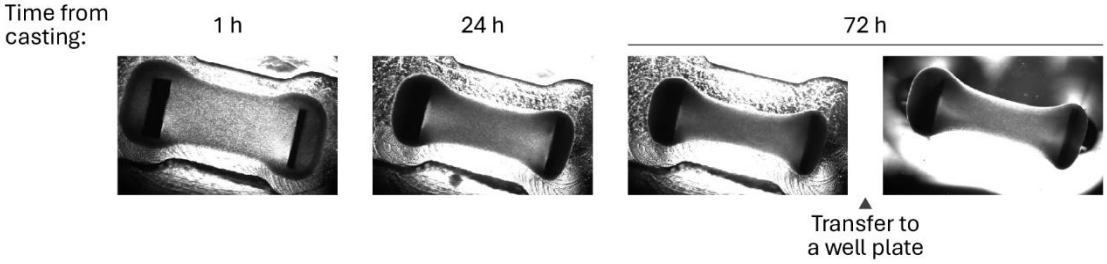
Note: *Maintaining the smart lid on top of a gently oscillating rocker (at 5-10 rpm) whenever not measuring preserves tissue viability over time. Make sure that the speed and angle of oscillation is such not to cause slippage of the smart lid or tilting above 25 degrees to prevent damage to the EHTs or spillage of medium.*

Following casting

For optimal viability and maturation of the EHTs, a complete medium refresh should be performed daily with freshly prepared medium. For the first 2 medium changes (day 1 and 2 after casting) 3D-AC+T should be used, to be substituted by 3D-AC from day 3 onwards. We recommend pairing this switch with the transfer of the EHTs from the casting mold to the 24 well plate, following the procedure below.

- Add 1.5 ml of warm 3D-AC to each well of a 24-well plate (Corning® Costar®, 3526).
- Disassemble the smart lid following handling instructions. Note that the tissues will briefly be out of the culturing medium, it is important to proceed with extreme care and work quickly to minimize any damage.
- Rapidly substitute the mold in the base frame with the previously prepared plate containing 3D-AC.
- Re-assemble the smart lid following same precautions indicated for assembly after casting. Note that at this step it is possible to add the stimulation plate to the smart lid, following handling recommendations from the *Cuore Manual* and *Handling video*.
- Move the assembled smart lid back to the incubator on top of the rocker and re-start gentle oscillation.

To monitor tissue compaction dynamics and morphological changes, it is good practice to regularly acquire pictures of the tissues. We recommend imaging approximately at 1 h, 24 h and 48 h after casting, before and after transferring from the casting mold to the 24 well culture plate, and at regular intervals throughout the experiment.



Typical compaction dynamics of EHTs cast following the present protocol.

Generally, EHTs show early signs of compaction already one hour after casting and are well compacted around the cantilevers by day 1 or 2. If tissues show slower compaction dynamics and the EHTs are not well detached from the mold and ready for transfer by day 3, the reagents and experimental procedure should be reviewed to identify any possible cause of failure.

It should be possible to detect spontaneous contractions of the EHTs already by day 2 or 3 from casting and forces increase progressively thereafter, plateauing around day 20-25. Note that in early days irregular spontaneous contractions, often unsynchronized across the tissue (resulting in double force peaks or very high apparent frequency of contraction), are common. Tissues contraction dynamics tend to get more regular after the first few days. Should forces not be detectable on day 3, or not increasing in the following days, this should be cause of concern and the reagents and experimental procedure should be reviewed.

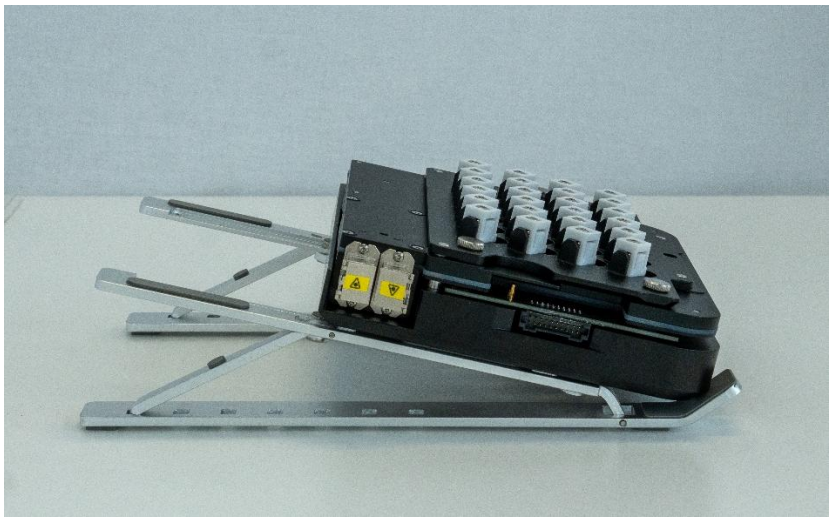
Note that the addition of fresh medium can acutely affect contraction kinetics. In order to maintain comparable conditions across days, we recommend recording contractions always at the same time in respect to medium change, and specifically either before or at least one hour after the change.

If electrical pacing of the EHTs is required by the experimental design, we recommend to time the daily change of medium after stimulation.

APPENDICES

1. Required equipment

- Biological safety cabinet
- Cell culture incubator
- Bench top centrifuge
- Vacuum aspirator
- Inverted microscope with 1x-2.5x objective
- Cell counter
- Standard cell culture ware: flasks, pipettes, pipette tips, sterile tubes
- Cuore smart lid, cleaned, autoclaved, and dried (see *Cuore Cleaning Protocol*)
- Corning® Costar® 24-well plates (cat. no. 3526)
- Heating/cooling block for well plates (optional but recommended; for example, cat. no. Z743493, Merck)
- Box for ice (recommended height 10-15 cm, to allow pipetting in the biological safety cabinet, and wide enough to fit a cooling block and a few conical centrifuge tube)
- Laptop stand with adjustable angle (optional but highly recommended)
- Incubator compatible rocker (for example ROCKER 2D basic 0004002000, IKA)



An example of a laptop stand used to place the Cuore smart lid under an angle, for better visibility of the tissues and safer pipetting. The angle should not exceed 25° to avoid spillage of the medium.

2. Required reagents

Reagent	Manufacturer	Cat. No.	Storage
Cuore cardiac tissue kit **containing:** • **axoCells™ hiPSC-derived ventricular cardiomyocytes (CM)** (2X 5 M vials) • **Cardiomyocyte maintenance basal medium** (3X 500 ml bottles) • **Cardiomyocyte maintenance medium supplements** (3X 10 ml)	Axol Bioscience X Optics 11 Life	Optics 11 Life 0040.0090.009	Liquid nitrogen vapor phase 4°C -20°C
Y-27632 ROCK inhibitor	Abcam	ab144494	4°C
Human cardiac fibroblasts (CFb)	Innoprot	P10452	Liquid nitrogen vapor phase
Fibroblast Medium-2 Kit **containing:** • **Fibroblast basal medium** • **Fetal bovine serum** • **Fibroblast growth supplement-2** • **Penicillin-streptomycin solution**	Innoprot	P60108-2	4°C -20°C -20°C -20°C
Poly-L-lysine (PLL)	Innoprot	PLL	-20°C
Primary Cells Detach Kit **containing:** • **DPBS (Dulbecco's phosphate-buffered saline)** • **Trypsin / EDTA solution (T/E)** • **Trypsin neutralization solution (TNS)**	Innoprot	P60305	Room temperature -20°C -20°C
Heat inactivated FBS (HI-FBS)	Gibco	A5256801	-20°C

Pluronic® F-127	Sigma-Aldrich	P2443	Room temperature
Collagen type I (TeloCol®-6 type I bovine collagen solution, 6 mg/ml)	Advanced Biomatrix	#5225	4°C
Collagen neutralization solution	Advanced Biomatrix	#5225 (Provided with Telo-Collagen)	Room Temperature
RPMI 1640 powder medium	Gibco	51800035	4°C
B-27™ supplement, minus insulin	Gibco	A1895601	-20°C
Bovine serum albumin	Sigma-Aldrich	A9647	4°C
Human IGF-I, animal-free recombinant protein (IGF-I)	Peprotech	AF-100-11	-20°C to -80°C
Human VEGF-165, animal-free recombinant protein (VEGF)	Peprotech	AF-100-20	-20°C to -80°C
Human TGF-beta 1, animal-free recombinant protein (TGF-β)	Peprotech	AF-100-21C	-20°C to -80°C
Human FGF-basic (FGF-2/bFGF) (154 aa), animal-free recombinant protein (bFGF)	Peprotech	AF-100-18B	-20°C to -80°C
Penicillin-streptomycin	Gibco	15070063	-20°C

3. Preparation and aliquoting of stock solutions

Reagent	Stock solution	Storage
PLL	Thaw on ice and aliquot in cold tubes at single use volumes (e.g. 150 µl).	-20°C
Trypsin / EDTA solution (T/E)	Thaw on ice and aliquot in cold tubes at single use volumes (e.g. 2 ml).	-20°C
Trypsin neutralization solution (TNS)	Thaw on ice and aliquot in cold tubes at single use volumes (e.g. 10 ml).	-20°C
5X RPMI	Dissolve in sterile ultrapure water at room temperature in constant agitation (it might take 1 hour or more). Lowering pH down to 4.0 with 1 N HCl might be necessary to enhance solubility. Re-adjust the pH to 7.2 with 1 N NaOH once the powder has completely dissolved and, if sterility has not been maintained, sterilize the solution by passing it through a 0.22 µm filter. Aliquot in single use tubes (e.g. 4 ml).	-20°C
Y-27632 ROCK inhibitor	10 mM Dissolve in sterile ultrapure water. Aliquot in single use tubes (e.g. 50 µl).	-80°C

Pluronic F-127	2.5% (w/v) in ultrapure water Gently shake/rock the solution to ensure complete dissolution (can take a few hours). Sterilize the solution by passing it through a 0.22 µm filter.	Room temperature, protected from light. Stable for at least a month.
IGF-I	100 µg/ml Reconstitute powder in 0.1% sterile, cell culture grade BSA. Working on ice, aliquot in cold tubes. We recommend making single use aliquots (e.g. 50 µl) and thawing fresh for medium preparation.	-20°C to -80°C
VEGF	5 µg/ml Reconstitute powder at an initial concentration of 100 µg/ml in 0.1% sterile, cell culture grade BSA. Working on ice, dilute to final concentration and aliquot in cold tubes. We recommend making single use aliquots (e.g. 50 µl) and thawing fresh for medium preparation.	-20°C to -80°C
TGF-β	5 µg/ml Reconstitute powder at an initial concentration of 100 µg/ml in 0.1% sterile, cell culture grade BSA. Working on ice, dilute to final concentration and aliquot in cold. We recommend making single use aliquots (e.g. 50 µl) and thawing fresh for medium preparation.	-20°C to -80°C
bFGF	10 µg/ml Reconstitute powder at an initial concentration of 100 µg/ml in 0.1% sterile, cell culture grade BSA. Working on ice, further dilute to final concentration and aliquot in cold tubes precoated with 0.2% BSA to minimize the protein adhesion to the plastic. We recommend making single use aliquots (e.g. 50 µl) and thawing fresh for medium preparation.	-20°C to -80°C

4. Media preparation

Fibroblast medium-2 (FM-2)

Reagent	Final concentration	For 500 ml
Fibroblast basal medium	-	500 ml
Fetal bovine serum	~5%	25 ml
Fibroblast growth supplement-2	~1%	5 ml
Penicillin-streptomycin solution	~1%	5 ml

Store medium at 4°C and use within a month.

Axol cardiomyocytes maintenance medium

Reagent	Final concentration	For 500 ml
Cardiomyocyte maintenance basal medium	98%	490 ml
Cardiomyocyte maintenance medium supplements	2%	10 ml

For long term storage, aliquot medium in smaller volumes and store at -80°C. Thaw overnight at 4°C before use and consume within 14 days.

2X RPMI+

Reagent	Final concentration	For 10 ml
5X RPMI	2X	4 ml
B-27™ supplement, minus insulin	-	0.8 ml
Ultrapure water (sterile)	-	5.2 ml

Aliquot into single use vials (400 µl aliquots are sufficient for the casting of 24 EHTs) and store at -20°C.

Axol cardiomyocyte plating medium (AC-PM)

Reagent	Final concentration	For 50 ml
Axol cardiomyocytes maintenance medium	90%	45 ml
HI-FBS	10%	5 ml
Y-27632 ROCK inhibitor 10 mM	10 µM	50 µl

The indicated volumes are indicative. Prepare the amount required for your experiment fresh on the day of casting (see recommended volumes in the step-to-step procedure).

Axol cardiomyocyte 3D medium with TGF-β (AC-3D+T)

Reagent	Final concentration	For 50 ml
Axol cardiomyocytes maintenance medium	~99%	49.3 ml
IGF-1 100 µg/ml	100 ng/ml	50 µl
VEGF 5 µg/ml	5 ng/ml	50 µl
bFGF 10 µg/ml	10 ng/ml	50 µl
TGF-β 5 µg/ml	5 ng/ml	50 µl
Penicillin-streptomycin	1%	500 µl

The volumes above are indicative. For example, for changing the medium of a full smart lid (24 EHT) 36 ml of medium are required, we therefore recommend preparing 38 ml or more, accounting for excess. Prepare the amount required for your experiment freshly on the day of casting or medium change. Always use freshly thawed aliquots of IGF-1, VEGF, bFGF and TGF-β and discard any leftovers.

Axol cardiomyocytes 3D medium without TGF-β (AC-3D)

Reagent	Final concentration	For 50 ml
Axol cardiomyocytes maintenance medium	~99%	49.35 ml
IGF-1 100 µg/ml	100 ng/ml	50 µl
VEGF 5 µg/ml	5 ng/ml	50 µl
bFGF 10 µg/ml	10 ng/ml	50 µl
Penicillin-streptomycin	1%	500 µl

The volumes above are indicative. For example, for changing the medium of a full smart lid (24 EHT) 36 ml of medium are required, we therefore recommend preparing 38 ml or more, accounting for excess. Prepare the amount required for your experiment freshly on the same day of medium change. Always use freshly thawed aliquots of IGF-1, VEGF and bFGF and discard any leftovers.

5. Cell mixture calculations

The final cell mixture that is incorporated into the collagen solution at EHT casting is obtained by combining the correct proportions of CFb and CM and resuspending them at high concentration into the volumes indicated in the Casting mixture composition section. In this section we provide an example of the calculations and procedure to obtain the correct cell concentration in the final cell mixture.

In the current example the planned experiment requires the casting of a full smart lid (24 EHTs) and to grant large excess the counts for 28 EHTs will be used. After cell resuspension, filtering, and counting the stock concentration of **viable** cells in each of the initial cell suspensions are as follows:

CFb: 7×10^5/ml in 10 ml

CM: 1.4×10^6/ml in 7 ml

The desired amount of cells in the final cell mixture, according to the table in Casting mixture composition section, is of 4.2 M CFb and 9.8 M CM. The volume of each cell suspension that must be combined into the mixture is calculated as:

$$Desired\ Volume\ (for\ each\ cell\ type) = \frac{Required\ cell\ amounts\ (M\ cells)}{Stock\ concentration\ (M\ cells/ml)}$$

For each cell type, given the stock concentrations above:

$$Desired\ Volume\ (CFb) = \frac{Required\ cell\ amounts\ (M\ cells)}{Stock\ concentration\ (M\ cells/ml)} = \frac{4.2\ M}{0.7\ M/ml} = 6\ ml$$

$$Desired\ Volume\ (CM) = \frac{Required\ cell\ amounts\ (M\ cells)}{Stock\ concentration\ (M\ cells/ml)} = \frac{9.8\ M}{1.4\ M/ml} = 7\ ml$$

Therefore 6 ml of the CFb initial resuspension and 7 ml of the CM initial resuspension will be combined into a conical tube, centrifuged and, once the supernatant medium has been removed, resuspended in 807.8 µl of AC-3D+T.

6. Culture of primary cardiac fibroblasts

Note: *The following protocols are derived from the supplier's recommendations. For further information please refer to: www.innoprot.com.*

When not explicitly stated otherwise, all steps should be performed in the biosafety cabinet, employing sterile technique and in compliance with biosafety regulations.

Coating the cell culture surface

- Thaw an aliquot of PLL on ice.
- In the biosafety cabinet, add 10 ml of sterile water to a T-75 flask and 150 µl of PLL (to a final concentration of 2 µg/cm^2). Transfer the flask for at least an hour or preferably overnight to the cell culture incubator at 37°C.
- After incubation, transfer the coated flask back to the biosafety cabinet and rinse twice with sterile water.

The coated flask can either be used directly for cell seeding or air-dried and stored at 4°C for up to a week. Bring back to room temperature before use.

Thawing

- Add 20 ml of room temperature fibroblast medium-2 (FM-2) to a room temperature PLL-coated flask.
- Quickly thaw a vial of CFb in a warm water bath, while gently moving the vial. Proceed to following step when most of the vial is thawed.
- Using a 1000 µl micropipette, gently resuspend the vial contents and transfer to the equilibrated flask. Rock gently to ensure homogeneous distribution of the cells.
- Move the flask containing the CFb to the cell culture incubator (5% CO_2, 37°C).
- At least 16 hours after plating, refresh the culture medium to remove residual DMSO and cell debris.

Maintenance and Passaging

After the first medium change, the medium should be refreshed either every third (for cultures below 70% confluence) or second day (for cultures above 90% confluence). The CFb should be passaged when they reach 90% confluency, preferably seeding at a 5000 cells/cm^2 concentration. Typically, this would result in a 90% confluent flask in 3-4 days. The following procedure describes the passaging procedure reporting the volumes required for a T-75 flask.

- Warm up to room temperature FM-2 (10 ml for resuspension + 20 ml for each flask to be seeded), 5 ml HI-FBS, 2 ml T/E and 10 ml TNS.
- Aspirate culture medium from the flask currently in culture.
- Rinse cells with 10 ml of DPBS, remove by aspiration.
- Add 8 ml DPBS and 2 ml T/E to the flask. Rock gently to ensure complete coverage.
- Move flask to the incubator for 1-3 minutes, monitoring at an inverted microscope from time to time.
- During incubation, add 5 ml of FBS to a 50 ml conical centrifuge tube.

- Once cells are rounded up and before a high percentage of them is detached, transfer the T/E solution from the flask to the tube containing FBS.
- Put back the flask without liquid into the incubator for an extra minute.
- Remove the flask from the incubator and gently tap the sides to favor cell detachment. Confirm detachment at the microscope and repeat if needed.
- Add 5 ml of TNS to the flask, pipette gently up and down to ensure rinsing the entire growth surface to collect all cells and transfer to the previously prepared 50 ml centrifuge tube containing FBS.
- Add an extra 5 ml of TNS to the flask, pipette gently up and down to ensure rinsing the entire growth surface once more and add to the same 50 ml centrifuge tube.
- Centrifuge the tube at 210 rcf for 5 minutes.
- During centrifugation, add 10 ml of room temperature FM-2 to the destination PLL-coated flask(s) and let equilibrate at room temperature.
- Aspirate supernatant from the centrifuged tube, paying attention not to disturb the cell pellet.
- Resuspend the cells in 10 ml of room temperature fibroblast medium-2 and count the cells employing your method of choice.
- Plate the required amount of cells into the equilibrated coated flask(s), top up the medium to a final volume of 20 ml and place in the cell culture incubator.

Note: *For best results, use CFb at passage 3 to 6 for casting EHTs in Cuore.*