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hiPSC-derived 3D cardiac microtissue models with integrated immune cells and vasculature

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Generation and characterization of hiPSC-derived vascularized-, perfusable cardiac microtissues-on-chip

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Abstract

In the heart *in vivo*, vasculature forms a semi-permeable endothelial barrier for selective nutrient- and (immune) cell delivery to the myocardium and removal of waste products. Crosstalk between the vasculature and the heart cells regulates homeostasis in health and disease. To model heart development and disease *in vitro* it is important that essential features of this crosstalk are captured. Cardiac organoid- and microtissue models often integrate endothelial cells (ECs) to form microvascular networks inside the 3D structure. However, in static culture without perfusion, these networks may fail to show essential functionality. Here, we describe a protocol to generate an *in vitro* model of human induced pluripotent stem cell (hiPSC)-derived vascularized cardiac microtissues on a microfluidic organ-on-chip platform (VMToC) in which the blood vessels are perfusable. First, prevascularized cardiac microtissues (MT) are formed by combining hiPSC-derived cardiomyocytes, ECs and cardiac fibroblasts in a pre-defined ratio. Next, these prevascularized MTs are integrated in the chips in a fibrin hydrogel containing additional vascular cells, which self-organise into tubular structures. The MTs become vascularized through anastomosis between the pre-existing microvasculature in the MT and the external vascular network. The VMToCs are then ready for downstream structural and functional assays and basic characterization. Using this protocol, cardiac MTs can be efficiently and robustly vascularized and perfused within 7 days. *In vitro* vascularized organoid and MT models have the potential to transition current 3D cardiac models to more physiologically relevant organ models that allow the role of the endothelial barrier in drug and inflammatory response to be investigated.

Basic Protocol: Generation of VMToC

Support protocol 1: Functional Characterization of VMToC

Support protocol 2: Structural Characterization of VMToC

Keywords:

human induced pluripotent stem cells; vessels-on-a-chip; cardiac microtissues; vascularization

Introduction

The healthy heart has one of the highest energy demands of all organs in the body. Vasculature is essential for regulating its function, providing (selective) nutrient-, cell- and drug delivery and removing waste products (Brutsaert, 2003). Interrupting vascular function can lead to cardiovascular disorders, a major cause of mortality worldwide (Berry and Duncker, 2020). Monitoring crosstalk between the vasculature and cells of the heart is therefore essential to address questions related to the heart development and disease and creating human models can be particularly beneficial for understating underlying

mechanisms. Human pluripotent stem cells are increasingly used as a basis for these models since they can differentiate to all cell types of the heart.

Endothelial cells (ECs) are often incorporated in organoids or microtissues (MTs) from stem cells so that they are pre-vascularized when used in *in vitro* studies (Cakir et al., 2019; Homan et al., 2019; Vargas-Valderrama et al., 2020; Giacomelli et al., 2020).Vascular networks in these tissues are often well-organized but usually not perfusable. Passive diffusion is then the only way small molecules, drugs and nutrients can penetrate organoids and MTs. Perfusion is also necessary for the stability and integrity of the microvascular networks and without it, these networks regress over time (Ryan et al., 2021). This has in part been rectified by transplanting these tissue constructs into living animals. This promotes the formation of functional vasculature through anastomosis where host vasculature and the microvascular network inside the transplanted tissues interconnect (Mansour et al., 2018; Takebe et al., 2013; Ryan et al., 2021). However, inter-species differences might alter vascularization and survival of the cells in MTs and organoids (Brady et al., 2023). We discovered that an alternative to transplantation is to place MTs in a microfluidic chip under flow: the presence of ECs in the chip outside the MT also supports anastomosis such that vasculature inside the MTs becomes lumenized (Arslan et al., 2023). Here we describe the protocol to generate these hiPSC-derived vascularized and perfusable cardiac MT on chip (VMToC) in detail.

In basic protocol 1, hiPSC-derived cardiomyocytes (CMs), ECs and cardiac fibroblasts (CFs) are thawed and combined to form pre-vascularized 3D cardiac MTs. The MTs are then combined with a fibrin hydrogel containing additional vascular cells and integrated within a channel of a commercially available microfluidic organ-on-chip platform. Vascular cells present in hydrogel start self-organizing into an external vascular network around the MTs and interconnect with the pre-existing internal vasculature in the MTs through anastomosis. Over the subsequent 5-7 days of culture in the microfluidic chips, continuous vascular networks become evident in- and around the MTs. The integrity and functionality of the vasculature formed in the VMToCs can be demonstrated by perfusing with fluorescent beads. This shows that vascular networks are lumenized and perfusable and is described in support protocol 1. Further functional characterization can be carried out by assessing either spontaneous- or (electronically) paced contractile kinetics of the beating MTs, as described in support protocol 1. Structural characterization of VMToCs can be performed by fixing and immunofluorescent staining, as described in support protocol 2.

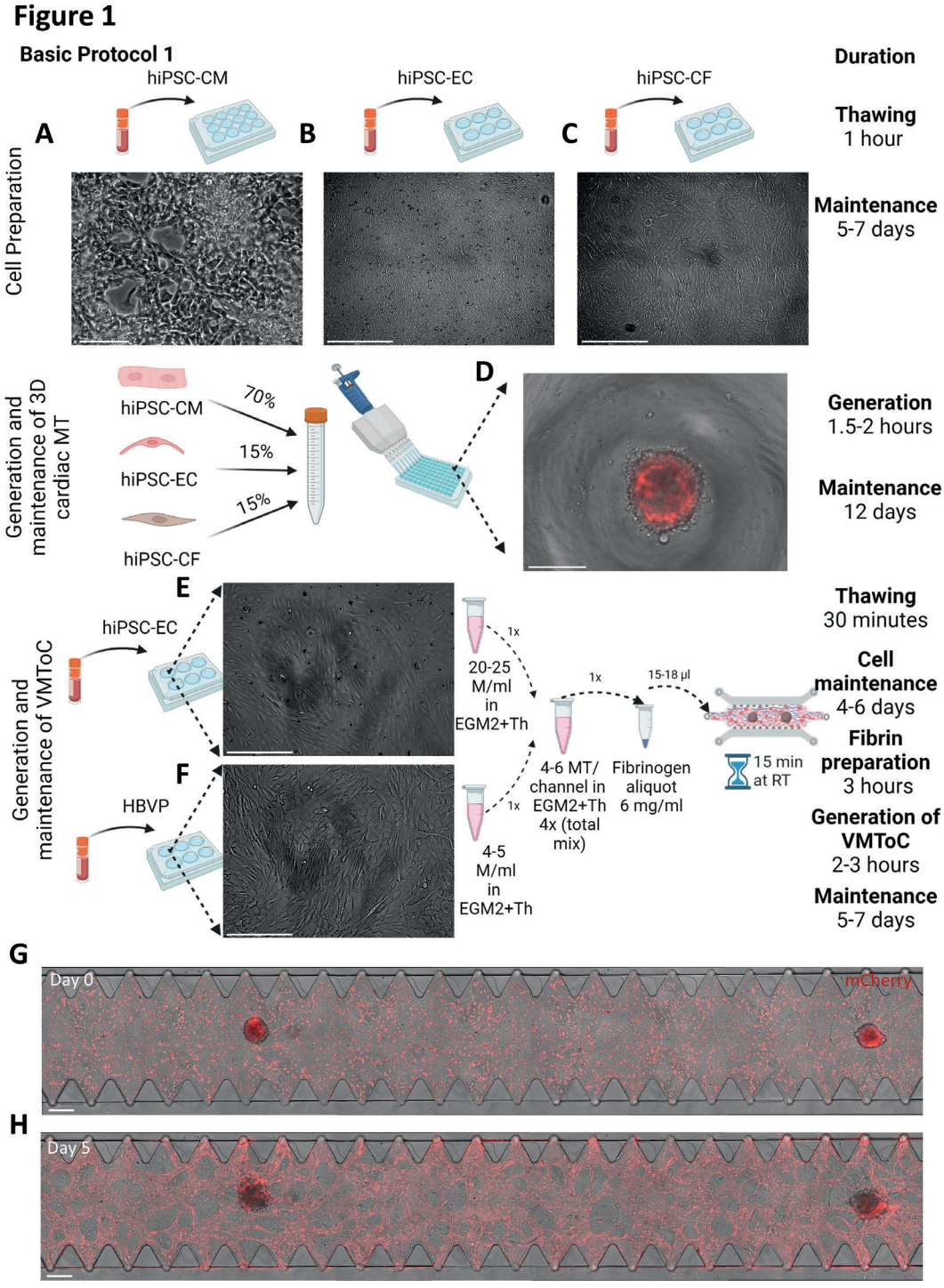

Figure 1: Schematic overview of the basic protocol 1 with representative images for each step. *(A) 10x Brightfield image of hiPSC-CMs on day 7 of thawing. (B-C) 4x Brightfield image of (B) hiPSC-ECs and (C) hiPSC-CFs on day 6 of thawing. (D) 10x Brightfield image of hiPSC-derived cardiac MTs on day 12 that shows prevascularization*

of MTs (mCherry, red). (E-F) 10x Brightfield image of (E) hiPSC-ECs and (F) HBVPs on day 6 of thawing. (G) Brightfield image of VMToCs on day 0 showing homogenous distribution of hiPSC-ECs (mCherry, red) and MTs. (H) Brightfield image of VMToCs on day 5 showing continuous vascular network developed in and around the MTs (mCherry, red). Scale bar – (A): 200 µm; (B, C): 675 µm; (D-F): 275 µm; (G, H): 250 µm. hiPSC, human induced pluripotent stem cells; CM: cardiomyocytes; EC, endothelial cells; CF, cardiac fibroblasts; HBVP, human brain vascular pericytes, MT, microtissues; VMToC, vascularized cardiac microtissue on a chip; EGM2, endothelial cell growth medium-2; Th, thrombin.

Basic Protocol

Generation of VMToC

Introductory paragraph

This protocol provides step-by-step methodology to generate hiPSC-derived 3D vascularized cardiac microtissues using a commercially available AIM Biotech IdenTx-9-Plate organ-on-chip platform. The protocol is divided into three sections. The first section describes the preparation of three different hiPSC-derived cardiac cell types to generate MTs (Fig. 1A-C). Cardiac MTs are then formed by combining the hiPSC-derived cardiac cells in a pre-defined ratio in V-shaped wells of a 96-well plate, as described previously, with minor modifications (Campostrini et al., 2021). The cells form single aggregates in each well and within 1 day, are compact and contractile cardiac MTs. hiPSC-ECs inside the MTs self-organize to form microvascular networks but without functional lumen (Fig. 1D). A mixture of hiPSC-derived endothelial cells (hiPSC-ECs, Fig. 1E), human brain primary pericytes (HBVPs, Figure 1F), the MTs and fibrinogen is pipetted into the middle channel of the AIM Biotech chips (Fig. 1G). Chips are maintained for 7 days under gravity-driven flow.

Starting from day 1 of seeding into the chip, hiPSC-ECs and HBVPs self-organize and form an external vascular network around the MTs. This external network begins interconnecting with the internal microvascular network inside the MTs in the chips (anastomosis). On day 3, vascular development continues and the anastomosis becomes more prominent. On day 5, vascular networks stabilize and a continuous vascular network in- and around the MTs is visible (Fig. 1H). Chips can be maintained until day 7. To improve vascular development further, chips can be placed at a later stage of culture (on day 3 (Fig. 2) or on day 5 (Fig. 3)) on a rocker platform to induce continuous perfusion. This promotes faster vascular development and shortens the total culture period. After the external network is developed, chips can be used for downstream applications such as modulating EC-CM crosstalk with external stimuli (Arslan et al., 2023) and testing cardiotoxicity of drugs (Di Cio et al., 2024). Characterization of these chips using structural and functional assays can be done as described in support protocol 1 and 2.

Figure 2

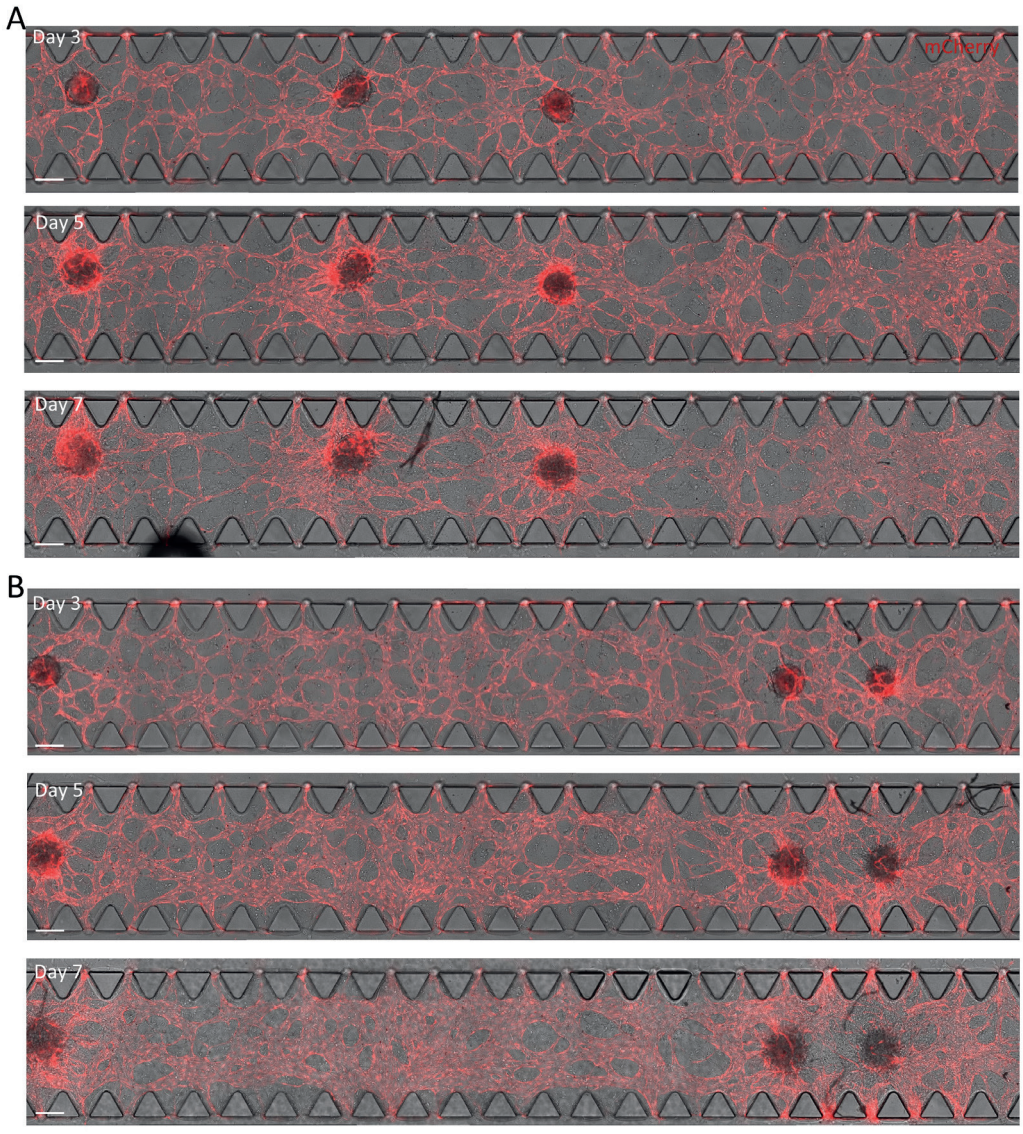

Figure 2: *(A-B) Brightfield images of 2 example VMToCs on day 3, 5 and 7 with continuous perfusion started on day 3 (mCherry, red). Scale bars: 250 μm*

Figure 3

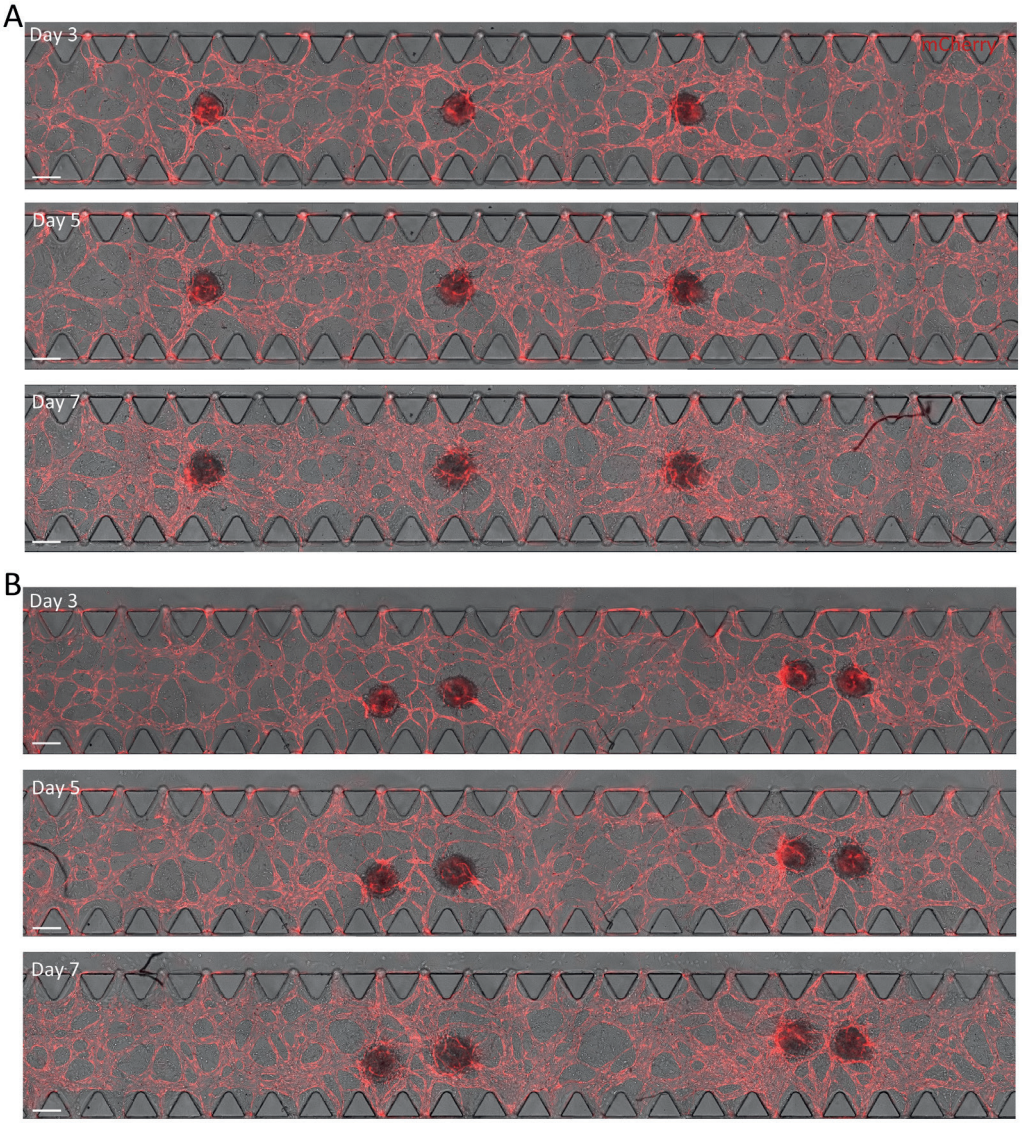

Figure 3: *(A-B) Brightfield images of 2 example VMToCs on day 3, 5 and 7 with continuous perfusion started on day 5 (mCherry, red). Scale bars: 250 µm*

Materials

- Cryopreserved hiPSC-derived CMs (Campostrini et al., 2021), ECs (Orlova et al., 2014) and CFs (Campostrini et al., 2021)
- Sterile biosafety cabinet (CleanAir)
- B(P)EL (see recipe) or MBEL (see recipe) medium, depending on the protocol used to differentiate hiPSC-CMs (Campostrini et al., 2021)
- L-Ascorbic acid 2-phosphate (AA2P), 5 mg ml–1 (see recipe)
- α-Monothioglycerol (αMTG) solution, 1.3% (v/v) (see recipe)
- Endothelial cell growth medium (EC-CGM) (see recipe)
- Fibroblast Growth Medium 3 (FGM3) (PromoCell, cat. no. C-23025)
- Matrigel coated 12 well plate (see recipe)
- Water bathe (Julabo TW20; MCO-18AIC Serial no. 08010017)
- Pipetman starter kit p20, p200, p1000 (Gilson international; cat. no. F167500S)
- 10-, 200-, and 1000-µl filter tips (Corning, cat. no. 4807, 4810, and 4809, respectively)
- Integra Pipetboy acu 2 (Boom B.V., cat. No. T155016)
- 5-, 10-, and 25-ml sterile plastic serological pipettes (Greiner Bio-One, cat. no. 606180, 607180, and 760180, respectively)
- 15- and 50-ml polystyrene conical tubes (Corning Falcon, cat. no. 352097 and 352098, respectively)
- Filtertips 1000 µl Biosphere® plus 96/Box (Sarstedt, cat. no. 70.3050.255)
- Centrifuge (Eppendorf, cat. no. 5810R)
- RevitaCell™ Supplement (100X (Thermo Fisher Scientific, cat.no. A2644501)
- CO2 cell culture incubator (Sanyo)
- Gelatin coated plate (see recipe)
- V-bottom 96 well microplates (Greiner Bio-one; cat. no. 651161)
- Dulbecco's phospohate-buffered saline without Ca+2 and Mg+2 (DPBS, Gibco, cat. no. 14190-094)
- TrypLE™ (5X) (see recipe)
- TrypLE™ Select Enzyme (1X), no phenol red (Gibco, cat. no. 12563029)
- Fuchs-Rosenthal Manual cell counter (VWR, cat. no. 631-0929)
- Trypan blue (Sigma-Aldrich, cat no, T8154-20ML)
- Human VEGF (165) IS, premium grade (Miltenyi Biotec, cat. no. 130-109-386)
- Human bFGF, premium grade (Miltenyi Biotec, cat. no. 130-093-842).

- 25-ml single use reservoirs (Avantor, cat. no. 613-1175)
- Finnpipette Multichannel Pipette p200 (ThermoFisher)
- Pericyte Medium (ScienCell, cat. no. 1201)
- Human Brain Vascular Pericytes (HBVPs; ScienCell, cat. no. SCC1200)
- Fibrinogen (Sigma-Aldrich, cat. no. F8630)
- Puradisc FP 30 Cellulose Acetate Syringe Filter, 0.2 µm, sterile (Cytiva, 10462200)
- BD Luer-Lok Syringe 50 ml (BD, cat. no. 300865)
- 1.5-ml microcentrifuge tubes (Eppendorf, cat. no. 0030 120.086)
- Eppendorf tubes 0.5ml (25-022-CI)
- EGM2 medium (see recipe)
- Thrombin (Sigma-Aldrich, cat. no. T4648-1KU)
- idenTx 9 Plate Box of 8 (Aim Biotech, cat. no. IDTX9)
- Chip medium (see recipe)
- DAPT (Sigma-Aldrich, cat. no. D5942)
- OrganoFlow® Rocker (Mimetas, cat. no. MI-OFPR-L)
- NIKON Eclipse Ti microscope

Protocol steps with step annotations

Cell preparation for MTs

This protocol is followed under aseptic conditions.

1. Follow the protocol of Campostrini et al (2021) for hiPSC differentiation, maintenance and cryopreservation of CM and CF.
2. Follow the protocol of Orlova et al (2014) for hiPSC differentiation, maintenance and cryopreservation of ECs.

If hiPSC derived cells are cryopreserved for later use, follow step 3 for thawing. Recovery period of hiPSC-CMs is 5-7 days, for hiPSC-ECs and hiPSC-CFs it is 4-6 days. Alternatively, freshly differentiated cells can be used to generate MTs. In this case, skip to step 32.

3. Pre-warm B(P)EL or MBEL, EC-CGM and FGM3 medium to room temperature (RT).
4. Prepare Matrigel coated plates (see recipe) to thaw hiPSC-CMs.
5. Thaw a vial of cryopreserved hiPSC-CMs in a hot water bath at 37 °C by moving it in a circular motion in the water until only a small piece of ice with a diameter of 1-2 mm is left inside the tube.
6. Transfer hiPSC-CMs into a 50-ml tube using 1000 µl Biosphere® filtertips.
7. Wash the vial with 1 ml B(P)EL or MBEL medium and add this on the cells drop by drop with a 1000 µl Biosphere® filtertips. Swirl the 50-ml tube with every droplet to mix and dilute the freezing medium of the cells.

Add the droplets every 6-8 seconds until the medium/freezing medium ratio reaches 10x. Then, add the droplets every 3-4 seconds until the medium/freezing medium ratio reaches 15-20x.

Swirling the tube gently is essential for higher yield of thawed hiPSC-CMs.

Using 1000 µl Biosphere® filtertips to thaw CMs is recommended (see materials). This will prevent hiPSC-CMs stress and adherence to the pipette tip walls and improve recovery.

8. Gently transfer cell/medium mix from the 50-ml tube to a 15-ml tube using a 5-ml pipette.
9. Centrifuge the 15-ml tube at 300x g for 3 mins.

After centrifugation, the pellet should be clear and compact at the bottom of the tube. If this is not the case, there might be a problem with centrifugation and a second round of centrifugation might be necessary.

10. Aspirate the supernatant.

Do not aspirate in close proximity to the pellet. There can be small amount (<20 µl) of medium left on the pellet.

11. Add 1 ml of B(P)EL or MBEL supplemented with RevitaCell (0.5x) on hiPSC-CM pellet and gently mix until there are no cell clumps visible.

Being gentle is essential in this step. Mix by pipetting up and down with a 1000 µl Biosphere® filtertips slowly until no cell clumps are visible.

12. Aspirate Matrigel from the coated plate and add the hiPSC-CMs in the coated 12-well in a circular move to distribute the cells.

Due to low recovery rate (30-40%) of hiPSC-CMs, it is recommended to thaw a vial of 3 million frozen hiPSC-CMs into one well of a 12-well plate. Recovery rate might vary between lines. With higher recovery rate (>60%), a vial can be thawed using a lower seeding density.

13. Place the plate in a 37 °C, 5% CO2 incubator for 24 hours. Refresh the medium after 24 hours with B(P)EL or MBEL medium. Then refresh the medium every 2-3 days.
14. Prepare Gelatin coated plates (see recipe) to thaw hiPSC-ECs.
15. Add 6 ml of pre-warmed EC-CGM in a 15-ml tube.
16. Thaw a vial of cryopreserved hiPSC-ECs in a water bath at 37 °C by moving the vials in a circular motion in the water until only a small piece of ice is left inside the tube.
17. Transfer hiPSC-ECs into a 15-ml tube with EC-CGM inside using a 1-ml pipette tip.
18. Wash the vial with 1 ml EC-CGM to collect all the cells and add this to the 15-ml tube.
19. Add 6 ml of pre-warmed FGM3 medium in a 15-ml tube.
20. Thaw a vial of cryopreserved hiPSC-CFs in a water bath at 37 °C by moving the vials in a circular motion in the water until only a small piece of ice is left inside the tube.
21. Transfer hiPSC-CFs to a 15-ml tube with a 1-ml pipette tip.
22. Wash the vial with 1 ml FGM3 to collect all the cells and add this to the 15-ml tube.
23. Centrifuge all tubes at 300x g for 3 mins.
24. Aspirate supernatants from each tube.
25. Add 1 ml of EC-CGM to hiPSC-ECs.
26. Mix gently by pipetting up and down with 1-ml pipette tip until no cell clumps are visible.
27. Aspirate 0.1% Gelatin from the coated 6-well plate.
28. Add hiPSC-ECs to these wells in 2 ml EC-CGM medium per well in desired ratio.

Seeding density highly depends on the proliferation and recovery rate of the cells and the culturing period. hiPSC-ECs are cryopreserved as 250-300K cells per vial. Seed in 1:4 ratio if hiPSC-ECs are used on day 3 of seeding and 1:6 if they are used on day 5 of seeding. With these ratios, at the end of the culture period, there should be between 250-300K hiPSC-ECs cells from 1 confluent (>90%) well of a 6-well plate.

29. Add 1 ml of FGM3 medium to hiPSC-CFs.
30. Add hiPSC-CFs to uncoated wells of a 6-well plate in 2 ml FGM3 medium per well in desired ratio.

Proliferation rate of hiPSC-CFs might change depending on the passage number. Cell seeding ratio should be adjusted accordingly.

31. hiPSC-ECs and hiPSC-CFs are incubated in a 37 °C, 5% CO_2 incubator. After 24-48 hours cells are refreshed with EC-CGM (ECs) or FGM3 (CFs). Then cells should be refreshed every 2-3 days.

In order to skip weekend refreshment, the medium can be topped up with 1 ml of extra medium on Friday.

MT formation

32. Pre-warm B(P)EL medium to room temperature.
33. Prepare V-bottom 96-well plates for MT seeding by labelling and adding 150 µl DPBS (-) in the outer wells of plates to prevent medium evaporation.
34. Aspirate the medium from the hiPSC-CMs and wash the well with 1 ml DPBS (-) before dissociation to remove dead cells.
35. Dissociate hiPSC-CMs with 0.5 ml TrypLE 5x per well for 10-12 mins at 37 °C, 5% CO_2.
36. Add 1.5 ml B(P)EL per well to a 15-ml tube.
37. After incubation with TrypLE 5x, dissociate hiPSC-CMs further by pipetting up and down with a 1000 µl Biosphere® filtertips until no clumps are visible.

After 10-12 mins incubation with TrypLE 5x, cells should be dissociated from the wells by pipetting up and down several times in TrypLE 5x until the mixture is completely uniform and no cell clumps are visible.

38. Add the hiPSC-CMs to the 15-ml tube with B(P)EL inside.
39. Wash the well with 1 ml of B(P)EL to collect all the cells.
40. Aspirate the medium from hiPSC-ECs and wash the well with 1 ml DPBS (-) before dissociation to remove dead cells.
41. Dissociate hiPSC-ECs with TrypLE 1x for 2-3 mins at RT.
42. Add 1 ml of B(P)EL to hiPSC-ECs to dilute the dissociation buffer.
43. Mix gently by pipetting up and down with a 1-ml pipette tip 3-4 times.
44. Add the cells to a 15-ml tube.
45. Aspirate the medium from hiPSC-CFs and wash the well with 1 ml DPBS (-) before dissociation to remove dead cells.
46. Dissociate hiPSC-CFs with TrypLE 1x for 3-5 mins (until the cells detach from the well and round up as single cells) at 37 °C, 5% CO_2.
47. Add 1 ml of B(P)EL to hiPSC-CFs to dilute the dissociation buffer.
48. Mix gently by pipetting up and down with a 1-ml pipette tip 3-4 times.
49. Add the cells to a 15-ml tube.
50. Take a 20 µl aliquot from each cell types to count using Fuchs-Rosenthal manual cell counter or any equivalent.

Aliquots can be diluted 1:2 or 1:3 either in B(P)EL or trypan blue to make counting easier.

51. Centrifuge 15-ml tubes with cells at 300x g for 3 mins.
52. Aspirate the supernatant from the tubes.
53. Add 1 ml B(P)EL in each tube and mix gently by pipetting up and down with 1000 µl Biosphere® filtertips until no clumps are visible in the tubes.
54. Prepare a mixture to form MTs containing 5000 cells in 50 µl of B(P)EL medium supplemented with 50 ng/ml VEGF and 5 ng/ml FGF for each well in a 96-well plate in the defined ratio of 70% hiPSC-CMS, 15% hiPSC-ECS and 15% hiPSC-CFS.

To ensure there is sufficient cell mixture for all wells of a 96-well plate, calculate medium and cell mixture for slightly higher number of wells. For example, there are 60 inner wells in a 96-well plate: make sufficient cell mixture for 66 wells i.e. 66x50 µl = 3300 µl of B(P)EL in total with cells for 1x 96-well plate. The outer wells are left empty.

55. Transfer the MT mixture in a 25-ml single use reservoir.
56. Seed 50 µl of MT mixture in the inner wells of a 96-well plate using a multichannel pipette.

During seeding of MTs, it is recommended to rock the reservoir with cells as this will ensure good mixing and cells will not sediment at the bottom of the reservoirs.

57. Centrifuge the plates at 300x g for 10 mins at RT.
58. Incubate MT plates at 37 °C, 5% CO_2 for 3 days without disturbing.
59. Refresh MT medium every 3-4 days with B(P)EL supplemented with 50 ng/ml VEGF and 5 ng/ml FGF until the chip experiments (step 82).

Microfluidic chips

Cell preparation for external network

60. Thaw hiPSC-ECs as described in step 14-18.
61. Add 6 ml of pre-warmed Pericyte medium in a 15-ml tube.
62. Thaw a vial of cryopreserved HBVPs in a water bath at 37 °C by moving the vials in a circular motion in the water until only a small piece of ice is left inside the tubes.
63. Transfer HBVPs to the 15-ml tube using a 1-ml pipette tip.
64. Wash the vial with 1 ml Pericyte medium to collect residual cells and add this to the 15-ml tube.
65. Centrifuge the 15-ml tube at 300x g for 3 mins.
66. Seed the cells in 1:4 ratio to use on day 3 and 1:6 to use on day 5.

In this protocol HBVPs were expanded until passage 3 and cryostored 1 M cells per vial.

67. Incubate the cells in a 37 °C, 5% CO_2 cell culture incubator for 48 h. Refresh with pericyte medium after 48 h and after that every 3-4 days.

Fibrinogen preparation

68. Prepare a 6 mg/ml fibrinogen/PBS solution in a 50-ml tube.
69. Incubate this solution for 3 h in a 37 °C water bath with a shaker.
70. After 3 h of incubation, filter the solution and collect it in a 15-ml tube. Place this tube on ice until use.
71. Prepare 10 µl aliquots of fibrin in 0.5 ml Eppendorf tubes on ice.

Chip seeding

72. Aspirate the medium from hiPSC-EC and HBVP and wash wells with DPBS (-) to remove dead cells.
73. Dissociate hiPSC-ECs and HBVPs with 1 ml of TryplE 1x for 2-3 mins until the cells detach from the wells and round up at RT.
74. Dilute the dissociation mix with EC-CGM and Pericyte medium for hiPSC-EC and HBVPs, respectively.
75. Transfer the cells to 15 ml tubes.
76. Wash the wells with respective medium to collect all the cells and transfer to 15-ml tube.
77. Take a 20 µl from each cell type to count.
78. Centrifuge the cells at 300x g for 3 min.
79. Aspirate the supernatant as much as possible without affecting the cell pellet.

It is important to remove the supernatant as much as possible for precise cell addition to the chips.

80. Prepare a 1.5 ml Eppendorf with EGM-2 medium supplemented with Thrombin (4U/ml).
81. Dilute hiPSC-EC and HBVP with EGM-2/Thrombin mix in separate 1.5 ml Eppendorf tubes.

In this protocol the cells are counted manually. Check for operator-to-operator variability. The cells are diluted as following: 25x10⁶ hiPSC-ECs per 1 ml and 5x10⁶ HBVPs per 1 ml (5:1 cell ratio).

82. Collect 30-40 MTs (for 1X idenTx 9 Plate) from the cultured plates (from step 59) using a 1-ml pipette tip from each well of a 96-well plate in a 15-ml tube.
83. Place the tube on a rack until MTs sediment.
84. When MTs are visible at the bottom of the tube, transfer MTs to a 1.5 ml Eppendorf tube using a 1-ml pipette tip.
85. When MTs are visible at the bottom of the Eppendorf tube, aspirate as much of the supernatant as possible.

86. Mix EGM2/thrombin mix with hiPSC-ECs and HBVP.

The amount of EGM-2/Thrombin to add to MTs should be decided according to the target number of channels to be loaded simultaneously. For example; each channel requires 10 µl of cell/MT mix. For 1 full plate with 9 channels, at least 90 µl of cell/MT mix is necessary. However, for precision it is recommended to add 20-30 µl extra cell/MT mix. 120 µl of cell/MT mixture is thus necessary for 1 full plate of chips. 1:1:2 (hiPSC-EC : HBVP : EGM-2/Thrombin) ratio should be followed to prepare this mixture. Following the example of 120 µl, 30 µl of hiPSC-EC, 30 µl of HBVP and 60 µl of EGM-2/Thrombin mix should be added on top of MTs, assuming all of the supernatant is removed from the MT vial.

87. Add EGM2/Thrombin and cell mix into the Eppendorf tube with MTs.
88. Mix the cell/MT/EGM-2/Thrombin mixture by pipetting up and down with a 20 µl pipette tip and take an aliquot with MTs.
89. Mix the cell/MT/EGM-2/Thrombin mix with fibrin in 1:1 ratio.

Example: Add 10 µl cell/MT/EGM-2/Thrombin mix to 10 µl fibrin aliquots.

90. Mix gently and fast by pipetting up and down with a 20 µl pipette tip not more than 3-4 times.

In order to increase the success of MT seeding in the gel channels, the cell/MT/fibrin mix should be added to one gel channel at a time. To semi-automate this process, a multi dispenser pipette can be used although it can be challenging to control the number of MTs; using this type of pipette is therefore not recommended.

91. Take 15-18 µl of this cell/MT/fibrin mix with a 20 µl pipette and seed via the gel inlet of the idenTx 9 plate chips (Fig 1).

Seeding of all channels should be performed in one continuous movement and the dispensing speed should be constant between channels to prevent channel-to-channel variability.

92. Incubate the chips for 15 mins at RT.
93. Prepare a chip medium (see recipe) during the incubation step 92
94. Slowly add 18 µl of chip medium to the inside of the left medium inlets (Fig 1).

See the medium inlets slowly filling. Medium should be visible from the right medium inlets.

95. Fill the left medium reservoir with 50 µl of chip medium and the right one with 100 µl to induce gravity-driven flow.
96. Incubate the chips in the 37 °C, 5% CO_2 cell culture incubator for 24 hours.
97. On day 1, refresh the medium with chip medium supplemented with DAPT (10 µM) for 24 hours.
98. Starting from day 2, refresh the medium with chip medium (without DAPT) daily until day 7.

In order to induce bi-directional continuous perfusion, chips can be incubated on a rocking platform using 8 degrees of inclination and 5 min intervals. Continuous perfusion by rocking can be induced as early as on day 0. However, since continuous perfusion accelerates vessel development, it is recommended to implement downstream assays on these chips earlier than day 7. Alternatively, chips can be placed on a rocker platform at a later stage (day 3 or 5) to further improve the vascularization process (See fig. 2 and 3).

Support Protocol 1
Functional Characterization of VMToC

Introductory paragraph

After generation of VMToCs, the structure and the integrity of vasculature can be assessed via a vessel perfusion assay with fluorescent beads to determine whether functional lumens have formed (Fig. 4A-B).

Figure 4

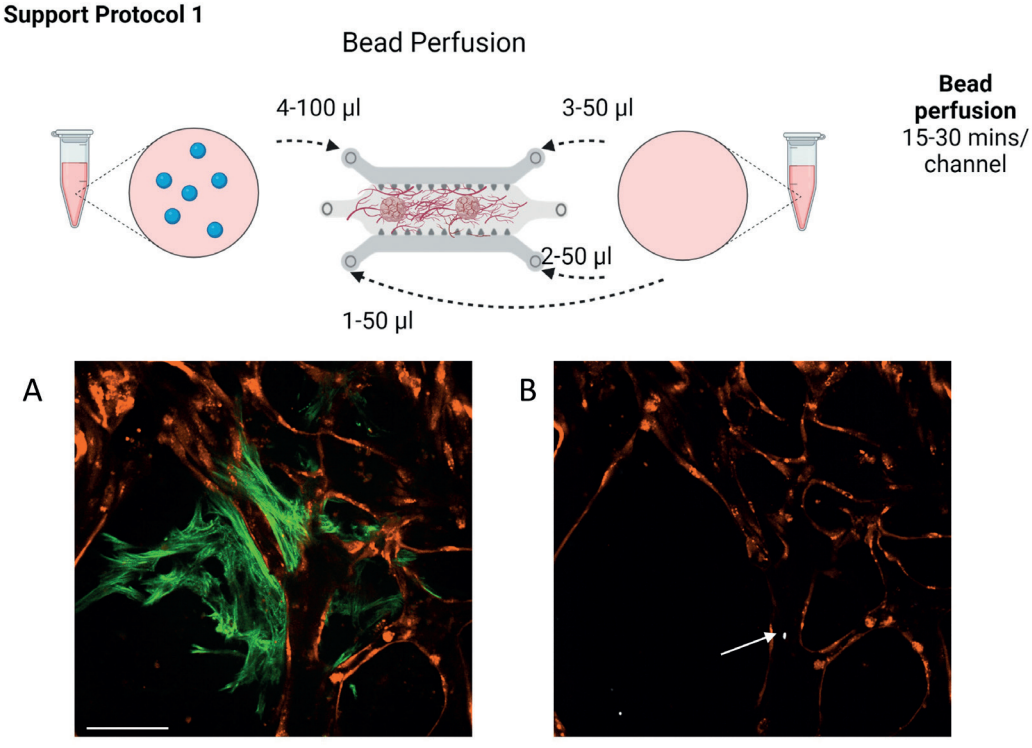

Figure 4: Schematic overview of the support protocol 1: Bead perfusion assay. *(A) Immunofluorescent imaging of VMToCs on day 5 (mCherry, orange; ACTN2, green) prior to the bead perfusion assay. (B) Immunofluorescent imaging of VMToCs with bead perfusion, arrow showing a bead moving in the microvasculature (mCherry, orange; 405 nm-beads, white). Scale bar: 100 µm*

In addition, VMToCs are contractile throughout the experiment. Therefore, it is important to follow their contractile dynamics live. In order to quantify the contractile parameters, VMToCs are recorded during spontaneous- or paced contractions. Pacing is performed using in-house electrodes that were designed to fit in the gel channels of the chips (Fig. 5). This protocol describes the pacing and recording videos for analysis with MuscleMotion software (Sala et al., 2018; van Meer et al., 2018) although other alternatives (Stebbeds et al., 2023; Huebsch et al., 2015a) can also be used.

Figure 5

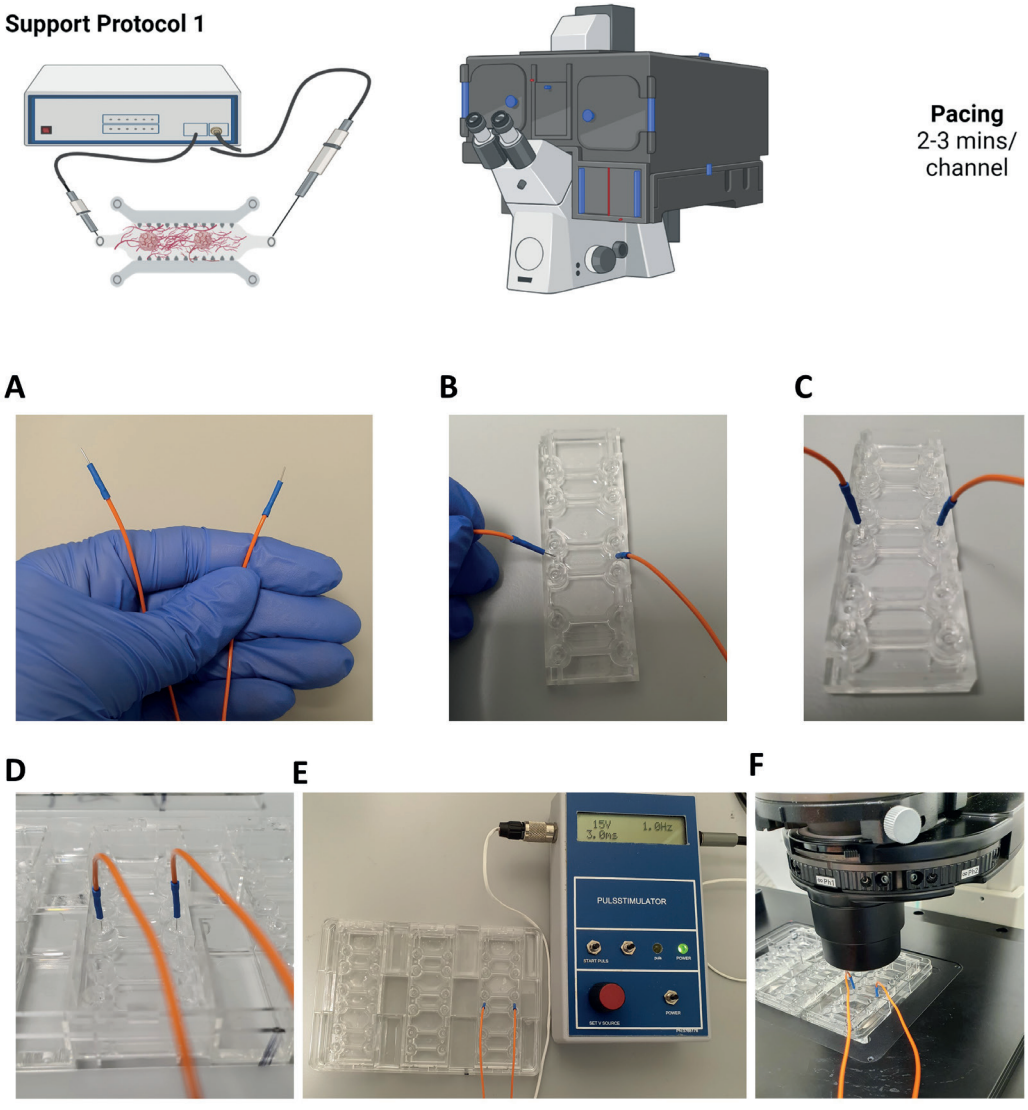

Figure 5: Schematic overview of the support protocol 1: Pacing. *(A) In-house electrodes that were used to pace VMToCs. (B) Picture of the electrodes and the chip showing integration of electrodes in the gel channel. (C) Side view of the chip with electrodes inside the gel inlet and outlet. (D) Bottom view of the plates with electrodes inside the gel inlet and outlet of a chip. (E) Pacing arrangement showing the settings for the experiment. (F) Picture of the plate with electrodes in the microscope stage.*

Materials:

- VMToC plates (Basic protocol 1)
- 405-beads (Fluoro-Max Dyed Blue Aqueous Fluorescent Particles, ThermoFisher Scientific, cat. No. B0200)
- EVOS M7000 imaging system (Thermo Fisher Scientific, cat.no. AMF7000)
- Andor Dragonfly 500 spinning disk confocal microscope
- Imaris 9.5 software (Bitplane, Oxford Instruments)
- Inverted microscope with a custom-built environmental chamber
- High-speed camera (DC3260C -High-Resolution Thorlabs)
- Custom-made pacing setup with platinum electrodes
- MUSCLEMOTION ImageJ macro (ImageJ v. 2.0.0-rc-49)

Protocol steps with step annotations

Bead perfusion assay

1. Prepare two 15-ml tubes with 10 ml chip medium (see recipe).
2. Add fluorescent bead droplets in one of the 15-ml tubes in a desired concentration (1 drop in 10 ml medium).

For bead tracking, low concentrations of beads are recommended. At low concentrations, such as 1 drop of beads mix for 10 ml chip medium, beads do not cluster together and enter the microvascular networks inside the MTs one- by-one. For higher concentrations, 1 drop of beads can be diluted in 5 ml of chip medium.

3. Prepare the appropriate microcopy station and insert the chip or the plate on imaging stage.

The use of an on-stage incubator with the microscope is recommended. MTs might slow down or stop beating when they are at room temperature for long periods. In this protocol, the Andor confocal spinning disk 500, 20x or 40x objectives are used to image dual wavelengths simultaneously. Alternatively, other confocal microscopes with similar features can be used.

4. Arrange the required field of view in which the lumen is visible with endothelial edges. This is where beads will move through.

It is recommended to take pictures of the desired field of views prior to running the bead perfusion assay (Fig. 4A).

5. A Add 50 µl of chip medium to 3 out of 4 medium reservoirs of the channel of interest.
6. Add 100 µl of chip medium with beads to the 4th medium reservoir of the channel.
7. Start recording of the video and save all the frames for analysis.

When using fluorescently tagged hiPSC-ECs in the channels, a microscope able to record 2 fluorescent channels simultaneously is recommended. If not available, it is recommended to overlay the recorded video of the fluorescent beads with the brightfield or fluorescent image taken prior to video recording in step 4.

Recording can take as long as a minute for beads to move inside the lumens. Extended recording might result in photobleaching of the fluorescently tagged cells. For video analysis, Imaris 9.5 software is used..

Pacing

8. Turn on the on-stage incubator of the inverted microscope to 37 °C, 5% CO2.
9. Place the chips inside the microscope stage when the temperature is stabilized.

With the change of temperature, contraction of MT might change. It is recommended to apply step 9 at least 30 mins before the start to allow MTs to adapt the stable temperature.

For optimal quality of the videos, they were recorded with high-speed camera capturing 100 frames per second, with 10x magnification and the field of view focused on the central MT part, avoiding the vessels or gel around. The same lighting settings were used for all the recordings to minimize the detection error in later video analysis..

10. Record movies of spontaneously beating MTs with 100 frames per second acquisition speed.

At this step there should be at least 2-3 contractions captured for optimal analysis. The duration of videos will depend on the cell line of origin.

11. Insert a pair of pacing electrodes into the inlet and outlet of the gel channel.
12. Perform the electric field stimulation of tissues with bipolar rectangular pulses of 3ms duration, 15V amplitude, using 0.8 or 1HZ frequency.

It might be necessary to optimize the voltage amplitude according to the gel channel size.

Pacing frequency should be determined according to the spontaneous contraction frequency. In order for tissues to follow the imposed rate, it is recommended to pace with at least 1-1.5x higher frequency than the spontaneous contraction frequency.

13. Record the videos.
14. Perform contraction analysis with the MUSCLEMOTION ImageJ macro as described previously (Sala et al., 2018)

Support Protocol 2
Structural Characterization of VMToC

Introductory paragraph

After the generation of VMToC, structure of the microtissues and vasculature can be characterized by various assays. This endpoint assay can be applied starting from day 4-5 depending on the vascular development in the chips. This protocol describes structural assessment of CMs and vascular networks by immunostaining (Fig. 6).

Figure 6

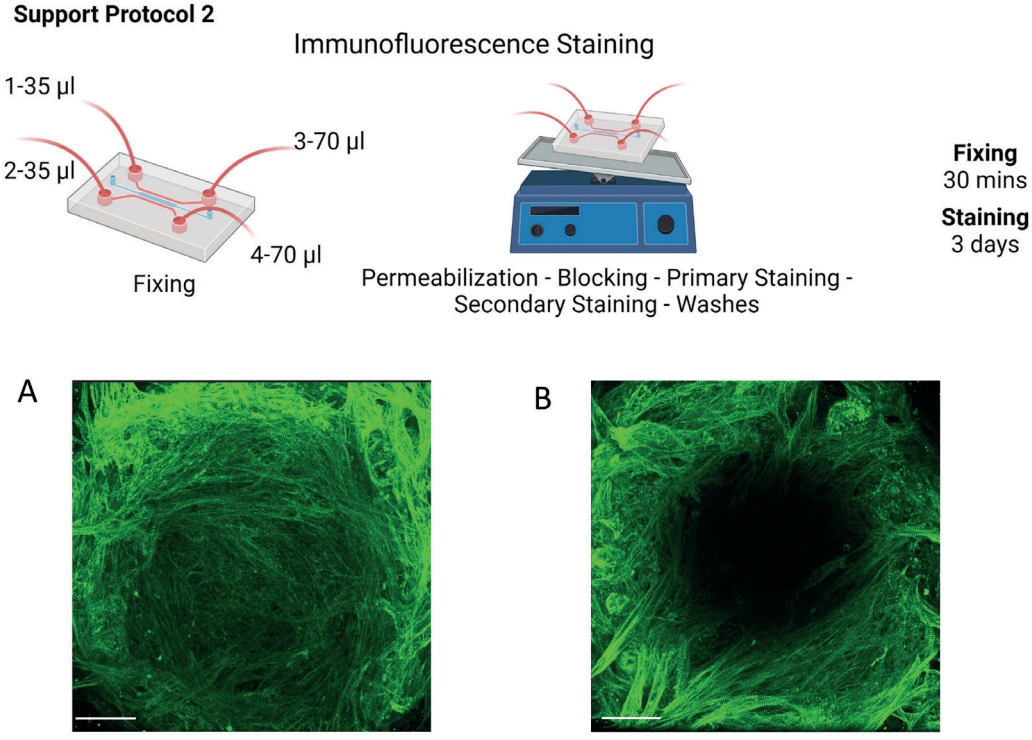

Figure 6: Schematic overview of the support protocol 2. *(A) Immunofluorescent imaging of VMToCs following the staining protocol showing proper staining of sarcomeres (ACTN2, green). (B) Immunofluorescent imaging of VMToCs following the shorter permeabilization protocol (2 h permeabilization/blocking; overnight staining with primary and secondary antibodies) showing improper staining of sarcomeres in the middle of the tissue (ACTN2, green). Scale bar: 50 µm*

Materials:

- VMToC plates (Basic protocol 1)
- Fixation solution (see recipe)
- Dulbecco's phospohate-buffered saline without Ca+2 and Mg+2 (DPBS, Gibco, cat. No. 14190-094)
- Penicillin/Streptomycin (Thermo Fisher Scientific, cat. no. 15070-063)
- Permeabilization/blocking solution (see recipe)
- Primary antibodies (see antibody table)
- Secondary antibodies (see antibody table)
- DAPI working solution (see recipe)
- EVOS M7000 imaging system (Thermo Fisher Scientific, cat. no. AMF7000)
- Andor Dragonfly 500 spinning disk confocal microscope

Protocol steps with step annotations

Fixation

1. Aspirate the medium from the inlets and outlets of the chips.
2. Fix the chips with fixation solution by adding 35 µl to the left medium inlets and 70 µl to the right medium outlets.
3. Incubate for 30 min at RT.
4. Aspirate the fixation solution.
5. Wash the chips by adding 35 µl of DPBS (-) to the left medium inlets and 70 µl to the right medium outlets.
6. Incubate for 10 min at RT.
7. Aspirate the DPBS (-).
8. Repeat steps 5-7 two more times for a total of three washes.

At this stage, the chips can be stored at 4 °C until further use. To store, add 50 µl of DPBS (-) to the left medium inlets and 100 µl in the right medium outlets.

In case the chips will be stored for a longer period, it is recommended to add penicillin streptomycin or any equivalent to the final DPBS solution to prevent contamination.

Immunostaining Protocol

9. Permeabilize and block the chips with one of the permeabilization/blocking solutions (see recipe) using the same volumes as in step 2 for 6 h at RT.
10. Dilute the primary antibodies (see antibody table) in the same permeabilization/blocking solution.
11. Add primary antibodies in the medium inlets and outlets using the same volumes as in step 2 and incubate overnight at RT.
12. Wash 3x with the same permeabilization/blocking solution following steps 5-7.

13. Prepare the secondary antibodies (see antibody table) in the same permeabilization/blocking solution.
14. Add secondary antibodies in the medium inlets and outlets using the same volumes as in step 2 and incubate overnight at RT.
15. Wash 3x with DPBS (-) following steps 5-7.
16. Prepare DAPI staining solution by diluting DAPI working solution (see recipe) in DPBS (-) (1:1000).
17. Add DAPI solution in chips following the same volume as step 2.
18. Incubate for 2 h at RT.
19. Wash 3x with PBS following steps 5-7.

It is recommended to perform the incubation steps in 9-19 on a rocker platform.

Depending on the tissue size, it might be necessary to optimize permeabilization/blocking concentration and duration with permabilization/blocking solutions for immunostaining (see recipe for alternatives). Bigger tissues might require higher concentration and/or longer incubation (see recipe for alternative 1).

20. Prepare microscopy stage with preferred filter and objective.
21. Perform imaging with EVOS M7000, Andor Dragonfly 500 spinning disk confocal microscope or other confocal microscopes.

Reagents and solutions

5X TrypLE Select solution

- Prepare a 1 mM EDTA by diluting 0.5M EDTA in DPBS (1:500). Dilute TrypLE Select 10X solution in 1:2 with 1 mM EDTA solution to prepare 5X diluted TrypLE Select solution. Store at room temperature up to 6 months.

α-Monothioglycerol (αMTG) solution, 1.3% (v/v)

- Prepare 9.87 ml Iscove's modified Dulbecco's medium (IMDM) (no phenol red) (Thermo Fisher Scientific, cat. no. 21056) and add 130 µl mono-thio glycerol (α-MTG; Sigma-Aldrich, cat. no. M6145-25 ml) in. Store up to 1 year at 4°C. Aliquots should be protected from light.

Antibodies

Antibodies	Concentration	Species	Brand	Cat no
ACTININ-2 (ACTN2)	1:800	Mouse	Sigma Aldrich	A-7732
Donkey anti mouse (488)	1:200		R&D Systems	A-21202

Basic fibroblast growth factor (FGF2) stock solution, 10 µg ml^{-1}

- Dilute FGF2 as 100 µg/ml in distilled water (dH2O; GibcoTM; Thermo Fisher Scientific, cat. no. 15230089). Dilute further as 10 µg/ml in 0.1% (w/v) Bovine Serum Albumin (BSA) in DPBS (see recipe). Prepare aliquots. Aliquots can be stored up to 1 year at –80°C. Repeated freeze-thaw cycles should be avoided.

DAPI working solution

- Reconstitute 10mg DAPI Nucleic Acid Stain, dilactate (Thermo Fisher Scientific, cat. no. D3571) in 2ml dH20. Prepare 40µl aliquots and store at -20°C. To prepare a 300µM working solution, add 1,4ml dH20 to the 40µl aliquot. Working solution can be stored at +4°C.

Permabilization/Blocking solutions for immunostaining

- Prepare permeabilization/blocking mix in 50 ml DBPS (-) following one of the recipes below. Mix the solution for a few hours by placing the tube on a roller bank at 4°C until the BSA is dissolved completely. Filter the solution using 0.22- µm-pore-size membrane filter. Store up to 4 weeks at 4°C.

Alternative 1

Composition	Volume	Concentration
Triton-X	250 µl	0.5%
Tween-20	250 µl	0.5%
BSA	0.5 g	1%
Fetal Bovine Serum (FBS)	1.5 ml	3%
Sodium deoxycholate mix	500 µl	0.01%

- Triton-x (Sigma-Aldrich, cat. No. T8787-100ml), tween-20 (Merck, cat. No. 8.22184.0500), FBS ultralow endotoxin (Biowest, cat. No. S1860-500), sodium deoxycholate (Sigma-Aldrich, cat. no. D6750-1006)

Alternative 2

Composition	Volume	Concentration
Triton-X	250 µl	0.5%
BSA	1g	2%

B(P)EL and MBEL

- Prepare B(P)EL or MBEL following the recipe below. Volumes are calculated for total 250 mL medium. Sterilize using a Stericup filter. Medium can be stored up to 3 weeks at 4°C.

Composition	B(P)EL Volume	Final concentration	MBEL Volume	Final concentration
IMDM	107.63 ml		118.125	
F12 nut. mix	113.88 ml		118.125	
PFHM-II	12.5 ml	5%		
10% (w/v) (see recipe) BSA/IMDM	6.25 ml	0.25%	6.25	0.25%
Lipids (100×)	2.5 ml	1×	2.5 ml	1×
ITS-X (100×)	250 µl	0.1×	250	0.1×
αMTG (1.3%; see recipe)	750 µl	450 µM	750	450 µM
AA2P (5 mg/ml; see recipe)	2.5 ml	0.05 m/ml	2.5 ml	0.05 m/ml
GlutaMAX (200 mM)	2.5 ml	2mM	2.5 ml	2 mM
Pen/Strep (5000 U/ml)	1.25 ml	0.5%	1.25 ml	0.5%
Trace elements B			25 µl	
Trace elements C			250 µl	

- IMDM, Iscove's modified Dulbecco's medium, no phenol red (Gibco™; Thermo Fisher Scientific, cat. no. 21056023); F12 nut. mix, Ham's F-12 nutrient mix, GlutaMAX supplement (Gibco™; Thermo Fisher Scientific, cat. no. 31765027); PFHM-II, Protein-Free Hybridoma Medium, 1× (Gibco™; Thermo Fisher Scientific, cat. no. 12040077); Lipids, chemically defined lipid concentrate(Gibco™; Thermo Fisher Scientific, cat. no. 11905031); ITS-X, insulin-transferrin-selenium-ethanolamine (100×; Gibco™, Thermo Fisher Scientific, cat. no. 51500056); GlutaMAX, GlutaMAX-1 supplement (Gibco™;

Thermo Fisher Scientific, cat. no. 35050038); Pen/Strep, Penicillin-Streptomycin (5000 U/ml; Gibco™; Thermo Fisher Scientific, cat. no. 15070063), trace Elements B 1000x solution (Thermo Fisher Scientific, cat. no. 25-022-CI), trace Elements C 1000x solution (Thermo Fisher Scientific, cat. no. 25-023-CI).

BSA, 10% (w/v) in IMDM

- Weigh 5 g of BSA (BovoStar) in a 50-ml tube and add 40 ml of IMDM on top. Mix the solution for a few hours by placing the tube on a roller bank at 4°C until the BSA is dissolved completely. Top up with 10 ml IMDM to reach final concentration of 10% (w/v) BSA. Filter using a 0.22-µm-pore-size membrane filter. Store for up to 4 weeks at 4°C.

Chip medium

- Prepare the chip medium by mixing EGM-2 with B(P)EL in 1:1 ratio. Supplement with 50 ng/mL VEGF.

EGM-2

- Add all supplements from the kit to the basal medium to prepare a 500-ml of EGM-2 following manufacturer's instructions. Add 2.5 ml Penicillin-Streptomycin (5000 U/ml; GibcoTM; Thermo Fisher Scientific, cat. no. 15070063). Store up to 6 weeks at 4°C.

Endothelial Cell Complete Growth Medium (EC-CGM)

- Prepare EC-CGM following the recipe below. Sterilize using a Stericup filter. Medium can be stored up to 2 weeks at 4°C.

Composition	Volume	Final concentration
EC-SFM	247.5 ml	
hPPS	2.5 ml	1%
VEGF, 50 µg/ml	150 µl	30 ng/ml
bFGF, 100 µg/ml	50 µl	20 ng/ml

- EC-SFM, Invitrogen, cat. no. 11111-044); hPPS, human serum from platelet-poor plasma (hPPS; Sigma-Aldrich, cat. no. P2918); VEGF, human VEGF (165) IS, premium grade (Miltenyi Biotec, cat. no. 130-109-386); bFGF, human bFGF, premium grade (Miltenyi Biotec, cat. no. 130-093-842).

Fixation solution

- Dilute 8% (wt/vol) PFA in 0.2 M phosphate buffer, pH 7.4 in order to prepare 4% (vol/vol) PFA solution. Store the solution up to 1 month at 4 °C.

Gelatin-coated 6-well plate

- Coat a or flask with 0.1% (w/v) gelatin solution 1ml/well of a 6x-well plate. Ensure that the whole surface is covered with gelatin. Incubate the plate with gelatin for 1 hr at 37°C before use. The coated plates can be stored up to 2 weeks at 4°C.

Gelatin solution, 0.1% (w/v)

- Weigh 1 g gelatin (from porcine skin, type A; Sigma-Aldrich, cat. no. G1890) and add 100 ml of dH2O on top to prepare a 1% (w/v) gelatin solution. Autoclave the solution. Aliquot in 10 mL and store this stock solution up to 1 year at –20°C.

- For 0.1% (w/v) gelatin solution, dilute stock solution in 1:10 with Dulbecco's phosphate-buffered saline (DPBS; Life Technologies, cat. no. 14190). Sterilize the solution using a 0.22-µm-pore-size membrane filter. Store up to 1 year at room temperature.

Matrigel-coated plates

- Keep Matrigel (Growth Factor Reduced; Corning, cat. no. 354230) aliquot on ice. Dilute Matrigel in cold DMEM/F12 (Gibco™; Thermo Fisher Scientific, cat. no. 31331028) in 1:120. Coat 12-well cell culture plates (Greiner Bio-one, cat. no. 665180) 1 mL/10cm². Incubate coated plates for at least 1 h at RT before use (Follow basic protocol step 12). Alternatively, coated plates can be stored at 4 °C up to 2 weeks for later use.

L-Ascorbic acid 2-phosphate (AA2P), 5 mg ml^{-1}

- Prepare AA2P by diluting 250 mg AA2P (Sigma-Aldrich, cat. no. A8960) in 50 ml dH2O. Prepare aliquots. Aliqouts can be stored up to 1 year at –20°C.

PFA, 8% (w/v)

- Weigh 40 g paraformaldehyde (PFA; Merck, cat. no. 1.04005.1000) and add 400 ml Milli-Q water on top. Heat this solution to 60°C and stir at medium speed. To dissolve the PFA, add ~10 drops of 1 N NaOH after few minutes of stirring until the solution is clear. Let the solution cool down. Add Milli-Q water until a total volume of 500 ml is reached. Store the solution up to 2 months at 4°C.

Phosphate buffer, 0.2 M (pH 7.4)

- For solution 1, weigh 8.28 g sodium dihydrogen phosphate dihydrate (NaH2PO4·2H2O; J.T. Baker, cat. no. 0326) and add 300 ml Milli-Q ultrapurified water (e.g., from a Millipore system) on top. For solution 2, weigh 10.78 g disodium hydrogen phosphate monohydrate (NaH2PO4·1H2O; Merck, cat. no. 1.06346.1000) and add 300 ml Milli-Q water on top. For 0.2 M phosphate buffer (pH 7.4), add solution 1 to solution 2 until a pH of 7.4 is reached. Store at room temperature indefinitely (no expiration date).

Sodium deoxycholate mix

- Reconstitute 0.5 g (1%) sodium deoxycholate (Sigma-Aldrich, cat. no. D6750-1006) in 50 ml DBPS. Mix well and store at RT up to 1 month.

VEGF stock solution

- Dilute VEGF as 100 µg/mL in distilled water. Dilute further as 50 µg/mL in 0.1% BSA in DPBS (Life Technologies, cat. no. 14190). Prepare 50-µL and 100- µL aliquots of VEGF. Aliquots can be stored at −80 °C.

Commentary

Background Information

Animal models such as rodents offer insights into heart development and disease. However, since there are physiological and genetic differences between rodent and human hearts, there is growing interest in using hPSC-based cardiac models (Mills and Hudson, 2019).

Many differentiation protocols described by us and others can be used to generate different types of hPSC-CMs (Zhang et al., 2012; Burridge et al., 2014; Campostrini et al., 2021; Wiesinger et al., 2022). These have proven valuable to model heart disorders like channelopathies (Giacomelli et al., 2017; Brandao et al., 2017). However, for modelling more complex disorders where more than one cell type might cause a disease state, studying hPSC-CMs alone may not be sufficient. In addition, hPSC-CMs under standard conditions are immature and whilst this is sufficient to study ion channel diseases, other conditions like cardiac myopathies, may need CMs in a more mature state. Moreover, cardiac disease may be exacerbated by inflammation or the presence of immune cells. More complex platforms with increased physiological relevance are therefore of interest.

3D cardiac MT/organoid/cardioid and engineered heart tissue models are based on the combination of self-assembly of cells, biomaterials and/or tissue engineering. Such models can (1) integrate multiple cell types and extracellular matrix components; (2) recapitulate cell-cell crosstalk between CMs and cardiac stromal (CF)- and vascular cells (EC); (3)

provide control over biomechanical and biochemical cues; and (4) consequently improve hPSC-CM maturation, at least to a postnatal state (Schmidt et al., 2023; Hofbauer et al., 2021; Mills et al., 2018; Beauchamp et al., 2020; Giacomelli et al., 2020; Richards et al., 2020; Goldfracht et al., 2020; Voges et al., 2023). However, one of the remaining hurdles has been to incorporate functional vessel-like structures which could improve oxygen, nutrient and drug transport and allow the introduction of inflammatory cells.

Organ-on-chip models are developing as one way to overcome this hurdle. MT/organoids can be cultured inside 3D microfluidic devices that enable functional vessel formation and perfusion following a similar strategy to transplantation studies where the MT/organoids are introduced into laboratory animals. In *in vitro* models, both the MTs/organoids and the vasculature can be of human origin eliminating the need for a living host (King et al., 2022; Arslan et al., 2023; Bonanini et al., 2022). In addition, human vasculature would support nutrient and drug delivery through the endothelial barrier, creating closer mimicry to the human heart.

Here, using organ-on-chip technology, we describe a protocol for an *in vitro* model of vascularized cardiac MTs all derived from hiPSCs. Cardiac MTs are first generated using a previously published protocol (Campostrini et al., 2021) with minor modifications. Specifically, to generate vascularized cardiac MTs we used hiPSC-ECs as described earlier (Orlova et al., 2014). However, depending on the application using cardiac-specific ECs might be an alternative (Campostrini et al., 2021). Cardiac-specific ECs have typical EC characteristics but express some cardiac transcription factors (Giacomelli et al 2020). We co-culture cardiac MTs here with hiPSC-ECs and HBVP in a fibrin hydrogel in the gel channel of a commercially available AIM Biotech chips as described in Basic Protocol 1. hiPSC-ECs and HBVPs self-organize into an external vascular bed around the MTs. Much as in the case of transplantation, branches of this vascular bed start anastomosis with the pre-existing internal microvasculature in the MTs. During the first 5 days of the culture, vascular development is evident. Around day 5, a continuous vascular network in- and around the MTs is formed and stabilized. Immunofluorescent imaging assays demonstrated that these vascular networks are lumenized.

In order to show whether these networks are also perfusable, beads are added in the medium channel. Beads spontaneously enter the external vascular networks from the medium-gel channel interface and end up inside the microvascular networks in the MTs. Spontaneous movement of the beads can be recorded as described in Support Protocol 1. With this, it is possible to estimate the fluid flow patterns such as speed and shear stress in the chips (Vila Cuenca et al., 2021). Alternatively, lumenized vessels can be perfused with beads as a surrogate for immune or other blood cells.

VMToCs are contractile throughout the whole experiment. Contractile parameters can be quantified either during spontaneous contraction or upon pacing. Pacing is preferred to eliminate the variability in the beat rates of different conditions and standardize it for comparison of other contraction parameters. In support protocol 1, custom-made platinum

electrodes are inserted in the gel channel inlets and outlets to stimulate VMToCs with bipolar rectangular pulses. Alternatively, commercial electrodes that fit in the gel inlet and outlet can be used. Contraction videos are recorded using a high-speed camera. These videos can be analyzed using open access software such as MuscleMotion (Sala et al., 2018) or other video based analysis tools (Huebsch et al., 2015b; Stebbeds et al., 2023) .

For further structural characterization of VMToCs, the chips are fixed and stained as described in Support Protocol 2. Staining protocol was optimized according to the tissue size, density and the specific antibody. Different permeabilization/blocking solutions were tested and the successful two alternatives were listed. However, for other tissue types, sizes and antibodies, the protocol might require further optimization. To allow easy live imaging and eliminate the staining hurdles, using fluorescently tagged hiPSC-derived cell types in tissues is highly recommended.

With this protocol, (1) we integrate 3D cardiac MTs that were well characterized structurally and functionally in terms of cell-cell crosstalk (Giacomelli et al., 2020); (2) these 3D cardiac MTs are partially mature before integration in chips; (3) the model is fully hiPSC-derived except for mural cell component in the external vascular network (HBVP) which could be replaced by hiPSC-derived smooth muscle cells (Vila Cuenca et al., 2021); (4) The number of cells required for MT formation is as low as 5000 cells, significantly reducing the number of hiPSC-derived cardiac cells required per experiment compared to other cardiac *in vitro* models. In addition, there are currently no animal- or *in vitro* models that can capture vascular defects such as endothelial dysfunction and coronary microvascular obstruction (Niccoli et al., 2016; Sorop et al., 2020). VMToCs can be useful to explore underlying mechanisms related to these defects by using patient-derived hiPSC lines.

We previously showed that vessel-on-chip without cardiac MTs can be cultured long-term (21 days) within a similar system (Vila Cuenca et al., 2021). However, VMToCs in this protocol are cultured until day 7. For longer culture, further optimization of culture conditions, such as inclusion of matrix metalloproteinase inhibitors, might be required. Another limitation is that the model is solely depend on self-organization. Therefore, it should be noted that certain levels of variability may be evident, such as the extent of vascularization. In addition, VMToCs are generated using 9x channel plates. For higher-throughput experiments, 40x channels or other high-throughput organ-on-chip platforms can be used. However, in that case, the seeding protocol should be optimized preferably for automated seeding or other more user-friendly methods.

In summary, this protocol can be used to generate vascularized and perfusable cardiac MTs efficiently in three-channel microfluidic chips and characterized within days. The model can be useful to study cell-cell crosstalk especially with the endothelial barrier, to model vascular defects and test drugs.

Critical Parameters

CMs used in this protocol reaches >85% purity. It is important to use high purity of CMs particularly when functional characterization is planned. In order to increase the purity upon thawing should it be below 80%, a short period of lactate treatment can be done (Campostrini et al., 2021). Depending on the line, the timing of thawing CMs until generation of MTs should be optimized to ensure recovery of thawed CMs.

In our protocol, MTs are integrated in the chips on day 12 after generation (Fig. 1D). This is critical as microvascular networks start self-organizing around day 8-10 in these MTs and longer culture without perfusion might lead to their regression. In order to ensure anastomosis between the external vascular network and internal microvasculature, it is important to make sure that the internal vascular structures are lumenized in the MTs at the time of integration in chips.

The number of additional vascular cells to integrate in chips with MTs is optimized according to the time necessary to form a proper vascular network around the MTs. This duration should be as short as possible to ensure the anastomosis and perfusion of MTs occur early. Higher vascular density is therefore preferred. However, the counting of cells is manual and could vary between operators. It is also noted that ECs from different hiPSC lines might have different proliferative capacities. Therefore, the number of vascular cells in chips should be optimized further for each operator and cell line.

The number of MTs inside the gel channels is critical. We observed that when multiple (more than 2) MTs are in close proximity in the gel channel, this affects the external vascular network between them and thus the vascularization of MTs (Fig. 3B). Therefore, it is recommended to have only 4-5 MTs per channel of this specific chip.

During the seeding of MT/cells/hydrogel, manual seeding is highly recommended to ensure control over the number of MTs inside the gel channels.

Seeding in chips should be performed as quickly as possible for a proper gelation of fibrin hydrogel. The plate should be kept horizontal during this process.

After seeding, to ensure proper gelation, it is critical to not move the plate for 15 mins. After this period, medium channels should be filled with medium either manually or in an automated way. In this process, we observed that bubbles can form in the medium channels. Bubbles can be removed by slow aspiration of the medium.

Notably, integration and vascularization of MTs here are robust and reproducible between lines. However, we observed that vascularization might be affected by the pre-vascularization of MTs. Even in the same multi-well plate, pre-vascularization levels of MTs might differ. This can lead to variability in vascularization levels in the chips.

In the bead perfusion assay, the beads spontaneously enter the vascular networks. If the bead concentration is too low (1 drop in 10 ml), they might not penetrate the vascular networks inside the MTs. This can be avoided by increasing the bead concentration (1 drop in 5 ml or less).

The staining protocol here is optimized according to the size and the thickness of MTs. We observed that using the same staining protocol of 3D cardiac MTs or VoC models failed to stain the middle of the MTs in VMToC (Fig. 6B). Therefore, we optimized this protocol following a staining protocol designed for denser and larger organoids like blood vessel organoids (Wimmer et al., 2019). We provided two alternative permeabilization/blocking solution and observed that alternative 1 is a stronger solution which might result in better staining with less gradient. It is recommended that this step is optimized according to the tissue size and thickness. Of note, the optimization of this protocol has been done using one antibody for CMs. Further optimization might be necessary for other antibodies. Alternatively, using fluorescently tagged hiPSC lines to derive cell types in tissues is recommended for easier imaging.

Troubleshooting

Table 1: Troubleshooting Guide for VMToC generation and characterization

Problem	Possible Cause(s)	Possible Solution(s)
MTs are not contracting in the gel channel	The seeding protocol takes too long, and the temperature changes might affect the contractility of MTs. CM yield is too low.	After providing medium in the medium channels, place the plate in the incubator for an hour. After this, MTs should start contracting. If the MTs do not start contracting after a day, discard the plate and start a new experiment. Use high purity of CMs.
Bubbles in the gel or medium channel	Rigorous mixing of the MT/cell/gel mix. Pipetting pre-existing bubbles from the Eppendorf tube.	Do not mix the MT/cell/gel mix more than 3-4 times. Do not pipette pre- existing bubbles from the tube.

Problem	Possible Cause(s)	Possible Solution(s)
MTs do not load in the gel channel	Not applying enough pressure. Too many MTs in the pipette tip. Gel channel inlet is blocked by the MTs.	Apply more pressure during the seeding of MTs. Decrease the MT number to seed per channel. Do not integrate MTs that are merged or bigger in size than the gel channel. Pipette the MT/cell/gel mix back and use the gel channel for the next round of mix.
Gel leaking to the medium channel	Applying more pressure during seeding than necessary. Improper bonding of the chips.	Keep the pressure applied during seeding stable. If this occurs in more than one channel, discard those chips.
Gel polymerization takes longer than expected	High concentration of Heparin in the cell culture growth medium	Reduce Heparin concentration in the cell culture growth medium used to prepare Thrombin and Fibrinogen solutions (Hajal et al., 2022)
Low or high vascular density in the gel channel	Difference in the final cell density caused by manual counting.	Optimize the final cell density according to the handler manual counting.
Vascular cells are not homogenously distributed	Tilting the plate during seeding. Improper mixing of the MT/cell/gel mix.	Keep the plate with chips in horizontal position during seeding. Pipette up and down gently 3-4 times before seeding in the gel channel.
Low efficiency in vascularization of MTs	The number of MTs per channel is high. The density of vascular cells is low. MTs in one channel are too close to each other.	Limit the number of MTs/channel to 4-5. Optimize the vascular cell density.

Problem	Possible Cause(s)	Possible Solution(s)
Vascular networks are not developing	The growth factors are not provided in the medium. Insuffiecient gelation of fibrin hydrogel.	Ensure 50 ng/ml VEGF is provided for the whole duration of the protocol and 10 ng/ml DAPT only on day 1. Incubate the gel for about 15 mins at room temperature after seeding. If the vascular networks are not developing 2 days after seeding, either use the rocker platform to improve vascularization or discard the plate.
Variability in contraction parameters	Recording of the moving debris around the MTs. Batch-to-batch and line- to-line variability.	Ensure that only MTs are recorded and limit recording of the surrounding areas.
Staining does not work	Too short permeabilization. Too short primary and secondary antibody incubation.	Ensure that the staining protocol here is followed. Alternative 1 as a permeabilization/blockin g solution is stronger. If MTs are larger, optimize the steps. Use fluorescently tagged lines in tissues.

Understanding Results

With regard to the seeding of MT/cell/gel mix, if the protocol is properly followed, each gel channel should have homogenously distributed vascular cells. There should be at least 1 MT in each channel. Vascular cells around the MTs will start self-organizing as early as day 1. On day 1, the internal microvascular networks will be prominent and start anastomosis with the external vascular network. Over the following days, the external vascular network will develop further and the anastomosis will become more evident. Around day 5, there will be continuous vascular network in- and around the MTs.

Chips can be used for structural and functional characterization assays as early as day 3-4 when the external vascular networks are fully developed. At this stage it is recommended to perform the bead perfusion assay if required.

In addition, MTs will be contractile throughout the whole period of the experiment. EC-CM crosstalk will modulate the contraction parameters of VMToCs, for example with increases in contraction duration and peak-to-peak times (Arslan et al., 2023). VMToCs can be used for pacing and drug testing as early as day 3-4.

Time Considerations

Basic Protocol 1

- Cell preparation takes 5-7 days in total depending on the CM recovery.
- Thawing of CMs - 30 mins.
- Thawing of ECs and CFs - 15 mins.
- MT formation - 1.5-2 hours.
- MT maintenance - 13 days in total including day 0.
- Cell preparation before chips - 6 days including the day 0.
- Fibrinogen preparation – 3-3.5 hours.
- Chip seeding in total - 2-3 hours depending on the plate numbers to seed.
- Chip maintenance - 5-7 days.

Support protocol 1

- Bead perfusion – 15 mins/MT
- Pacing – 1-1.5 hours depending on the number of MTs to be paced.

Support protocol 2

- Fixing and staining – 3 days

Conflict Of Interest Statement

The authors declare no competing interests.

Data Availability Statement

The data supporting the findings of this study are available on request from the corresponding author.

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(mandatory for NIH, optional for all others)

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