TECHNICAL MANUAL

Generation and Maturation of Blood Vessel Organoids Using STEMdiff™ Blood Vessel Organoid Kit

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1.0 Introduction

Blood vessels are a fundamental part of all organ systems and have critical roles in multiple diseases, including diabetes, atherosclerosis, and cancer. The blood vasculature is composed of endothelial cells that form luminal tubes and pericytes covering the endothelial wall. In vitro models of vascular biology involve co-culturing endothelial cells with pericytes but do not fully recapitulate their three-dimensional (3D) organization and functionality. A novel culture system in which human pluripotent stem cell (hPSC)-derived blood vessel organoids (BVOs) are used to model the structural and functional features of blood vasculature was recently reported by Wimmer et al.[1,2] Based on these publications, we have developed STEMdiff™ Human Blood Vessel Organoid Kit to enable robust and reproducible generation of BVOs in culture.

STEMdiff™ Blood Vessel Organoid Kit is a serum-containing kit for the robust differentiation of hPSC-derived BVOs in a five-stage protocol. BVOs are a 3D in vitro model of blood vessel architecture. They model in vivo vessel architecture through endothelial cells forming tube structures with attachment of perivascular cells (e.g. pericytes) to blood vessels. BVOs make functional vessels with lumen formation and deposition of the basement membrane. BVO formation is initiated through hPSC aggregate formation followed by mesoderm induction and subsequently vascular induction. Vascular-induced aggregates are embedded in a collagen/Matrigel® sandwich to form a blood vessel structure. BVOs generated using this kit have CD31+/CD34+/CD144+/KDR+ endothelial cells and PDGFR-β+/CD146+/SMA+/NG-2+ pericytes.

2.0 Materials, Reagents, and Equipment

2.1 STEMdiff™ Blood Vessel Organoid Kit (Catalog #100-0651)

The components listed below are available as STEMdiff™ Blood Vessel Organoid Kit; STEMdiff™ Blood Vessel Organoid Maturation Medium (Catalog #100-0658) is also available for individual sale.

Refer to the Product Information Sheet (PIS; Document #10000010330) for component storage and stability information, available at www.stemcell.com, or contact us to request a copy.

COMPONENT NAME	COMPONENT #	SIZE
STEMdiff™ Blood Vessel Organoid Aggregation Basal Medium	100-0652	60 mL
STEMdiff™ Blood Vessel Organoid Aggregation Supplement*	100-0653	15 mL
STEMdiff™ Blood Vessel Organoid Induction Basal Medium	100-0654	100 mL
STEMdiff™ Blood Vessel Organoid Induction Supplement*	100-0655	1 mL
STEMdiff™ Blood Vessel Organoid Mesodermal Induction Supplement**	100-0656	1 mL
STEMdiff™ Blood Vessel Organoid Vascular Induction Supplement**	100-0657	1 mL
STEMdiff™ Blood Vessel Organoid Maturation Medium*	100-0658	100 mL

*This product contains material derived from human plasma. Donors have been tested and found negative for hepatitis B surface antigen (HBsAg), HIV-1 antibodies, and/or HIV-1 antigen. However, this product should be considered potentially infectious and treated in accordance with universal handling precautions.

**Please refer to the Safety Data Sheet (SDS) for hazard information. This product contains components dissolved in dimethyl sulfoxide (DMSO). DMSO is a strong solvent and skin penetrant and can transport many substances through the skin. DMSO can also penetrate some protective glove materials including latex and silicone. Extra caution should be utilized when handling this product.

2.2 Additional Required Materials and Reagents

PRODUCT	CATALOG #
3 cc Syringes	28230
ACCUTASE™	07920
Collagen Solution (3 mg/mL)	04902
Conical tubes, 15 mL and 50 mL	e.g. 38009 and 38010
Corning® Matrigel® Growth Factor Reduced Basement Membrane Matrix	Corning 356231
Corning® Matrigel®, Growth Factor Reduced, Phenol Red Free*	Corning 356231*
Corning® Matrigel® hESC-Qualified Matrix	Corning 354277
Costar® 12-Well Flat-Bottom Plate, Tissue Culture-Treated	38052
DMEM/F-12 with 15 mM HEPES	36254
DMEM/HIGH with L-glutamine, without sodium pyruvate	Cytiva SH30003.03

PRODUCT	CATALOG #
Ethylenediaminetetraacetic acid (EDTA) disodium salt solution (0.5 M)	Millipore Sigma E7889-100mL
Gibco™ GlutaMAX™ Supplement	Fisher Scientific 13462629
Gibco™ Ham's F12 Nutrient Mix	Fisher Scientific 11765054
Gibco™ Sodium Bicarbonate 7.5% solution	Fisher Scientific 25-080-094
HEPES Buffer Solution (1 M)	07200
mTeSR™1 OR mTeSR™ Plus OR TeSR™-E8™	85850 OR 100-0276 OR 05990
Needles, 26 G x ½" length	VWR CABD305111
PrimeSurface® 3D Culture Spheroid plates: Ultra-low Attachment Plates, 96 well, U bottom, clear plates	Sumitomo Bakelite MS-9096UZ
Serological pipettes, 10 mL	e.g. 38004
Sodium hydroxide solution (1 N)	Millipore Sigma S2770-100mL
Spatula	e.g. VWR 82027-532
Tissue culture dish lid (plastic or glass), 100 mm	e.g. 100-0082
Trypan Blue	07050
Ultra-Low Adherent Plate for Suspension Culture	38071
Y-27632 (Dihydrochloride)	72302

*Optimal Matrigel® lots will have a protein concentration > 8.5 mg/mL.

For a complete list of products available from STEMCELL Technologies Inc., visit www.stemcell.com.

2.3 Equipment

- Biohazard safety cabinet certified for Level II handling of biological materials
- Incubator with humidity and gas control to maintain 37°C and 95% humidity in an atmosphere of 5% CO_2 in air
- Low-speed centrifuge with a swinging bucket rotor

  Note: All centrifugation protocols described in this manual can be performed with the brake on.
- Inverted microscope with 2X, 4X, 10X, and 20X phase objectives
- -20°C freezer
- Refrigerator (2 - 8°C)

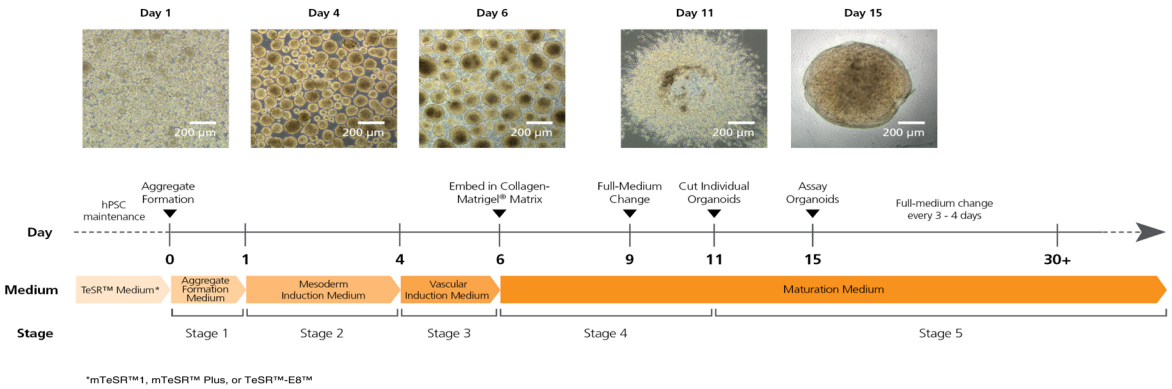
3.0 Generation and Maturation of Blood Vessel Organoids

The protocol for generation and maturation of blood vessel organoids is divided into five sections as follows:

- Section 3.3 Aggregate Formation (Day 0 - 1)
- Section 3.4 Mesoderm Induction (Day 1 - 4)
- Section 3.5 Vascular Induction (Day 4 - 6)
- Section 3.6 Blood Vessel Network Formation (Day 6 - 11)
- Section 3.7 Blood Vessel Organoid Self-Assembly and Maturation (Day 11 - 30+)

Refer to section 3.1 for the protocol diagram, and section 3.2 for preparation of media. Use sterile technique in all protocols. For Troubleshooting, refer to section 4.0.

3.1 Protocol Diagram



3.2 Preparation of Media

Use sterile technique to prepare media. Prepare each medium as needed in the protocols. Refer to Table 1 for medium components, volumes, and in-use storage and stability.

1. Thaw Supplement(s) at room temperature (15 - 25°C). Mix thoroughly.

   Note: If not used immediately, aliquot and store at -20°C. Do not exceed the shelf life of the supplement. After thawing aliquots, use immediately. Do not re-freeze.

2. Add Supplement(s) to Basal Medium as indicated in Table 1. Mix thoroughly. Warm media to 37°C before use.

   Note: If not used immediately, store media as indicated in Table 1.

Table 1. Preparation of Media

MEDIUM	COMPONENT	VOLUME*	PREPARATION & STORAGE
Aggregate Formation Medium (25 mL)	STEMdiff™ Blood Vessel Organoid Aggregation Basal Medium	20 mL	Mix thoroughly. If not used immediately, store at 2 - 8°C for up to 2 weeks.
	STEMdiff™ Blood Vessel Organoid Aggregation Supplement	5 mL	
Mesoderm Induction Medium (20 mL)	STEMdiff™ Blood Vessel Organoid Induction Basal Medium	19.4 mL	
	STEMdiff™ Blood Vessel Organoid Induction Supplement	200 µL	
	STEMdiff™ Blood Vessel Organoid Mesodermal Induction Supplement	400 µL	
Vascular Induction Medium (20 mL)	STEMdiff™ Blood Vessel Organoid Induction Basal Medium	19.4 mL	
	STEMdiff™ Blood Vessel Organoid Induction Supplement	200 µL	
	STEMdiff™ Blood Vessel Organoid Vascular Induction Supplement	400 µL	

*These volumes are sufficient to initiate and differentiate a full 6-well plate of hPSCs to generate vascular aggregates.

3.3 Aggregate Formation (Day 0 - 1)

This protocol is for the formation of cell aggregates (6 wells) from human embryonic stem (ES) or induced pluripotent stem (iPS) cells maintained in TeSR™-E8™, mTeSR™1, or mTeSR™ Plus on Corning® Matrigel® hESC-Qualified Matrix. Warm cultureware, media, and reagents to either room temperature (15 - 25°C) or 37°C, as indicated in the protocol below.

Note: Human ES/iPS cell cultures are ready to passage when the majority of the colonies are large, compact, and display dense multi-layered centers. Passage human ES/iPS cell cultures when they are no more than 70 - 80% confluent and exhibit < 10% differentiation.

For complete instructions on coating cultureware with Corning® Matrigel®, refer to the Technical Manual for TeSR™-E8™, mTeSR™1, or mTeSR™ Plus, available at www.stemcell.com, or contact us to request a copy.

Day 0

1. Dilute 0.5 M EDTA solution to 0.5 mM in PBS. Warm the 0.5 mM EDTA solution to 37°C in an incubator. Warm ACCUTASE™ to room temperature.
2. Prepare 25 mL of Aggregate Formation Medium (see section 3.2).
3. Prepare Aggregate Seeding Medium as follows:
   a. Add 90 µL of 10 mM Y-27632 to 18 mL of Aggregate Formation Medium (final concentration 50 µM).
      Note: Set aside the remaining Aggregate Formation Medium to be used for rinsing in step 12.
   b. Mix thoroughly. Warm to 37°C.
4. Use a microscope to visually identify regions of differentiation in the ES/iPS culture. Remove regions of differentiation by scraping with a pipette tip or by aspiration.
5. Aspirate medium from ES/iPS culture.
6. Add 0.6 mL/well of warm 0.5 mM EDTA (prepared in step 1). Incubate at 37°C and 5% CO_2 for 5 minutes.
7. Carefully remove the plate from the incubator.
8. Aspirate EDTA. Add 0.6 mL/well of room temperature ACCUTASE™.

9. Incubate at 37°C and 5% CO_2 for 5 minutes.
10. Monitor cells under the microscope; cells should be round and completely detached from the plate.
11. Using a 1 mL pipettor, gently resuspend cells by pipetting up and down slowly 3 - 5 times. Transfer the cell suspension from each well to a sterile 50 mL conical tube.
12. Rinse each well with 1 mL of Aggregate Formation Medium and add this rinse to the tube containing cells.
13. Count cells using Trypan Blue and a hemocytometer.
14. Calculate the volume of this cell suspension required to seed 3 wells per density as follows:
    a. 2 x 10^5 and 4 x 10^5 cells/well in TeSR™-E8™ or mTeSR™1, or
    b. 1 x 10^5 and 2 x 10^5 cells/well in mTeSR™ Plus
15. Transfer the calculated volumes to 2 x 15 mL conical tubes.

    Note: It is recommended to test both cell densities to determine the optimal density for each cell line.
16. Centrifuge the tubes at 300 x *g* for 5 minutes.
17. Remove and discard the supernatant. Add 9 mL of Aggregate Seeding Medium to each tube to resuspend the cells.
18. For each cell suspension, add 3 mL/well to 3 wells of a 6-well ultra-low adherent plate.
19. Shake the plate to distribute cells evenly, then incubate at 37°C and 5% CO_2. Do not disturb the plate for at least 24 hours.

Day 1

20. Observe aggregates under a microscope. Aggregates should reach a diameter of < 50 µm and exhibit round and smooth edges (see Figure 1).

    Note: If not many aggregates are observed, incubate for one additional day at 37°C and 5% CO_2. Do not disturb the plate.
21. Proceed to section 3.4.

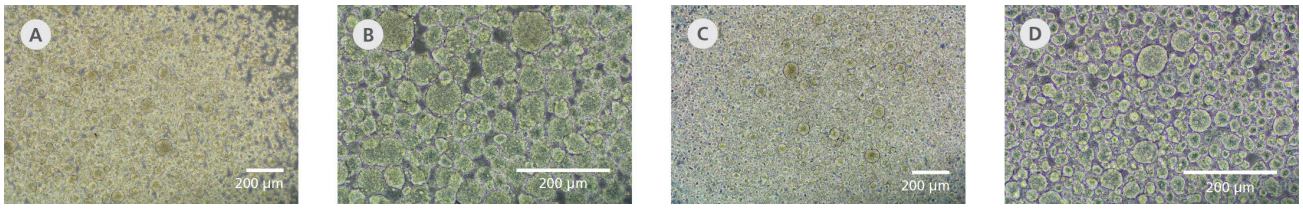


Figure 1. hPSC Aggregates on Day 1 in Aggregate Seeding Medium

Bright-field images show aggregates with a diameter < 50 µm generated from **(A&B)** iPS (WLS-1C) cells and **(C&D)** ES (H9) cells. Magnification: 4X (A&C) and 10X (B&D).

3.4 Mesoderm Induction (Day 1 - 4)

Day 1

1. Prepare Mesoderm Induction Medium (see section 3.2) and warm to 37°C.
2. Collect the aggregates and medium from the 6-well plate using a 10 mL serological pipette on **slow**. Transfer the aggregate suspension to a sterile 50 mL conical tube. Set aside the 6-well ultra-low adherent plate (to be used in step 6).

   Note: This step normalizes the number of aggregates per well to correct for differences in aggregation efficiency between wells.

3. Divide the aggregates evenly among 6 x 15 mL conical tubes. Let the aggregates settle by gravity for 20 - 30 minutes until all aggregates have settled to the bottom of the tubes.

   Note: Always collect aggregates by gravitation; centrifugation of cell aggregates is not recommended.
4. Using a 10 mL serological pipette on **slow**, carefully remove medium, leaving behind 200 - 400 µL in each tube.
5. Add 3 mL of Mesoderm Induction Medium to each tube containing aggregates.
6. Using a 10 mL serological pipette, transfer the aggregates of one 15 mL tube to one well of the 6-well ultra-low adherent plate set aside in step 2. Repeat for the remaining tubes, using all 6 wells of the plate.
7. Shake the plate to distribute aggregates evenly along the bottom of the wells. Incubate at 37°C and 5% CO_2. Minimize movement of the plate during incubation.

Day 2 - 4

8. Once per day, quickly pipette each well of aggregates 3 - 4 times with a P1000 micropipette set to 1 mL to prevent/reduce aggregate fusion.
9. Shake the plate to distribute aggregates evenly along the bottom of the wells. Incubate at 37°C and 5% CO_2. Minimize movement of the plate during incubation.

Day 4

On day 4, observe aggregates under a microscope. Aggregates should have round and smooth edges (see Figure 2). Proceed to section 3.5, using only aggregates with round and smooth edges (one seeding density may not display the desired morphology).

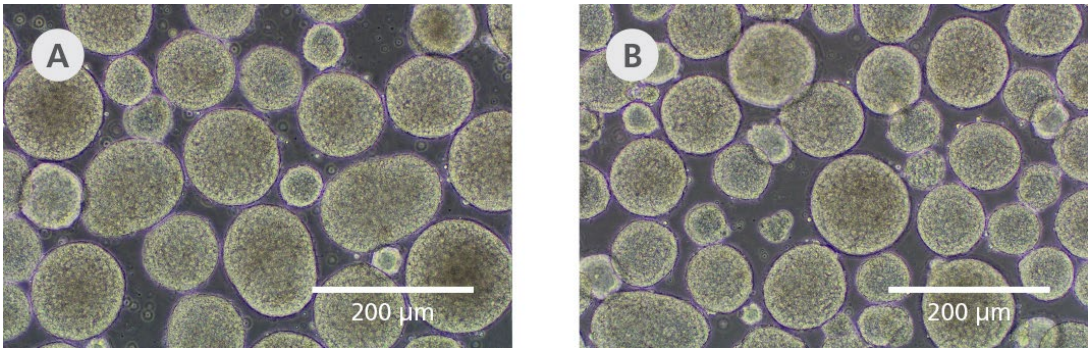


Figure 2. Aggregates on Day 4 with Round and Smooth Edges in Mesoderm Induction Medium

Bright-field images of aggregates generated from **(A)** WLS-1C iPS and **(B)** H1 ES cell lines. Magnification: 10X.

3.5 Vascular Induction (Day 4 - 6)

Day 4

1. Prepare Vascular Induction Medium (see section 3.2) and warm to 37°C.
2. Using a 10 mL serological pipette on **slow**, collect the aggregates from one well. Transfer to a sterile 15 mL conical tube. Repeat for remaining wells, for a total of 6 x 15 mL conical tubes.
3. Let aggregates settle by gravity for 5 - 10 minutes, until all aggregates have settled to the bottom of the tubes.
4. Using a 10 mL serological pipette on **slow**, carefully remove medium, leaving behind 200 - 400 µL in each tube.
5. Using a 200 µL pipettor, remove as much of the remaining medium as possible.
6. Add 3 mL of Vascular Induction Medium to each tube containing aggregates.

7. Transfer aggregates from one tube to one well of the 6-well ultra-low adherent plate. Repeat for the remaining tubes, for a total of 6 wells.
8. Using a 1 mL pipettor, quickly pipette each well of aggregates 3 - 4 times.
9. Shake the plate to distribute aggregates evenly along the bottom of the wells. Incubate at 37°C and 5% CO_2. Minimize movement of the plate during incubation.

Day 5 - 6

10. Once per day, quickly pipette each well of aggregates 3 - 4 times using a P1000 micropipette to prevent/reduce aggregate fusion. Shake plate to distribute aggregates evenly along the bottom of the wells. Minimize movement of the plate during incubation.
11. On days 5 and 6, observe aggregates under a microscope. Aggregates should start to grow slightly larger and exhibit rough edges (see Figure 3). The presence of single cells (shed from the aggregates) indicates good differentiation.
12. On day 6, proceed to section 3.6, using only aggregates with rough edges (one seeding density may not display the desired morphology).

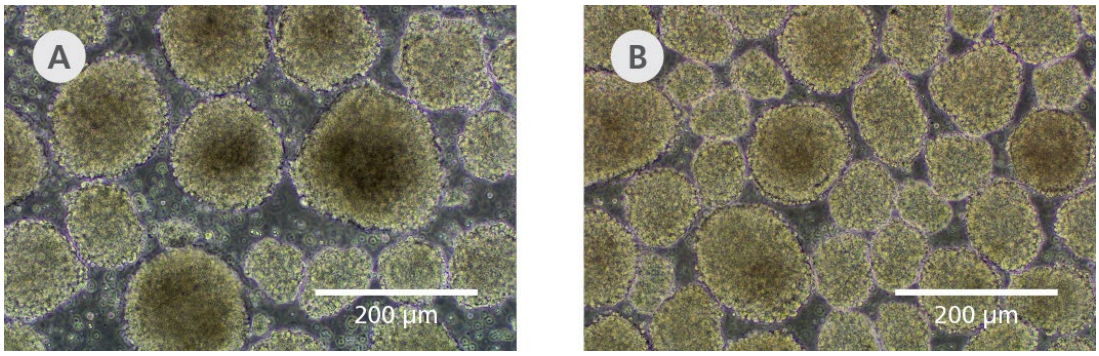


Figure 3. Aggregates on Day 6 in Vascular Induction Medium

Bright-field images of aggregates on day 6; aggregates become larger and show rough edges. Aggregates were generated from **(A)** WLS-1C iPS and **(B)** H1 ES cell lines. Magnification: 10X.

3.6 Blood Vessel Network Formation (Day 6 - 11)

Day 6

1. Thaw STEMdiff™ Blood Vessel Organoid Maturation Medium at room temperature (15 - 25°C).

   Note: If not used immediately, store at 2 - 8°C for up to 2 weeks. Alternatively, aliquot and store at -20°C. Do not exceed the shelf life of the medium. After thawing aliquots, use immediately. Do not re-freeze.
2. Warm STEMdiff™ Blood Vessel Organoid Maturation Medium to 37°C.
3. Determine the number of wells of 12-well plates required for embedding aggregates as follows: Count the number of aggregates per well under a microscope. **Do not** count very small aggregates; these will be lost during washing and **will not** generate organoids. One full 6-well plate of aggregates can be embedded into two or three 12-well plates. This usually corresponds to ~60 aggregates per well.

   Note: The density of aggregates per well is important. Seeding the aggregates too densely will lead to excessive fusion of blood vessel networks; a sparse seeding density will result in inefficient vascular network formation.

3.6.1 Preparation of Collagen/Matrigel® Matrix

Aggregates must be embedded between two layers of Collagen/Matrigel® Matrix. The following example is for preparing 12 wells of embedded aggregates (12 mL of Collagen/Matrigel® Matrix). For other amounts, adjust accordingly.

1. Determine the total amount of Collagen/Matrigel® Matrix required, based on 1 mL per well of embedded aggregates (0.5 mL/well for Layer 1 + 0.5 mL/well for Layer 2). Each layer is composed of 75% Collagen/Medium Solution and 25% Corning® Matrigel® Growth Factor Reduced Basement Membrane Matrix.

   Example: For 12 wells of embedded aggregates, 12 mL of Collagen/Matrigel® matrix is required (6 mL per layer). Each layer will be composed of 4.5 mL of Collagen/Medium Solution and 1.5 mL of Matrigel®.

2. Thaw the required Matrigel® aliquots on ice.
3. For Layer 1, pipette ONLY the '**Medium Components**' in the order listed in Table 2 into a 15 mL conical tube and let sit on ice. Repeat for Layer 2 in a second 15 mL conical tube.

Table 2. Preparation of Collagen/Medium Solution

	REAGENT	VOLUME (for one layer in 12 wells)*
Base	Sodium hydroxide solution (1 N)	41.7 µL
Collagen	Collagen Solution (3 mg/mL)	3.33 mL
Medium Components	5X DMEM/HIGH (1.34 g per 20 mL of sterile-filtered water)	626 µL
	GlutaMAX™ Supplement	32 µL
	Ham's F12 Nutrient Mix	795 µL
	HEPES Buffer Solution (1 M)	63 µL
	Sodium bicarbonate 7.5% solution	49 µL
	Sterile-filtered water	61 µL
Total Volume (for one layer)		**4997.7 µL**

*Do not scale volumes lower, as this will affect polymerization.

4. Place 4 x 15 mL empty conical tubes on ice.
5. Immediately before use, reverse pipette Collagen Solution for Layer 1 into a cold 15 mL conical tube as follows:
   a. Set a P1000 micropipette to 666 µL.
   b. Depress micropipette to the second stop and pipette up the Collagen Solution.
   c. Pipette the Collagen Solution into the cold 15 mL conical tube, stopping once you reach the first stop on the micropipette.
   d. Repeat four additional times to obtain a total of 3.33 mL (666 µL x 5).

   Note: Always place Collagen Solution bottle on ice and keep 15 mL conical tubes on ice at all times.
6. Keeping the tubes on ice, add the contents of the tube containing Medium Components for Layer 1 (prepared in step 3) to the Collagen Solution tube (prepared in step 5) and pipette up and down several times to mix. Ensure that the mixed solution is uniformly yellow and place back on ice.

   Note: Do not introduce air bubbles while pipetting.
7. Add sodium hydroxide solution ('Base' in Table 2) to the tube containing Collagen + Medium Components. Pipette up and down repeatedly until the solution is mixed thoroughly and appears uniformly red. This is the **Collagen/Medium Solution**.

8. Reverse pipette 750 µL x 6 of the Collagen/Medium Solution into an empty, cold 15 mL conical tube on ice to obtain a total volume of 4.5 mL. Add 1.5 mL of Corning® Matrigel® Growth Factor Reduced Basement Membrane Matrix to the tube, then use a P1000 micropipette to mix. This is the **Collagen/Matrigel® Matrix** solution (6 mL).
9. Proceed to section 3.6.2.

3.6.2 Embedding Aggregates in Collagen/Matrigel® Matrix

1. To prepare Layer 1, add 0.5 mL of Collagen/Matrigel® Matrix solution per well of a 12-well plate. Shake the plate horizontally to evenly distribute the solution across the wells.
2. Incubate the plate at 37°C (atmospheric CO_2) for 20 - 30 minutes to solidify the solution.
3. From one well of the 6-well plate containing aggregates, use a 10 mL serological pipette to transfer the aggregates to a 15 mL conical tube. Repeat for remaining wells, for a total of 6 x 15 mL conical tubes.
4. Let aggregates settle by gravity for 5 - 10 minutes. Remove medium.
5. Add 2 mL of DMEM/F-12 to each tube. Collect aggregates by gravitation. Remove medium.
6. Pool the aggregates from all tubes into one 15 mL conical tube as follows:
   a. Add 1 mL DMEM/F-12 to one 15 mL conical tube containing aggregates, then transfer this volume between all conical tubes until all aggregates are pooled in one tube.
   b. Pipette the aggregates up and down with a 1 mL pipettor to prevent excessive aggregate sticking.
   c. Let aggregates settle by gravity for 5 - 10 minutes. Remove medium using a 10 mL serological pipette on **slow**, leaving behind 200 - 400 µL of medium in each tube.
   d. Use a 200 µL pipettor to remove all remaining medium.
   e. Place aggregates on ice for at least 10 minutes.
7. Prepare Collagen/Matrigel® Matrix solution for Layer 2 by repeating section 3.6.1 steps 5 - 8. Add the solution (6 mL) to the aggregates and keep on ice.
8. Remove the 12-well plate containing Layer 1 from the incubator. Pipette the Layer 2 aggregate/matrix solution up and down to evenly distribute aggregates. Carefully add 0.5 mL of aggregate/matrix solution to the middle of each well.

   Note: To get even distribution of aggregates, add solution to the middle of well, dispensing quickly. The aggregates should evenly spread out across the bottom of the wells. If they are not evenly distributed, finish dispensing all wells of one plate, then move the plate in a figure-8 motion to distribute aggregates evenly.
9. Let the plate sit at room temperature (15 - 25°C) for 5 minutes to begin polymerization. Then, slowly move the plate to a 37°C incubator. Incubate for 1 - 2 hours for matrix solidification.
10. Add 1 mL of warm (37°C) STEMdiff™ Blood Vessel Organoid Maturation Medium dropwise to the middle of each well.
11. Observe matrix; ensure that the matrix is intact, and the layers have not separated.
12. Incubate at 37°C and 5% CO_2 for 24 hours. Proceed to section 3.6.3.

3.6.3 Blood Vessel Network Sprouting

Day 7 - 9

1. Observe the blood vessel networks under a microscope. Radially sprouting vessels should be visible 24 hours after embedding. By day 9, the middle of the organoids should not appear too dense.

2. On **day 9**, perform a full-medium change as follows:
   a. Tilt the plate forward and remove medium by keeping the pipette tip at the interface of the medium and air. Ensure not to pipette up the matrix.
   b. Add warm (37°C) Maturation Medium dropwise to the center of the well.
   c. Incubate at 37°C and 5% CO_2 for 2 days.

Day 11

3. Observe vascular networks sprouting under a microscope. Vascular networks should have extensive radial sprouting, and little to no denseness in the center of the organoids (see Figure 4). Organoids should be ~500 µm to 1 mm in diameter. Proceed to section 3.7, using only organoids with the desired morphology.

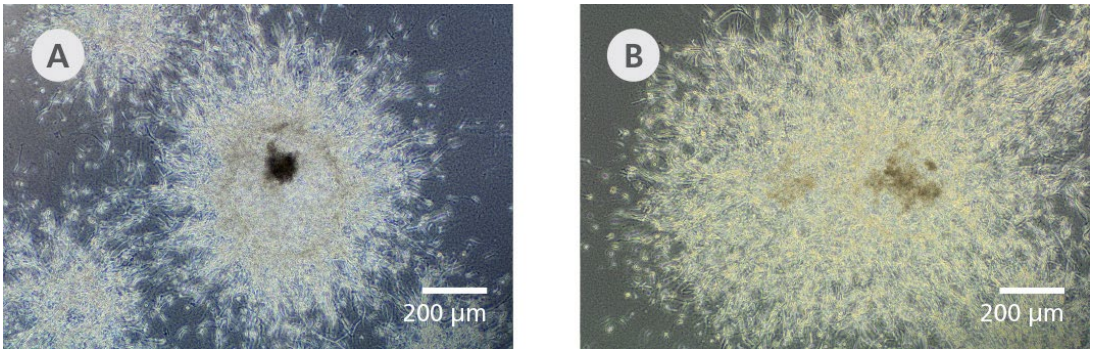


Figure 4. Blood Vessel Networks Sprouting on Day 11 in STEMdiff™ Blood Vessel Organoid Maturation Medium

Representative bright-field images of blood vessel networks sprouting on day 11 in STEMdiff™ Blood Vessel Organoid Maturation Medium, generated from **(A)** WLS-1C iPS and **(B)** H1 ES cell lines. Magnification: 4X.

3.7 Blood Vessel Organoid Self-Assembly and Maturation (Day 11 - 30+)

Note: Warm STEMdiff™ Blood Vessel Organoid Maturation Medium to room temperature (15 - 25°C) before use.

Day 11

1. Equip a biosafety cabinet with a tissue culture microscope. Ensure to spray surfaces to sterilize and wipe down the microscope with a Kimwipe™ and 70% isopropyl alcohol.
2. For blood vessel organoid cultures, the entire matrix layer must be detached from the plate well bottom using the round end of a sterile spatula. Move the spatula along the edges of the well to detach the matrix, then move the spatula toward the well center along the bottom of the well.
3. Using the sterile spatula, transfer the matrix onto a lid of a 100 mm dish.
4. Using two sterile 27G needles, cut out single blood vessel networks and try to reduce the amount of matrix surrounding the organoids. Depending on initial seeding densities, you should expect a full 96-well plate of organoids from 2 - 3 wells of the 12-well plate.

5. Transfer single organoids to individual wells of a 96-well 3D culture spheroid plate as follows:
   a. Using a cut 1 mL pipette tip, pipette up 150 µL of Maturation Medium.
   b. Dispense a small amount of medium on the organoid, then pipette up the organoid + medium. Dispense into one well of the 96-well plate.

      Note: If you cut quickly (i.e. cut 20 - 30 organoids in ~15 minutes), then it is best to cut 20 - 30 organoids at a time before transferring organoids. Cut organoids will dry over time and stick to the dish lid, so it is best to transfer organoids quickly to the 96-well plate.
6. Incubate the 96-well plate at 37°C and 5% CO_2 for 4 days.

Day 15

7. Observe organoids under a microscope. Organoids should be completely spherical and blood vessels should be visible (see Figure 5). Organoids will be ~500 µm to 2 mm in length.

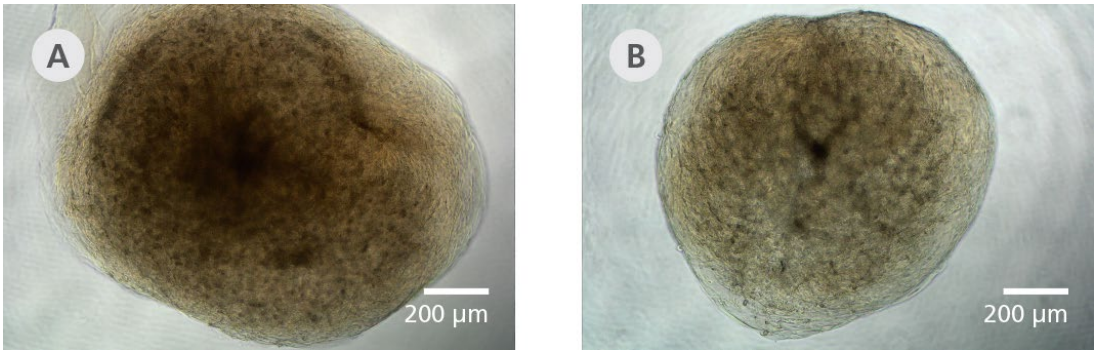


Figure 5. Mature, Spherical Blood Vessel Organoids on Day 15 in STEMdiff™ Blood Vessel Organoid Maturation Medium and a 96-Well Plate

Bright-field images of blood vessel organoids generated from **(A)** WLS-1C and **(B)** H9 cell lines. Magnification: 4X.

8. Using a multi-channel pipettor, perform a full-medium change as follows:
   a. Remove medium from organoids, then add 150 µL of fresh Maturation Medium.
   b. Incubate at 37°C and 5% CO_2.

Day 18/19 - 30+

9. Perform a full-medium change with Maturation Medium every 3 - 4 days.

4.0 Troubleshooting

PROBLEM	POSSIBLE CAUSE	RECOMMENDED ACTION
Aggregate Formation (Day 0 - 1)		
Many small and fragile aggregates	Starting hPSCs were of poor quality	• Check pluripotency by observing morphology and labeling markers of undifferentiated state (OCT3/4 and TRA-1-60) to ensure hPSCs are of high quality. If OCT3/4 and TRA-1-60 are low (< 90%), restart with high-quality hPSCs • Start with high-quality hPSCs from an earlier passage number • Passage cells when they are no more than 70 - 80% confluent
	6-well ultra-low attachment plate was disturbed during culture	• Ensure 6-well plate is not disturbed for at least 24 hours after seeding (section 3.3 step 18)
	Poor-aggregating cell line	• Increase the initial cell seeding density (section 3.3 step 14) • Increase aggregation time to a maximum of 2 days (section 3.3 step 19)
Many large and dense aggregates	Starting hPSCs were of poor quality	• As recommended above
	hPSCs were not dissociated sufficiently	• Use fresh ACCUTASE™ and EDTA (section 3.3 steps 6 & 8) • Ensure hPSCs dissociate into a single-cell suspension and are not in clumps after pipetting (section 3.3 steps 10 & 11) • Increase duration of incubation with ACCUTASE™ (section 3.3 step 9)
Mesoderm Induction (Day 1 - 4) and Vascular Induction (Day 4 - 6)		
Many large and dense aggregates	Starting hPSCs were of poor quality	• Do not continue experiment; restart with high-quality hPSCs
	6-well ultra-low adherent was disturbed during culture	• Ensure 6-well plate is not disturbed during culture (section 3.4 steps 7 - 9)
	Fusion of aggregates	• Increase pipetting to 2 times per day, pipette with more force, and ensure the aggregates are evenly distributed along the bottom of the well (section 3.4 steps 7 - 9 & section 3.5 steps 8 - 10) • Decrease the initial cell seeding density (section 3.3 step 14)
Aggregates are not spherical on Day 4	Pipetting of aggregates is too harsh	• Pipette with less force (section 3.4 step 8)
Vascular Induction (Day 4 - 6)		
Aggregates do not get rough edges and there is little cell debris in the wells	Starting hPSCs were of poor quality	• Do not continue experiment; restart with high-quality hPSCs
	Initial cell seeding density was too high or aggregation duration was too long	• There should be only ~120 - 250 aggregates/well after vascular induction • Decrease the initial cell seeding density (section 3.3 step 14) • If aggregation step was optimized for 2 days, decrease duration of aggregation to 1 day (section 3.3 steps 18 - 19)

PROBLEM	POSSIBLE CAUSE	RECOMMENDED ACTION
Blood Vessel Network Formation (Day 6 - 11)		
There is poor vessel sprouting	Inefficient mesoderm/vascular induction	• Ensure there is not excessive aggregate fusion • Aggregates should display correct morphology during differentiation
	Matrigel® lot was suboptimal	• Use alternative lot of Matrigel® (protein concentration > 8.5 mg/mL)
Matrix detaches from culture dish OR Layer #1 and Layer #2 split after medium addition	Poor collagen polymerization	• Ensure Collagen Solution and all other solutions are kept on ice and are not exposed to temperatures above 2 - 8°C, as polymerization ability will be reduced • Always pipette viscous Collagen Solution using reverse pipetting • Ensure Medium Components, Collagen Solution, and Base are combined immediately before use (section 3.6.1 Table 2) • Ensure 5X DMEM HIGH has not precipitated • Ensure sodium hydroxide solution is 1 M • Test pH of Collagen/Medium Solution using pH indicator strips after addition of 1 M sodium hydroxide; the pH should be ~7 - 7.5 for polymerization to occur
Blood Vessel Organoid Maturation (Day 11 - 30+)		
Organoids do not have self-encapsulated, spherical morphology	Organoids cut had too much matrix left around them	• Organoids should have as much excessive matrix cut off as possible, without cutting blood vessels
	Organoids have sub-optimal composition of pericytes	• It is best to use flow cytometry (FACS) analysis to determine the cell composition of organoids and ensure both endothelial cells and pericytes are present
Organoids have increased cell debris/cells released during culture	Organoids are reaching the optimal timepoint for use	• Reduce culture time
	Organoids are releasing/generating other cell types	• This can spontaneously occur in culture; usually these cells will stay viable for a short period of time and co-stain for hematopoietic marker CD45 and endothelial marker CD144

5.0 Fixation, Immunostaining, and Mounting

5.1 Materials, Reagents, and Equipment

5.1.1 Materials Required

PRODUCT	CATALOG #
1.7 mL microcentrifuge tubes	e.g. 38089
3 cc Syringes	28230
Bovine serum albumin (BSA)	e.g. Millipore Sigma 10735078001
Clear nail polish	e.g. Sally Hansen Insta-Dri Top Coat
Conical tubes	e.g. 38009 (15 mL) and 38010 (50 mL)
Cover glasses, rectangular (24 mm x 50 mm)	e.g. Fisher Scientific 12-543D
DAPI (Hydrochloride)	75004
D-PBS (Without Ca++ and Mg++)	37350
Fetal bovine serum (FBS)	e.g. 100-0179
Fine forceps	e.g. Fine Science Tools 11254-20
Fluorescence Mounting Medium	Agilent S3023
iSpacer® 1.0 mm	SUNJin Lab IS003
Needles, 27 G x ½" length	VWR CABD305109
Paraformaldehyde (PFA)	e.g. Thermo Fisher 28908
RapiClear® 1.47 (water-soluble clearing reagent)	SUNJin Lab RC147001:10ml
Sodium deoxycholate	e.g. Millipore Sigma D6750-10G
Spatula	e.g. VWR 82027-532
Tissue culture dish lid (plastic or glass), 100 mm	e.g. 100-0082
Triton™ X-100	e.g. Millipore Sigma X100-5ML
Tween® 20	e.g. Millipore Sigma P1379-25ML

5.1.2 Equipment Required

- Biohazard safety cabinet certified for Level II handling of biological materials
- Rocking platform shaker

5.1.3 Preparation of Reagents

Prepare the following reagents before proceeding to section 5.2.

Blocking Buffer

Prepare Blocking Buffer by combining components as indicated in Table 3. Let BSA dissolve, then mix thoroughly and store at 2 - 8°C for up to 2 months.

Note: Blocking Buffer components may vary with the type of antibody used.

Table 3. Preparation of Blocking Buffer

COMPONENT	AMOUNT	FINAL CONCENTRATION
Bovine serum albumin (BSA)	0.5 g	1%
D-PBS	~30 mL	--
Fetal bovine serum (FBS)	1.5 mL	3%
Tween® 20	250 µL	0.5%
Triton™ X-100	250 µL	0.5%
Sodium deoxycholate solution (1% wt/vol)*	500 µL	0.01%
D-PBS	Top up to final volume of 50 mL	--

*Add 0.5 g sodium deoxycholate to 50 mL of D-PBS. Mix thoroughly and store at 15 - 25°C for up to 1 month.

4% PFA Solution

Prepare a 4% solution of paraformaldehyde (PFA) in D-PBS. Mix thoroughly and store at 2 - 8°C for up to 1 month.

Wash Buffer (PBS-T)

Dilute Tween® 20 in D-PBS to a final concentration of 0.05%. Mix thoroughly and store at 2 - 8°C for up to 1 month.

DAPI Solution

Add DAPI to D-PBS to a final concentration of 1 µg/mL. Mix thoroughly.

5.2 Fixation, Immunostaining, and Mounting Protocols

The following protocols are for immunofluorescence fixing, staining, and mounting of either vascular networks (day 11) or blood vessel organoids (day 15 - 30+). Carefully follow the volumes indicated, depending on whether vascular networks or blood vessel organoids are being stained.

5.2.1 Fixation of Vascular Networks

1. Equip a biosafety cabinet with a tissue culture microscope. Spray surfaces to sterilize and wipe microscope with 70% isopropyl alcohol on a Kimwipe™.
2. For staining of vascular networks, the entire matrix must be detached from the well bottom using the round end of a sterile spatula. Move the spatula along the edges of the well to detach the matrix. Then, move spatula toward the well center along the bottom of the well until the matrix is freely floating.
3. Under sterile conditions, transfer the matrix using a sterile lab spatula onto the lid of a 100 mm dish.
4. Using two sterile 27G needles, divide the matrix into halves or quarters, ensuring not to cut through any vascular networks.
5. Using a round-ended spatula, transfer the pieces of vascular networks to a 15 mL conical tube.

6. Add 5 mL of D-PBS to the tube. Let vascular networks settle to the bottom of the tube, then remove D-PBS, taking care not to pipette up the vascular networks. Repeat this wash step.
7. Add 5 mL of 4% PFA Solution to the tube (fixation). Incubate on a rocking platform at room temperature (15 - 25°C) for 20 minutes. Remove the fixation solution.
8. Add 2 mL of D-PBS to the tube. Let vascular networks settle to the bottom of the tube, then remove D-PBS. Repeat this wash step.

   Note: If immunostaining will not be performed immediately, add 2 mL of D-PBS to the 15 mL conical tube containing the vascular networks, wrap the lid with Parafilm®, and store at 2 - 8°C for up to 2 months.
9. Proceed to section 5.2.3 for immunostaining.

5.2.2 Fixation of Blood Vessel Organoids

1. Using a cut 1 mL pipette tip, transfer blood vessel organoids to a 15 mL conical tube.

   Note: A maximum of 32 organoids can be fixed and stained in one 15 mL conical tube.
2. Let organoids settle to the bottom of the tube, then remove medium using a serological pipette.
3. Add 5 mL of D-PBS to the tube. Let organoids settle to the bottom of the tube, then remove D-PBS, taking care not to pipette up the organoids. Repeat this wash step.
4. Add 2 mL of 4% PFA Solution to organoids (fixation). Incubate on a rocking platform at room temperature (15 - 25°C) for 60 minutes. Remove the solution.
5. Wash blood vessel organoids 2X with 2 mL D-PBS. Remove D-PBS.

   Note: If immunostaining will not be performed immediately, add 2 mL D-PBS to the 15 mL conical tube containing the blood vessel organoids, wrap the lid with Parafilm®, and store at 2 - 8°C for up to 2 months.
6. Proceed to section 5.2.3 for immunostaining.

5.2.3 Immunostaining Vascular Networks & Blood Vessel Organoids

1. Remove all D-PBS from vascular networks/blood vessel organoids.
2. Add 2 mL of Blocking Buffer to each 15 mL conical tube and incubate on a rocking platform at room temperature (15 - 25°C) for 3 hours.
3. During Blocking Buffer incubation, prepare primary antibody mix by diluting each primary antibody in Blocking Buffer. Refer to Table 4 for recommended antibodies and dilutions. Keep primary antibody mix cold (2 - 8°C).

Table 4. Recommended Primary Antibodies and Dilutions

PRIMARY ANTIBODY	DILUTION	CATALOG #
Actin Smooth Muscle	1 in 100	Thermo Fisher Scientific 14-9760-80
Calponin 1	1 in 100	Abcam AB46794
CD144	1 in 50	Santa Cruz SC9989
CD31	1 in 100	Agilent DAKO M0823
CD31	1 in 100	R&D Systems AF806
Collagen-IV	1 in 200	Millipore Sigma AB769
PDGFRβ	1 in 100	R&D Systems AF385
PDGFRβ	1 in 100	Cell Signaling 3169S
SMA	1 in 100	Millipore Sigma 2547

4. Remove tubes from the rocking platform and allow vascular networks/blood vessel organoids to settle to the bottom of the tubes.
5. Transfer vascular networks/blood vessel organoids to 1.7 mL microcentrifuge tubes as follows:
   - Vascular networks: Remove ~1 mL of Blocking Buffer, then invert the 15 mL conical tube over microcentrifuge tube.
   - Blood vessel organoids: Use a cut 1 mL pipette tip to pipette organoids into microcentrifuge tubes.
6. Let vascular networks/blood vessel organoids settle to the bottom of the tubes, then remove any remaining Blocking Buffer.
7. Add cold primary antibody mix as follows:
   - Vascular networks: Add 250 µL
   - Blood vessel organoids: Add 100 µL
8. Incubate tubes at 2 - 8°C on a rocking platform overnight (> 16 hours). Remove primary antibody mix.
9. Add 1 mL of Wash Buffer and incubate on a rocking platform at room temperature for 30 minutes. Allow the vascular networks/blood vessel organoids to settle to the bottom of the tubes, then remove the Wash Buffer. Repeat this wash step 2X for a total of 3 washes.
10. During Wash Buffer incubation, prepare secondary antibody mix by diluting each secondary antibody in Blocking Buffer (use manufacturer's recommended dilution). Keep secondary antibodies at room temperature in the dark. Remove any remaining Wash Buffer.
11. Add secondary antibody mix as follows:
    - Vascular networks: Add 625 µL
    - Blood vessel organoids: Add 250 µL
12. Incubate tubes on a rocking platform at room temperature for 3 hours in the dark. Remove secondary antibody mix.
13. Add 1 mL of Wash Buffer and incubate on a rocking platform at room temperature for 30 minutes in the dark. Allow the vascular networks/blood vessel organoids to settle to the bottom of the tube, then remove the Wash Buffer. Repeat this wash step for a total of 2 washes.
14. Add 1 mL of DAPI Solution and incubate vascular networks/blood vessel organoids on a rocking platform at room temperature for 10 minutes in the dark. Remove DAPI Solution.
15. Add 1 mL of D-PBS and incubate vascular networks/blood vessel organoids on a rocking platform at room temperature for 10 minutes in the dark.
16. Proceed to section 5.2.4 (vascular networks) or section 5.2.5 (blood vessel organoids).

5.2.4 Mounting Vascular Networks

1. Using fine forceps, transfer one piece of vascular networks to a 24 x 50 mm cover glass.
2. Remove excess D-PBS from networks with a Kimwipe™, ensuring not to touch the networks as the Kimwipe™ will stick.
3. Add 1 - 2 drops of Fluorescence Mounting Medium.
4. Place a 24 x 50 mm cover glass on top and gently squeeze to spread the mounting medium evenly. Do not use excessive force, as the networks can slip out.
5. Let slides sit in the dark on top of a Kimwipe™ for several minutes to allow even distribution of mounting medium.
6. Place slides on top of Parafilm® and incubate at room temperature (15 - 25°C) overnight (> 16 hours) in the dark.
7. Seal edges of cover glass with clear nail polish, then proceed to immunofluorescent imaging.

   Note: Slides can be stored in the dark for several months at 2 - 8°C. However, it is best to image as soon as possible after staining.

5.2.5 Clearing and Mounting Blood Vessel Organoids

Clearing Blood Vessel Organoids

1. Remove D-PBS from stained organoids.
2. Warm RapiClear® 1.47 to room temperature (15 - 25°C), then invert to mix. Using a cut 1 mL pipette tip, add 500 µL of RapiClear® 1.47 to stained organoids.
3. Incubate organoids at room temperature overnight (> 16 hours) in the dark.

Mounting Blood Vessel Organoids

4. Add adhesive strip to the side of the iSpacer® that does not already have adhesive.
5. Using forceps, remove the protective film covering the adhesive on one side of the iSpacer®.
6. Attach iSpacer® to a 24 x 50 mm cover glass via the uncovered adhesive. Seal the iSpacer® to the slide; the back end of forceps can be used to push the iSpacer® down to make a tight seal. Be careful not to break the cover glass while sealing the iSpacer®.
7. Using a cut 1 mL pipette tip, pipette organoids up and down several times, then pipette into the iSpacer®.
8. Warm RapiClear® 1.47 to room temperature (15 - 25°C), then invert to mix. Using a cut 1 mL pipette tip, pipette a sufficient volume of RapiClear® 1.47 to fill the iSpacer® (containing the organoids) to the top level for mounting, taking care not to overflow.
9. Using forceps, remove the protective film covering the adhesive on top of the iSpacer®.
10. Place a 24 x 50 mm cover glass on top by lying the left side down first, then slowly lowering the right side.

    Note: This ensures no bubbles form in the clearing medium.
11. Seal edges of cover glass with clear nail polish, then proceed to immunofluorescent imaging.

    Note: Slides can be stored in the dark for several months at 2 - 8°C. However, it is best to image as soon as possible after staining.

6.0 Flow Cytometry (FACS) Staining

6.1 Materials, Reagents, and Equipment

6.1.1 Materials Required

PRODUCT	CATALOG #
D-PBS (Without Ca++ and Mg++)	37350
Dulbecco's Phosphate Buffered Saline with 2% Fetal Bovine Serum (D-PBS with 2% FBS)	07905
Ethylenediaminetetraacetic acid disodium salt solution (EDTA, 0.5 M)	Millipore Sigma E7889-100mL
Fetal bovine serum (FBS)	e.g. 100-0179
DMEM with 4500 mg/L D-Glucose OR DMEM/F-12 with 15 mM HEPES	36250 OR 36254
Liberase™ TH Research Grade	Millipore Sigma 5401135001
Dispase II, powder	Thermo Fisher 17105041
DNase I Solution (1 mg/mL)	07900
Conical tubes	e.g. 38009 (15 mL) and 38010 (50 mL)
Round-bottom polystyrene tubes, 5 mL	e.g. 100-0088
96-well round-bottom microplate	e.g. 38018
12 mL syringes	e.g. Cardinal Health 8881512878
MiniSart® Syringe Filter, PES, 0.22 µm	Sartorius 16532-GUK
70 µm cell strainer	e.g. 27260
Falcon® Round-Bottom Tubes with Cell Strainer Cap, 5 mL	100-0087
Tissue culture dish lid (plastic or glass), 100 mm	e.g. 100-0082
Scissors	Fine Science Tools 14083-08
Trypan Blue	07050

6.1.2 Equipment Required

- Rocking platform shaker
- Low-speed centrifuge with a swinging bucket rotor

6.1.3 Preparation of Reagents

Prepare the following reagents before proceeding to section 6.2:

FACS Buffer

To D-PBS with 2% FBS, add 0.5 M EDTA to a final concentration of 2 mM. Mix thoroughly and store at 2 - 8°C.

Inhibition Medium

To DMEM or DMEM/F-12, add 10% FBS. Mix thoroughly and store at 2 - 8°C.

Liberase™ TH Solution

1. Place 2 x 5 mg vials of Liberase™ TH on ice.
2. Add 2 mL of D-PBS (Without Ca++ and Mg++) to each vial and let enzyme dissolve. Do not let sit on ice for longer than 20 minutes.
3. Once dissolved, pipette to mix, then prepare 1.6 mL aliquots (4 mg).

   Note: If not used immediately, store at -20°C.

6.2 Enzymatic Digestion of Vascular Networks/Blood Vessel Organoids

1. Immediately before vascular networks/blood vessel organoids are ready for digestion, prepare Enzymatic Mix as indicated in Table 5 in a 15 mL conical tube. Thaw all frozen aliquots at room temperature (15 - 25°C). Prepare Dispase II fresh.

   Note: 10 mL of Enzymatic Mix is sufficient for one well of vascular networks, or ~32 blood vessel organoids. For other amounts, adjust volumes in Table 5 accordingly.

   Table 5. Preparation of Enzymatic Mix (10 mL)

COMPONENT	VOLUME
Liberase™ TH Solution	1.6 mL
Dispase II (dissolve in D-PBS to 10 mg/mL)	3 mL
DNase I Solution (1 mg/mL)	500 µL
D-PBS (Without Ca++ and Mg++)	Top up to final volume of 10 mL

2. Incubate Enzymatic Mix on a rocking platform at 37°C for 25 minutes. Do not over-incubate.
3. For vascular networks: Use a lab spatula to transfer one well of vascular networks to a 100 mm dish lid. Remove medium.

   OR

   For blood vessel organoids: Use a cut 1 mL pipette tip to transfer ~32 organoids to a 100 mm dish lid. Remove medium.
4. Mince vascular networks/blood vessel organoids with scissors until they are a slurry. Once minced, set aside until the Enzymatic Mix has finished incubating.

   Note: Proper mincing is important, as this will influence how well the enzymatic digestion works. Lift one edge of the 100 mm dish lid, such that vascular networks/blood vessel organoids are pooled to one area, along the lip of the lid; this allows for easier mincing of samples.
5. Sterile filter the Enzymatic Mix into a new 15 mL conical tube through a 12 mL syringe equipped with a 0.22 µm PES syringe filter.

6. Using a cut 1 mL pipette tip, transfer 1 mL of Enzymatic Mix to the lid containing minced sample. Pipette up and down to resuspend, then transfer the suspension to the tube containing Enzymatic Mix.
7. Incubate on a rocking platform at 37°C and 5% CO_2 for 30 minutes.
8. Assess whether vascular networks/blood vessel organoids are digested. If there are still many minced pieces of sample, incubate for an additional 10 - 15 minutes.
9. Triturate sample several times by pipetting up and down, then pipette the sample through a 70 µm cell strainer into a cold 50 mL conical tube on ice.

   Note: For the remainder of the protocol, keep samples on ice.
10. Rinse sample tube with 10 mL of Inhibition Medium and pipette through the 70 µm cell strainer.
11. Pipette 30 mL of Inhibition Medium through the 70 µm cell strainer.
12. Remove the strainer from the 50 mL conical tube.
13. Centrifuge cells at 300 x *g* for 10 minutes at 2 - 8°C.
14. Observe the tube. If there is a cell pellet, proceed to step 15. If there is a viscous solution rather than a cell pellet at the bottom of the tube, the digestion has not worked. Repeat the digestion with a fresh sample, ensuring Enzymatic Mix components are at the correct concentration, not over-incubated, and all medium is removed from samples before mincing.
15. Aspirate medium and resuspend cells in 500 µL of FACS Buffer.
16. Filter sample through a Falcon® round-bottom tube with cell strainer cap and place on ice.
17. Proceed to section 6.3.

6.3 FACS Staining of Vascular Networks/Blood Vessel Organoids

1. Count cells using Trypan Blue and a hemocytometer.
2. Aliquot 1 x 10^5 cells/FACS staining panel into either 5 mL FACS round-bottom tubes or 96-well round-bottom plates.
3. Centrifuge cells at 300 x *g* for 5 minutes at 2 - 8°C.
4. While the samples are centrifuging, prepare a sufficient quantity of antibody mix (100 µL/sample) using the appropriate antibody at the predetermined optimal working dilution.
5. Carefully remove the supernatant without disrupting the cell pellet, then resuspend cells in the antibody mix. Gently mix and incubate at 2 - 8°C for 30 - 60 minutes. Protect samples from direct light.
6. If using 5 mL FACS tubes, add 1 mL of FACS Buffer to each tube; if using 96-well round-bottom plates, add 200 µL of FACS Buffer to each well. Gently mix, then centrifuge at 300 x *g* for 5 minutes at 2 - 8°C.
7. Carefully remove the supernatant without disturbing the cell pellet.
8. Repeat steps 6 and 7.
9. Resuspend cells in 200 µL of FACS Buffer. Transfer to a 5 mL round-bottom tube if necessary.
10. Optional nuclear stain: DAPI (Catalog #75004) can be added at a final concentration of 0.2 µg/mL to assess viability (e.g. add 1 mg/mL DAPI at a 1 in 5000 dilution to the cell suspension from step 9).
11. Place samples on ice, while avoiding exposure to direct light, and analyze by flow cytometry as soon as possible.

7.0 Cryopreservation & Thawing of Blood Vessel Organoids

7.1 Materials, Reagents, and Equipment

7.1.1 Materials Required

PRODUCT	CATALOG #
Conical tubes, 15 mL	38009
Corning® Cryogenic Vials with Orange Caps	100-0091
Dimethyl Sulfoxide (DMSO)	e.g. Fisher Scientific D128-500
DMEM/F-12 with 15 mM HEPES	36254
Fetal bovine serum	e.g. 100-0179
PrimeSurface® 3D Culture Spheroid Plates: Ultra-low Attachment Plates, 96 well, U-bottom, Clear Plates	Sumitomo Bakelite MS-9096UZ
STEMdiff™ Blood Vessel Organoid Maturation Medium	100-0658
Ultra-Low Adherent Plate for Suspension Culture	38071

7.1.2 Equipment Required

- Biohazard safety cabinet certified for Level II handling of biological materials
- Incubator with humidity and gas control to maintain 37°C and 95% humidity in an atmosphere of 5% CO_2 in air
- Inverted microscope with phase objectives

7.2 Preparation of Media

Use sterile technique to prepare media. Prepare each medium as needed in the protocols. Refer to Table 1 for medium components, volumes, and in-use storage and stability.

Table 6. Preparation of Media

MEDIUM	COMPONENT	VOLUME	PREPARATION & STORAGE
Cryopreservation Medium (10 mL)	Fetal Bovine Serum	9 mL	Use immediately
	DMSO	1 mL	
Recovery Medium (10 mL)	DMEM/F-12 with 15 mM HEPES	9 mL	Use immediately
	Fetal Bovine Serum	1 mL	

7.3 Cryopreservation of Blood Vessel Organoids

1. Mature organoids in 96-well spheroid plates until at least Day 15 of the protocol.
2. Prepare Cryopreservation Medium and chill on ice for 30 minutes.
3. Using a cut P1000 tip, transfer 20 blood vessel organoids to a 15 mL conical tube and let sit on ice for 10 minutes.
4. Remove the majority of the medium with a 10 mL serological pipette, then use a P1000 tip to remove any remaining medium.
5. Add 1 mL of Cryopreservation Medium to the blood vessel organoids.
6. Place the tube on ice for 5 minutes.
7. Transfer the blood vessel organoids with a cut P1000 tip to Corning® Cryogenic Vial(s).
8. Place the vial(s) in an isopropanol freezing container at -80°C to -150°C overnight.
9. Transfer the vial(s) to liquid nitrogen the next day.

7.4 Thawing of Cryopreserved Blood Vessel Organoids

1. Prepare Recovery Medium and STEMdiff™ Blood Vessel Organoid Maturation Medium and warm both at room temperature (15 - 25°C) for 1 hour.
2. Add 10 mL of Recovery Medium into a 15 mL conical tube.
3. Thaw a Corning® Cryogenic Vial containing blood vessel organoids in a 37°C water bath.
4. Using a cut P1000 tip, transfer the entire contents of the vial into the 15 mL conical tube containing recovery solution (prepared in step 2).
5. Wait 5 minutes for the thawed blood vessel organoids to settle to bottom of the 15 mL conical tube.
6. Remove the majority of medium with a 10 mL serological pipette, then use a P1000 tip to remove any remaining medium.
7. Add 3 mL of STEMdiff™ Blood Vessel Organoid Maturation Medium to blood vessel organoids. Use a cut P1000 tip to transfer the organoids into one well of a 6-well Ultra-Low Adherent Plate for Suspension Culture.
8. Shake the plate to distribute organoids, then incubate at 37°C and 5% CO_2. Do not disturb the plate for at least 24 hours.
9. After 24 hours, warm STEMdiff™ Blood Vessel Organoid Maturation Medium at room temperature for 1 hour.
10. Remove the plate from the incubator and transfer the organoids to a 15 mL conical tube using a cut P1000 tip.
11. Wait 5 minutes for the organoids to settle to the bottom of the conical tube.
12. Remove the majority of medium using a 10 mL serological pipette, then use a P1000 tip to remove any remaining medium.
13. Add 3.2 mL of STEMdiff™ Blood Vessel Organoid Maturation Medium to the organoids.
14. Using a cut P1000 tip, pipette up and down several times to suspend organoids. Transfer individual organoids in 150 µL of STEMdiff™ Blood Vessel Organoid Maturation Medium to each well of a PrimeSurface® 3D Culture Spheroid plates: Ultra-low Attachment Plates, 96 well, U-bottom, Clear Plates.

Note: It is easier to transfer the organoids when they are in the 15 mL tube than transferring them to a petri dish. You have more control by pipetting single organoids into wells so you don't accidentally add more than one.

15. Incubate at 37°C and 5% CO_2.

16. Perform a full-medium change with 150 µL/well of STEMdiff™ Blood Vessel Organoid Maturation Medium every 3 - 4 days. After 4 - 5 days, post-thaw organoids are ready to use for downstream assays.

8.0 References

1. Wimmer RA et al. (2019a) Human blood vessel organoids as a model of diabetic vasculopathy. Nature 565(7740): 505–10.
2. Wimmer RA et al. (2019b) Generation of blood vessel organoids from pluripotent stem cells. Nature Protocols 14: 3082–100.

TECHNICAL MANUAL

Generation and Maturation of Blood Vessel Organoids Using STEMdiff™ Blood Vessel Organoid Kit

STEMCELL™ TECHNOLOGIES

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