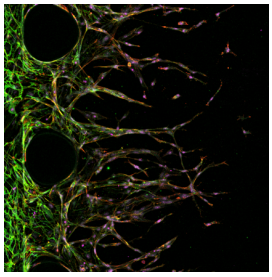
APPLICATION SPECIFIC PROTOCOL – ANGIOGENESIS

AIM 3D Cell Culture Chips offer a new perspective in studying angiogenesis by allowing the growth of new vascular sprouts in a 3D matrix from a pre-existing endothelial monolayer. With the transparent laminate at the bottom, the dynamic process of angiogenesis can be monitored in detail from a viewing angle that is perpendicular to the direction of sprouting. The two-media- channel design not only enables the generation of concentration gradient of angiogenic stimuli but also the application of interstitial flow that is very useful in inducing angiogenesis. This protocol covers the techniques to obtain consistent angiogenic sprouting in AIM chips.



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MONOLAYER FORMATION ⏱ TIMING 20 min

MATERIALS

Reagents

- 1X PBS (Life Technologies, Cat. No. 70011044)
- 0.25% trypsin with EDTA (Lonza, Cat. No. CC5012)
- Cell culture medium (Lonza, Cat. No. CC3202)

Others

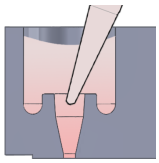
- Collagen-filled and fibronectin-coated AIM chips

1. Trypsinize endothelial cells as per protocol and re-suspend the cells at 1.5 M cells/ml.

2. Add an additional 20 µl of medium into one of the ports at the media channel that is to be seeded with cells.

   Reminder *Ports must be filled with medium before seeding cells into the media channels.*

3. Use a micropipette to withdraw 10 µl of endothelial cell suspension. Position the tip near the inlet of a media channel and inject the cell suspension. Wait for 2 min and then repeat the same procedure for the opposite connected inlet. In total, 20 µl of endothelial cell suspension is seeded per media channel. The additional 40 µl of fluid (20 µl of cell suspension and 20 µl of medium) creates a height difference between the two media channels thus generating interstitial flow across the gel. This helps the attachment of endothelial cells on the gel interface.

   Position the pipette tip near inlets while inject cell suspension

   ! Critical *Do not insert the tip completely into the inlets to avoid introducing cells into the media channels at a high flow rate. High flows will not allow cells to settle along the channel, resulting in uneven distribution.*

   ! Critical *Lay chips (on AIM holders or in humidified chambers) on a flat surface while seeding cells into AIM chips. Inclination of the chips affects the cell distribution.*

4. Visual inspection under a microscope is recommended. If the cell distribution is not optimal for your application, adjust the concentration of the cell suspension and repeat the seeding steps.

   ? Troubleshooting (see Table 1 for troubleshooting advice)

5. (Optional) Change medium 2 h after the cells have been seeded to remove unattached cells.

6. Keep the chips in an incubator. Endothelial cells should form a confluent monolayer covering the channel in 1 d

   ? Troubleshooting (see Table 1 for troubleshooting advice)

OPTION 1: APPLICATION OF ANGIOGENIC STIMULI ⏱ TIMING 10 min

MATERIALS

Reagents

- Medium A: Cell culture medium enriched with 40 ng/ml Vascular Endothelial Growth Factor (VEGF) and 250 nM Sphingosine 1-phosphate (S1P) and (optional) 50 ng/ml Fibroblast Growth Factor (FGF) (See REAGENT SETUP)
- Medium B: Cell culture medium enriched with 20 ng/ml VEGF and 250 nM S1P and (optional) 10 ng/ml FGF (See REAGENT SETUP)
  (Optional: Addition of FGF in the presence of VEGF and S1P can improve the sprouting frequency in AIM chips but it is not mandatory)

Others

- AIM Chips with a complete endothelial monolayer

7. Remove medium from all 4 ports by carefully aspirating medium out from the troughs.

8. Add 70 µl of Medium B into one port of the EC-populated channel and then add 50 µl to the opposite connected port. Repeat this for the other channel but use Medium A instead to create the VEGF and FGF gradients across the collagen gel.

Reminder *The differential volumes in the two ports allow the replacement of medium to take place in the channel. The minimum volume of medium is 30 µl to ensure the inlets are covered and the troughs are wetted. If less than 30 µl of medium is used, the surface tension at the inlets will prevent the medium from flowing through the channel. We recommend using at least 50 µl of medium for easier handling.*

9. Replace the biomolecule-enriched media daily to avoid the drying of media in the ports and also to reset the concentration gradients. We recommend keeping the cells in culture for 2-3 d (starting from the application of angiogenic stimuli) to obtain optimum extent of angiogenic sprouting.

Reminder *The gradient formed that is across the collagen gel will dissipate over time if it is not reset by a medium change or is not maintained by continuous flows of media in both channels.*

? Troubleshooting (see Table 1 for troubleshooting advice)

OPTION 2: COMBINATION OF ANGIOGENIC STIMULI & INTERSTITIAL FLOW ⏱

TIMING 10 min

In combination, angiogenic stimuli (VEGF and S1P) and interstitial flow can reliably induce angiogenic sprouting in AIM chips. This is suitable for applications that require consistent sprout formation while the generation of a biomolecule concentration gradient is not needed.

MATERIALS

Reagents

- Medium C: Cell culture medium enriched with 40 ng/ml VEGF and 125 nM S1P (See REAGENT SETUP)

Others

- AIM Chips with a complete endothelial monolayer

10. Remove medium from all 4 ports by carefully aspirating medium out from the troughs.

11. Add 50 µl of Medium C into one port of the EC-populated channel and then add 30 µl to the opposite connected port (80 µl per channel). Repeat this for the other channel but increase the volume of medium to70 µl and 50 µl respectively (120 µl per channel).

Reminder *The differential volumes in the two media channels generate a basal to apical interstitial flow that lasts for approximately 4 h.*

Reminder *A daily short exposure to interstitial flow with diminishing flow rate in the presence of VEGF and S1P is sufficient to induce angiogenesis consistently. However, you may refer to the Connector Handling Protocol for methods to maintain the interstitial flow for extended period.*

12. Replace the biomolecule-enriched medium daily to avoid the drying of medium in the ports and also to re-introduce the interstitial flow. We recommend keeping the cells in culture for 2-3 d (starting from the application of angiogenic stimuli and interstitial flow) to obtain optimum extent of angiogenic sprouting.

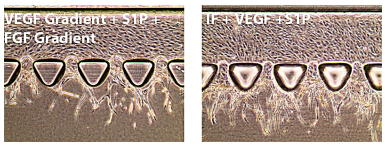


Figure 1 Phase contrast images of endothelial sprouting as induced by the application of: VEGF + S1P + FGF as shown on the left (Option 1); interstitial flow (IF) + VEGF + S1P as shown on the right (Option2) for 3 d.

? Troubleshooting (see Table 1 for troubleshooting advice)

QUANTIFICATION OF ANGIOGENIC SPROUTING ⏱ TIMING Variable

In order to quantify the extent of angiogenesis in AIM chips, we recommend labelling the cells with appropriate fluorophores (such as VE-cadherin staining that is specific to endothelial cells) to visualize them. Bright field, phase contrast and epifluorescence microscopy are all compatible with AIM chips but three-dimensional imaging techniques such as confocal microscopy is preferred due to the nature of this assay. The following quantification methods use images taken from confocal microscopy as illustrative examples.

SPROUT LENGTH AND NUMBER OF BRANCHING POINTS

The sprout length is an informative metric that can describe how fast (when the period of culture is taken into consideration) and how far the sprouts have extended into the 3D gel region. It can also tell the complexity of the sprouts especially when the sprout length is analysed together with the number of branching points. The higher number of branching points indicates the greater complexity of a sprout.

13. We recommend using confocal images of endothelial cells that are either fluorescently tagged or stained to quantify the sprout length and the number of branching points.

14. Pre-process the images if necessary. Depending on the image quality, you may apply a Gaussian filter to smoothen the edges and fill up the gaps between the bright signals around the cell membrane if the endothelial cells are stained for cell-cell junction protein (e.g. VE-cadherin).

Reminder *You need to optimize the pre-processing steps based on the stained proteins/organelles and the image quality. Other pre-processing techniques that may help achieve more accurate estimation of sprout length and number of branching points including noise reduction through despeckle and background subtraction. You should always pre-process your images as a whole.*

15. Try all threshold methods on at least three individual stacks of images. Choose the method that segments your data best and produces the closest estimation to the original images. Threshold the pre-processed images to binarize the images.

16. Use Skeletonize3D plugin (http://fiji.sc/ Skeletonize3D) in ImageJ to find the centerlines (also known as skeleton) of objects in the input image.

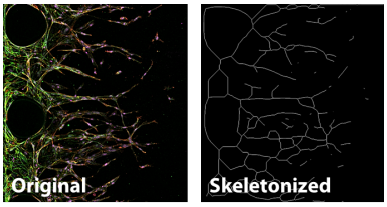


Figure 2 A 3D projection of a stack of confocal images (left) and its corresponding skeletonized image (right). The skeletonized image can be used for subsequent analysis.

17. Use AnalyzeSkeleton plugin (http://fiji.sc/ AnalyzeSkeleton) in ImageJ to analyze the skeletons you have generated. This yields information including the average and maximum branch length (sprout length), the number of branches and the number of actual junctions (branching points).

Reminder *You may perform a 3D projection on a stack of images to generate a 2D image before the image binarization only if the information across the different planes along the z-axis is not important for your application.*

CELL NUMBBER

On its own, the total cell number in the sprouts is less indicative on the extent of angiogenic sprouting. But when combined with other metrics such as the number of branching points and sprout length, it is informative and can help determine if the sprouts formed are more matured and constituted of multiple cells.

18. We recommend using confocal images with nuclear stain for cell counting.

19. Pre-process the images such as cropping the media channels out while retaining the 3D gel region is necessary if only the cells that form sprouts in the 3D gel region are of interest to your application.

20. Use 3D Objects Counter plugin (http://fiji.sc/3D_Objects_Counter) in ImageJ to count the total number of nuclei that are captured in the images. Depending on the image quality, you may adjust the threshold level and size filter to make sure every nucleus in the region of interest is counted.

Reminder *We recommend validating this semi-automated counting method with manual counting (by using Point Picker plugin) as image quality can affect the accuracy of this counting method.*

SPROUT AREA

The sprout area measures the extent of endothelial cell coverage. Similar to the total cell number, the sprout area is best analysed together with the number of branching points and sprout length to understand the sprout morphologies. In combination, the sprout area and sprout length can help derive the mean tubular diameter which is another useful metric for angiogenesis studies.

21. We recommend using confocal images of endothelial cells that are either fluorescently tagged or stained in the cytoplasm to quantify the sprout area.

22. Pre-process the images if necessary. Depending on the image quality, you may apply a Gaussian filter to smoothen the edges and fill up the gaps between the bright signals around the cell membrane if the endothelial cells are stained for cell-cell junction protein (e.g. VE-cadherin).

Reminder *You need to optimize the pre-processing steps based on the stained proteins/organelles and the image quality. Other pre-processing techniques that may help achieve more accurate estimation of sprout area including noise reduction through despeckle and background subtraction. You should always pre-process your images as a whole.*

23. Try all threshold methods and choose the method that segments your data best and produces the closest estimation to the original images based on at least three individual stacks of images. Threshold the pre-processed images to binarize the images.

24. Use Isosurface function in BoneJ plugin (http://bonej.org/) in ImageJ to estimate the 3D surface area.

Reminder *The 3D visualization of the images and the estimation of the 3D surface area are greatly affected by the resampling and threshold values that are chosen for BoneJ plugin. You should vary these two values systematically and choose the combination based on the rendered images that are closest to your actual data.*

25. You can then derive the mean tubular diameter by dividing the sprout area by the sprout length.

Reminder *Be cautious when deriving a number based on two estimated values as the discrepancies may be high. We recommend using this method ONLY IF the diameters of lumina are largely homogenous as validated through analyzing the cross-sectional images.*

TROUBLESHOOTING

Table 1 Troubleshooting advice

Step	Problem	Possible Reason	Solution
4.	Cells do not distribute evenly	The interval between the injections of cell suspension is short thus the flow of cells in the channel may be disrupted	Wait for at least 2 min before seeding cells into the opposite connected inlet
4.	Too many cells in a channel	Concentration of cell suspension is too high	Flush out unattached cells with culture medium immediately and repeat the seeding steps with cell suspension that is less concentrated
4.	Too few cells in a channel	Concentration of cell suspension is too low	Increase the concentration of cell suspension or repeat the seeding steps (without modifying the concentration of cell suspension) until the target cell density is obtained
4.	Cells do not adhere to the gel interface	The pressure head applied is insufficient	Increase the volume of cell suspension
6.	Fail to form endothelial monolayer within 1d	Seeding density is too low	Increase the seeding density
			Wait for another 24 h
		Passage number of cell is too high	Use cells with earlier passage number
		Channel is not properly coated thus affecting cell attachment	Increase fibronectin incubation time
9.	Fail to form sprouts	Passage number of cell is too high	Use cells with earlier passage number
		The cells are too confluent in the culture flask before seeded into AIM Chips	Trypsinize the cells when they are 80 - 90% confluent
		Hydrogel is too stiff: Cells cannot invade into the gel ; Hydrogel is too soft: Gel degradation by cells is faster than the sprout formation	Optimize the concentration, gelling time and pH (only for collagen I) of the hydrogel
		Inappropriate amount of angiogenic stimuli	Optimized the concentrations of angiogenic stimuli

REAGENT SETUP

CHEMOATTRACTANT-ENRICHED MEDIA

Reagents

- $VEGF_{165}$ (R&D Systems, Cat. No. 293-VE-010)
- S1P (Sigma-Aldrich, Cat. No. S9666)
- FGF (ReproCELL, Cat. No. RCHEOT003)
- Culture medium (Lonza, Cat. No. CC3202)
- BSA (Sigma Aldrich, Cat. No. A9647)
- Fatty acid-free BSA (Sigma Aldrich, Cat. No. A7030)
- Methanol (VWR, Cat. No. PL230ZA)

Others

- 0.2 µm 250 ml bottle top filter or 0.2 µm syringe filter

1. Add S1P powder in methanol:water (95:5) to 0.5 mg/ml, then sonicate at 45 °C until S1P is suspended in the solution.

2. Aliquot into desired volumes then remove the solvent by using a stream of dry nitrogen until a thin film of S1P is formed or freeze dry them and then store them at -20 °C as primary stocks. Maximum storage for primary stocks is up to 1 year.

3. Use fatty acid-free BSA with a minimum concentration of 4 mg/ml in sterile PBS as the S1P carrier to make a 125 µM secondary stock solution. This secondary stock solution can be kept at 4 °C for 3 months.

4. Reconstitute recombinant human $VEGF_{165}$ at 100 µg/ml in sterile PBS containing at least 0.1 % BSA. Aliquot it into smaller volumes and store them at -20 °C. Dilute the aliquots to 10 µg/ml with PBS as secondary stock solutions before use.

5. Dilute FGF to 50 µg/ml with sterile PBS containing at least 0.1 % BSA as a secondary stock solution.

6. **Medium A**: Add 4 µl of VEGF (10 µg/ml), 2 µl of S1P (125 µM) and 1 µl of FGF (50 µg/ml) per ml of medium to get cell culture medium enriched with 40 ng/ml VEGF and 250 nM S1P and 50 ng/ml FGF.

7. **Medium B**: Add 2 µl of VEGF (10 µg/ml), 2 µl of S1P (125 µM) and 0.2 µl of FGF (50 µg/ml) per ml of medium to get cell culture medium enriched with 20 ng/ml VEGF and 250 nM S1P and 10 ng/ml FGF.

8. **Medium C**: Add 4 µl of VEGF (10 µg/ml) and 1 µl of S1P (125 µM) per ml of medium to get cell culture medium enriched with 40 ng/ml VEGF and 125 nM S1P and 10 ng/ml FGF.