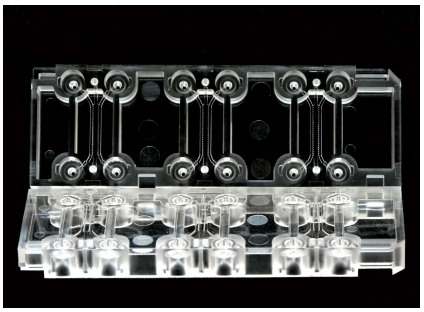
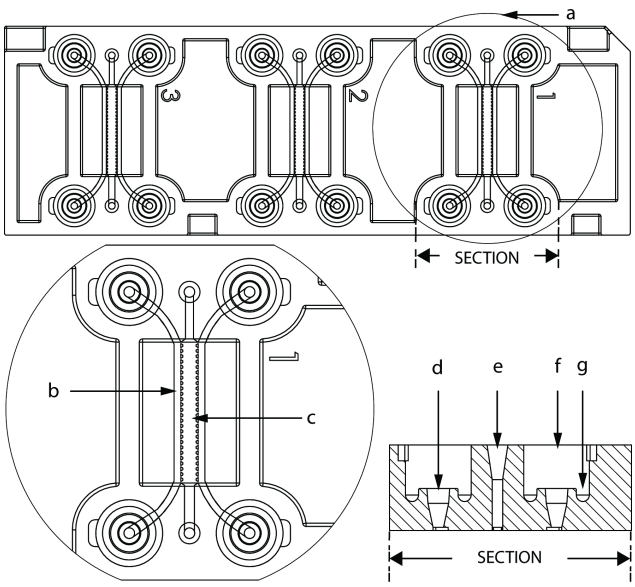
GENERAL PROTOCOL

AIM 3D Cell Culture Chip serves as a flexible platform for various applications in biological research. This general protocol covers the basic techniques that are essential across various applications to prepare & fill collagen gel, coat media channels, seed cells, change medium and stain cells. The formation of an endothelial monolayer in the AIM chip is used here as an illustrative example.



SCHEMATIC

The following schematic shows the top view and section view of an AIM chip. This nomenclature will be used extensively in this protocol.



Nomenclature:

a : site (a DAX-1 chip contains 3 sites)
b : media channel
c : gel channel
d : media inlet
e : gel inlet
f : port
g : trough

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PREPARING & FILLING COLLAGEN GEL ⏱ TIMING 50 min

MATERIALS

Reagents

- 10 X PBS with phenol red (see REAGENT SETUP; Life Technologies, Cat. No. 70011044)
- Sodium hydroxide solution, 0.5 M (see REAGENT SETUP; Sigma-Aldrich, Cat. No. 221465)
- Sterile deionized water (Thermo Water Purifying System)
- Collagen type I, rat tail (Corning Life Science, Cat. No. 354236)
- Cell culture medium (Lonza, Cat. No. CC3202)

Others

- 1.5 ml microcentrifuge tube
- Pocket size pH meter (Hach, Cat. No. H138) or pH papers (Sigma Aldrich, Cat. No. 37144)
- Ice bucket or styrofoam box
- Ice
- AIM chips
- AIM holders or humidified chambers

Calculations before Experiments

i. The following steps are calculated based on a collagen stock solution of 3.75 mg/ml, but any other concentration in the range of 3 - 4.5 mg/ml may still be used to make 200 µl of 2.5 mg/ml collagen solution at pH 7.4. This amount is sufficient for filling at least 4 AIM chips (in total 12 sites).

Reminder *The concentration, pH value and total volume of the final collagen solution can be adjusted for different applications. Please see Table 1 for a list of publications that use different formulations of collagen gel for different experiments.*

ii. The volume of 10 X PBS with phenol red is always one tenth (1/10) of the final volume (200 µl), A = 20 µl

iii. The volume of collagen, B is calculated based on the equation below, B = 133.3 µl

$$3.75\frac{mg}{ml} \times B = \frac{2.5mg}{ml} \times 200\mu l$$

iv. The volume of 0.5 M NaOH needed to adjust the pH of the solution is a variable that usually falls between 4 to 8 µl and the exact number can be determined through iterative method (see Table 1). For a collagen stock solution of 3.75 mg/ml, the volume of 0.5 M NaOH solution needed, C = 7.8 µl

v. Add sterile deionized water to dilute the collagen solution to the desired concentration without affecting the pH value. The volume of deionized water, D is calculated last by using the equation below.

$$D = Total\ Volume - A - B - C$$

$$D = 200\mu l - 20\mu l - 133.3\mu l - 7.8\mu l = 38.9\mu l$$

Table 1 List of applications with different formulations of collagen gel (or other hydrogel)

Main Category	Sub-category	Collagen I Gel Concentration	References
Vascular functions	Angiogenesis	2.5 mg/ml at pH 7.4	[1]
		2.5 mg/ml at pH 7.4	[2]
		2.5 mg/ml at pH 7.4	[3]
		2.0 mg/ml at pH 7.4	[4]
	Anti-angiogenesis	3.0 mg/ml at pH 7.4	[5]
	Vasculogenesis	2.0 mg/ml, 2.5 mg/ml and 3.0 mg/ml at pH 7.4; Fibrinogen only and mixture of both	[6]
	Flow response	2.5 mg/ml at pH 7.4	[7]
	Transendothelial migration	2.0 mg/ml at pH 5, pH 7.4 and pH 11	[8]
Cancer	Spheroid dispersion	2.5 mg/ml at pH 7.4	[9]
	Extravasation	2.0 mg/ml	[10]
		Fibrinogen only: 2.5 mg/ml and 5 mg/ml	[11]
		6.0 mg/ml	[12]
	Intravasation	2.5 mg/ml	[13]
	Flow response	2.0 mg/ml at pH 8.9	[14]
		2.0 mg/ml at pH 8.9	[15]
	Invasion and Migration	2.0 mg/ml at pH 7.4	[16]
		2.0 mg/ml at pH 11, Matrigel only and mixture of both	[17]
		2.0 mg/ml at pH 6.0, pH 7.4 and pH 11	[18]
Neurobiology	Neurite guidance	2.0 mg/ml at pH 7.4	[19]
	Differentiation of NSC	2.0 mg/ml at pH 7.4 + Matrigel (1:1)	[20]
Stem cell Biology	Differentiation of ESC	2.0 mg/ml at pH 7.4	[21]

Iterative method to determine the volume of 0.5 M NaOH solution

1. Perform the following steps in a laminar flow hood and every item should be sterilized beforehand.

2. Keep 10X PBS with phenol red, 0.5 M NaOH solution, deionized water and collagen stock solution on ice throughout the process.

Reminder *Make sure the color of 10X PBS with phenol red is homogeneous before use as the color of 10X PBS with phenol red changes when it is about to freeze.*

3. Add 20 µl of 10 X PBS with phenol red into a microcentrifuge tube (on ice).

4. Add the calculated volume of collagen, B into the microcentrifuge tube.

5. Add an estimated amount of 0.5 M NaOH solution into the microcentrifuge tube. The recommended starting volume is 4 µl to make 200 µl of 2.5 mg/ml collagen solution at pH 7.4.

6. Mix thoroughly with a micropipette.

! Critical *Pipette up and down until the color is homogeneous throughout (if unsure, pipette at least 100 times).*

! Critical *Pipette up and down with care to avoid the generation of bubbles.*

! Critical *Keep the microcentrifuge tube on ice while mixing to prevent any unwanted polymerization of the collagen gel.*

7. Check the color of the mixed solution. Phenol red is a pH indicator itself and the desired color for pH7.4 is faint pink.

8. Discard the mixed solution if the mixed solution is purple or red, which indicates a high pH value. Start again with a lower volume of 0.5 M NaOH solution.

9. Add 0.5 M NaOH solution into the mixed solution in a small step wise manner (each addition is not more than 0.5 µl) if the mixed solution is yellow, which indicates a low pH value. Mix thoroughly with each addition of 0.5 M NaOH solution. Repeat this until the color changes to faint pink.

! Critical *Make sure no residue is left in the pipette tip as the pH value is highly sensitive to the addition of 0.5 M NaOH solution.*

10. If a pocket size pH meter is available, add a drop of mixed solution (~10 µl depends on the model of pH meter) to measure the pH value. Otherwise, add 10 µl to 20 µl of mixed solution on a pH paper and match it to the color chart.

11. Confirm the pH value is within ±0.1 of the target value with a pH meter. Otherwise, make sure the color of mixed solution is faint pink and the pH paper readout lies between pH7 and pH8.

12. Calculate the volume of deionized water required and add it into the mixed solution.

13. The collagen gel recipe may be used for future experiments as long as the same reagents are used. For any change of reagent, a new recipe has to be developed again.

Table 2 An example of collagen gel recipe

Reagents	Volume
10X PBS	20 µl
Collagen	133.3 µl
0.5 M NaOH	7.8 µl
Deionized water	38.9 µl

Preparing collagen gel ⏱ **TIMING 10 min**

14. Keep 10X PBS with phenol red, collagen stock solution, 0.5 M NaOH solution and deionized water on ice and add them into a microcentrifuge tube (on ice) sequentially according to the pre-determined collagen gel recipe.

15. Mix the solution thoroughly by using a micropipette to get a collagen solution with homogeneous faint pink color.

Filling collagen gel ⏱ **TIMING 40 min**

16. Assemble AIM chips into an AIM holder (see Instructions For Use for the AIM holder included in the package).

17. Draw 10 µL of collagen solution with a 1- 10 µL micropipette. Make sure the collagen solution is kept on ice at all times.

! Critical *Limit the volume of collagen solution to 10 µl to prevent the collagen solution from overflowing into media channels.*

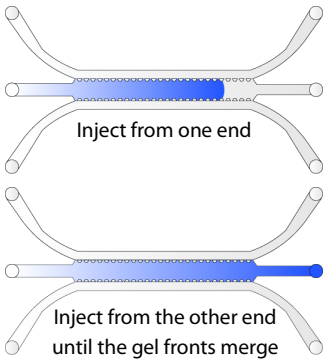
18. Fill the chip from either gel inlet:

! Critical *Hold the plunger firmly while removing the micropipette from the inlets, otherwise the negative pressure will suck the collagen up.*

! Critical *Avoid discharging collagen solution abruptly to prevent the collagen solution from overflowing into media channels.*

? Troubleshooting (see Table 3 for troubleshooting advice)

a. **Option 1**: Fill collagen solution from either one of the inlets and stop near the end of posts. Fill from the other inlet until the gel fronts merge. This method is recommended for new users.



b. **Option 2**: Fill collagen solution from one side all the way to the other side. Continue to push the collagen solution in gently until it reaches the other inlet. This method ensures that the gel is being filled homogeneously but it requires greater control over pipetting pressure (especially when the collagen solution reaches the opposite inlet) to prevent the collagen solution from overflowing into the flanking media channels.

19. Add 6 ml of water into the reservoirs of the AIM holder. Alternatively, prepare a humidified chamber to house the chips (e.g. by adding water into a pipette tip box until approximately 1/3 is filled; both water and pipette tip box should be sterile).

! Critical *AIM chips are laminated with a gas-permeable film that enables gas exchange to take place. The bottom of the chips should therefore be exposed to allow for air circulation.*

20. If humidified chambers are used, pre-warm the humidified chambers to 37 °C by putting them in an incubator.

Reminder *Pre-warming of AIM holders is not necessary as the AIM holders allow direct contact of AIM chips with warm air when placed in an incubator.*

21. Place the gel-filled chips (on AIM holders or in humidified chambers) into a 37 °C incubator and incubate for half an hour to allow the polymerization of collagen to take place.

Reminder *The polymerization time can be optimized to suit your specific application.*

! Critical *Temperature will affect collagen polymerization and 37 °C is recommended for most applications.*

! Critical *Chips with unpolymerized gel must be handled with care. Excessive agitation or impact may cause the unpolymerized gel to leak out of the gel channel.*

HYDRATING & COATING MEDIA CHANNELS ⏱ TIMING 70 min

MATERIALS

Reagents

- Fibronectin (Sigma-Aldrich, Cat. No. F0895) or any coating reagent for your specific application

! Critical *Collagen coating is not suitable for collagen-filled AIM chips as the solvent of the collagen coating solution may dissolve the polymerized collagen.*

Others

- Collagen-filled AIM chips

22. After incubation, insert a pipette tip into either inlet of the media channel that requires coating and push gently until the tip fits. Inject 15 µl of coating solution (e.g. 50 µg/ml fibronectin solution diluted in culture medium or 1X PBS) into the channel. Due to surface tension, the injected coating solution will form a spherical cap at the opposite inlet. Repeat this step for the other channel. Use culture medium to hydrate the media channels if coating is not required.

Insert a tip into an inlet until it fits. Inject medium till it reaches the opposite inlet.

! Critical *Hold the plunger firmly while removing the micropipette from the inlets, otherwise the negative pressure will suck the coating solution/medium up.*

! Critical *Do not inject more than 20 µl of coating solution/medium at this step or the high injection pressure may disrupt the collagen gel.*

? Troubleshooting (see Table 3 for troubleshooting advice)

23. Incubate the media-channel-hydrated chips (on AIM holders or in humidified chambers) for 1 h in a 37°C incubator.

24. Add 70 µl of medium into one port and then add 50 µl into the opposite port of the same media channel to flush out the coating solution. Repeat this for the other channels. If the coating solution has to be removed completely, wash the media channels with culture medium by repeating this step twice (see CHANGING MEDIUM).

Reminder *Media channels have to be hydrated with culture medium/coating solution after the polymerization of collagen. This is to prevent the collagen gel in the chips from drying up.*

▪ **PAUSE POINT** *The media-channel-hydrated chips can be kept in an incubator for not more than 2 days before the seeding steps depending on your application (In a single experiment, do not mix the media-channel-hydrated chips that are prepared at different time points because the properties of collagen may change over time).*

SEEDING CELLS ⏱ TIMING 40 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
- Cells
- Centrifuge

25. Trypsinize cells as per protocol. Briefly, remove medium from the cell culture flask/dish and wash the endothelial cells with sterile 1X PBS for twice. Add enough 0.25 % trypsin with EDTA solution to cover the bottom of the culture flask/dish and incubate them for 2 min in a CO_2 incubator.

26. Perform a visual inspection to make sure the cells have detached from the substrate.

27. Add medium with FBS, at least 5 times the volume of trypsin, into the culture flask/dish to neutralize the activity of trypsin.

28. Transfer the cell suspension to a 15 ml tube and pellet the cells by centrifuging at 250 x*g* for 5 min at RT.

29. Re-suspend the cells in culture medium with densities ranging from 0.5 M to 3 M cells/ml, depending on cell types and applications. For example, endothelial cells at a density of 1.5 M cells/ml is suitable for obtaining a confluent monolayer overnight.

30. If the cells need to be seeded on the gel interface, add an additional 20 µl of medium into one of the ports at the media channel that is to be seeded with cells. Otherwise, proceed to step 31.

Reminder *Ports must be filled with medium before seeding cells into the media channels. Filled ports allow the cell suspension to flow into the media channels.*

31. Use a micropipette to withdraw 10 µl of endothelial cell suspension. Position the tip near the inlet of media channels 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 that helps the attachment of endothelial cells on the gel interface.

Position the pipette tip near inlets while injecting 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 flow in the media channel, thus disturbing cell distribution.*

32. 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 3 for troubleshooting advice)

33. If another cell type B is to be seeded in the opposite media channel, incubate the chips for at least 30 min after cell type A has been seeded to allow proper attachment of cell type A on the substrates. Repeat the seeding steps for cell type B.

34. Keep the chips in an incubator and proceed to step 35.

CHANGING MEDIUM ⏱ TIMING 10 min

MATERIALS

Reagents

- Cell culture medium (Lonza, Cat. No. CC3202)

35. (Optional) Change medium 2 to 4 h (or longer for less adhesive cell types) after the cells have been seeded.

36. Remove medium from all 4 ports by carefully aspirating the medium out from the troughs. To replace the medium in a media channel, add 70 µl of medium into one port and then add 50 µl into the opposite connected port. Repeat this for the other channel.

Always remove medium from troughs

! Critical *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 50 µl of medium for easier handling.*

! Critical *Do NOT aspirate medium from inlets to avoid accidental removal of medium from the channels.*

37. Keep the chips in an incubator. Endothelial cells should form a confluent monolayer covering the channel in 1 d. If the cells need to be kept longer in culture, change medium daily as described in step 36.

STAINING CELLS ⏱ TIMING 2 d

MATERIALS

Reagents

- 4% Formaldehyde (See REAGENT SET UP; Sigma Aldrich, Cat. No.158127)
- 0.1% Triton X-100 (See REAGENT SET UP; Sigma Aldrich, Cat. No. T8787)
- Blocking buffer (Life Technologies, Cat. No. B-10710)
- 1X PBS (Life Technologies, Cat. No. 70011044)
- Primary Antibody: VE-cadherin (Enzo Life Sciences, Cat. No. ALX-210-232-C100)
- Corresponding Secondary Antibody (Life Technologies, Cat. No. A11034)
- Rhodamine Phalloidin (Life Technologies, cat. No. R415)
- Hoechst (Life Technologies, Cat. No. H1399)

Others

- Parafilm

Cell Fixation ⏱ TIMING **10 min**

38. Remove the medium from all 4 ports carefully by placing the tip of an aspirator at the troughs.

39. Add 70 µl of 1X PBS into one port and then add 50 µl into the opposite connected port of a media channel. Repeat this for the other channel. The differential volumes in the two connected ports create flow in the channels to replace the medium.

40. Remove 1X PBS from all ports. Add 70 µl of 4% formaldehyde into one of the ports and then add 50 µl into the opposite connected port. Repeat this for the other channel.

41. Incubate for 15 min at RT.

42. Remove 4% formaldehyde from all ports by using a micropipette and wash the channels twice with 1X PBS as described in step 39.

! Critical *Dispose 4% formaldehyde and the 1st subsequent wash in a designated waste bottle.*

▪ **PAUSE POINT** *Fixed cells can be kept for up to 1 month (depending on target antigens as some antigens may degrade over time after fixation) at 4°C as long as the PBS in the ports does not dry up.*

Cell Permeabilization ⏱ TIMING **15 min**

(Optional: Permeabilization is only necessary when cell-impermeable fluorescent probes are used, e.g. phalloidin)

43. Remove PBS from all 4 ports carefully by placing the tip of an aspirator at the troughs.

44. Add 70 µl of 0.1 % Triton X-100 into one of the ports and then add 50 µl into the opposite connected port. Repeat for the other channel.

45. Incubate for 10 min at RT.

46. Wash once with PBS as described in step 39.

Blocking ⏱ **TIMING 2.5 h**

(Optional: Blocking is only necessary for immunofluorescent staining)

47. Remove PBS from all 4 ports carefully by placing the tip of an aspirator at the troughs.

48. Add 70 µl of blocking buffer into one of the ports and then add 50µl into the opposite connected port. Repeat this for the other channel.

49. Incubate for 2 h at RT.

Primary Antibody Staining ⏱ **TIMING 18 h**

(Optional: Primary antibody staining is only necessary for immunofluorescent staining)

50. Prepare antibodies according to the manufacturer's recommendations. For example, dilute the anti-VE-cadherin antibody with 1X PBS in a 1:100 ratio.

51. Remove blocking buffer from all 4 ports carefully by placing the tip of an aspirator at the troughs.

52. Add 70 µl of antibodies into one of the ports and then add 50 µl into the opposite connected port of the cell-populated channel. Repeat this for the other channel. If the target cells are embedded within the 3D hydrogel, apply differential volumes of antibodies between media channels (e.g., 120 µl in one channel and 60 µl in the other) to generate interstitial flow that can transport the antibodies to the cells.

! Critical *Do not wash with 1X PBS after the blocking step.*

53. Seal the AIM holders with parafilm. Incubate overnight at 4°C.

54. Remove the primary antibodies from all 4 ports carefully by placing the tip of an aspirator (or using a micropipette to recycle the antibodies) at the troughs. You may reuse the antibodies depending on the antigen and antibody.

55. Wash the channels with 1X PBS 5 times, with a 5 min incubation between each wash, as described in step 39.

Secondary Antibody Staining ⏱ **TIMING 1.5 h**

(Optional: Secondary antibody staining is only necessary for immunofluorescent staining)

56. Prepare the secondary antibodies according to the manufacturer's recommendations. For example, dilute the Alexa Fluor-conjugated goat anti rabbit-secondary antibody with 1X PBS in a 1:100 ratio.

57. Add 70 µl of antibodies into one of the ports and then add 50 µl into the opposite connected port. Repeat this for the other channel. If the target cells are embedded within the 3D hydrogel, apply differential volumes of antibodies between media channels (e.g., 120 µl in one channel and 60 µl in the other) to generate interstitial flow that can transport the antibodies to the cells.

58. Incubate for 1 h at RT.

59. Remove the secondary antibodies from all 4 ports carefully by placing the tip of an aspirator (or using a micropipette to recycle the antibodies) at the troughs. You may reuse the antibodies depending on the antigen and antibody.

60. Wash the channels with 1X PBS 3 times, with a 5 min incubation between each wash, as described in step 39.

! Caution *Cover the AIM chips (on AIM holders or in secondary containers) with aluminium foil to minimize photobleaching.*

Fluorescent Staining ⏱ **TIMING 1.5 h**

61. Remove PBS from the ports and then add 70 µl of Hoechst (10 µg/ml)/Rhodamine Phalloidin (3 U/ml) into one of the ports and then add 50 µl into the opposite connected port. Repeat this for the other channel.

Reminder *The fluorescent staining of nuclei and actin by using Hoescht and Rhodamine Phalloidin can be carried out either concurrently or separately.*

! Caution *Hoechst (or other nucleus staining reagents) should always be handled using protective gloves and clothing.*

62. Incubate for 1 h at RT.

Reminder *Incubation time for actin staining can be increased if stronger fluorescent intensity is needed.*

63. Wash the channels with 1X PBS 5 times, with a 5 min incubation between each wash as described in step 39. Seal the AIM holders that carry the stained chips with parafilm and keep them in 4 °C and protect them from light until imaging.

Reminder *The staining protocol should be optimized for your specific application (with different cell types and different proteins of interest).*

▪ **PAUSE POINT** *Stained cells can be kept for up to 1 month (depending on your application) at 4°C in dark as long as the PBS in the ports does not dry up.*

TROUBLESHOOTING

Table 3 Troubleshooting advice

Step	Problem	Possible Reason	Solution
18.	Collagen solution overflows into the media channels	Filling pressure is too high	Inject collagen solution smoothly
		Filling volume is more than required	Use 10 µl of collagen solution only
		Collagen polymerizes thus blocking flow	Handle collagen solution on ice and avoid prolonged injection
22.	Medium leaks from one channel to the other channel	Insufficient polymerization time	Increase the polymerization time
		Collagen quality may vary from manufacturer to manufacturer and from batch to batch	Change to a new collagen stock from a different batch or manufacturer
22.	Air bubbles are trapped in the media channels	Humidity level is not maintained high during hydrogel polymerization	Ensure the reservoirs in holders are filled and avoid prolonged polymerization time
32.	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
32.	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
32.	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
32.	Cells do not adhere to the gel interface	The pressure head applied is insufficient	Increase the volume of cell suspension

REAGENT SETUP

0.5 M SODIUM HYDROXIDE (NaOH) SOLUTION

Reagents

- Sodium Hydroxide (Sigma Aldrich, Cat. No. 221465)
- Deionized water (Thermo Fisher Water System)

Others

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

1. Dissolve 2 g of NaOH (molecular weight: 40 g/mol) pellets in 90 ml of deionized water by stirring at room temperature. Top up the solution with deionized water to yield final volume of 100 ml (final concentration is 0.5 M). Sterilize NaOH solution by passing through a 0.2 µm bottle top filter or syringe filter in a sterile laminar flow hood.

2. Seal and keep the filtered NaOH solution at 4 °C for collagen gel preparation. Aliquot the solution into smaller volume (e.g. 1 to 5 ml) is recommended as the amount of NaOH solution needed for each experiment is small (typically ranging between 5 µl to 20 µl, depending on the total amount of collagen gel).

! Caution *Wear protective clothing and gloves while working with NaOH. Avoid the addition of large amount of NaOH into water at once to prevent the generation of excessive heat from this exothermic reaction.*

10 X PBS WITH PHENOL RED

Reagents

- 10X PBS (Life Technologies, Cat. No. 70011044)
- Phenol red sodium salt (Sigma-Aldrich, Cat. No. 114537)

Others

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

1. Dissolve 63.6 mg of phenol red sodium salt into 40 ml of 10X PBS as primary stock solution through vortexing. Properly sealed primary stock solution is stable at room temperature for up to a year.

2. Dilute primary stock solution with 10X PBS in a 1: 10 ratio (5 ml of primary stock solution + 45 ml of 10X PBS) to yield the working concentration. Sterilize the 10X PBS solution (with phenol red) by passing the solution through a 0.2 µm bottle top filter or a syringe filter in a sterile laminar flow hood.

3. Seal and keep the filtered 10X PBS with phenol red at 4 °C for collagen gel preparation. Aliquot the solution into smaller volume (e.g. 1 to 5 ml) is recommended as the amount of 10X PBS with phenol red needed for each experiment is small (typically ranging between 20 µl to 40 µl, depending on the total amount of collagen gel).

4% FORMALDEHYDE

Reagents

- Paraformaldehyde powder (Sigma-Aldrich, Cat. No. 158127)
- 1X PBS (Life Technologies, Cat. No. 70011044)

Others

- Hot plate with magnetic stirrer

1. Add 40 g of paraformaldehyde (PFA) powder into 800 ml of 1X PBS and heat it up to 60 °C by using a hot plate.

   ! Caution *Wear protective clothing and gloves while working with PFA. Prepare this solution in a ventilated hood.*

2. Stir for approximately 6 h.

   Reminder *pH can be adjusted by using 1 M NaOH to facilitate the dissolution of PFA. If so, neutralize the pH back to approximately pH 7.0 by using dilute HCL after the PFA is dissolved.*

3. Adjust the volume to 1L with 1X PBS and then filter the solution and make aliquots.

0.1% TRITON-X

Reagents

- Triton X-100 (Sigma Aldrich, Cat. No. T8787)
- 1X PBS (Life Technologies, Cat. No. 70011044)

1. Dilute Triton X-100 with 1X PBS to yield 0.1 % (v/v) working concentration.

2. Aliquot the solution and store them at room temperature.

1. Lim, S.H., et al., *Complementary effects of ciclopirox olamine, a prolyl hydroxylase inhibitor and sphingosine 1-phosphate on fibroblasts and endothelial cells in driving capillary sprouting.* Integrative Biology, 2013. **5**(12): p. 1474-1484.
2. Kim, C., et al., *In vitro angiogenesis assay for the study of cell-encapsulation therapy.* Lab on a Chip, 2012. **12**: p. 2942-2950.
3. Chan, J.M., et al., *Engineering of In Vitro 3D Capillary Beds by Self-Directed Angiogenic Sprouting.* PLoS ONE, 2012. **7**(12): p. e50582.
4. Shin, Y., et al., *In vitro 3D collective sprouting angiogenesis under orchestrated ANG-1 and VEGF gradients.* Lab on a Chip, 2011. **11**(13): p. 2175-2181.
5. Yamamoto, K., et al., *The stabilization effect of mesenchymal stem cells on the formation of microvascular networks in a microfluidic device.* Journal of Biomechanical Science and Engineering, 2013. **8**(2): p. 114-128.
6. Park, Y., et al., *In Vitro Microvessel Growth and Remodeling within a Three-Dimensional Microfluidic Environment.* Cellular and Molecular Bioengineering, 2014. **7**(1): p. 15-25.
7. Vickerman, V. and R.D. Kamm, *Mechanism of a flow-gated angiogenesis switch: Early signaling events at cell-matrix and cell-cell junctions.* Integrative Biology (United Kingdom), 2012. **4**(8): p. 863-874.
8. Han, S., et al., *A versatile assay for monitoring in vivo-like transendothelial migration of neutrophils.* Lab on a Chip, 2012. **12**(20): p. 3861-3865.
9. Aref, A.R., et al., *Screening therapeutic EMT blocking agents in a three-dimensional microenvironment.* Integrative Biology, 2013. **5**(2): p. 381-389.
10. Jeon, J.S., et al., *In Vitro Model of Tumor Cell Extravasation.* PLoS ONE, 2013. **8**(2).
11. Chen, M.B., et al., *Mechanisms of tumor cell extravasation in an in vitro microvascular network platform.* Integrative Biology, 2013. **5**(10): p. 1262-1271.
12. Bersini, S., et al., *A microfluidic 3D in vitro model for specificity of breast cancer metastasis to bone.* Biomaterials, 2014. **35**(8): p. 2454-2461.
13. Zervantonakis, I.K., et al., *Three-dimensional microfluidic model for tumor cell intravasation and endothelial barrier function.* Proceedings of the National Academy of Sciences, 2012.
14. Polacheck, W.J., J.L. Charest, and R.D. Kamm, *Interstitial flow influences direction of tumor cell migration through competing mechanisms.* Proc Natl Acad Sci U S A, 2011. **108**(27): p. 11115-20.
15. Polacheck, W.J., et al., *Mechanotransduction of fluid stresses governs 3D cell migration.* Proceedings of the National Academy of Sciences, 2014. **111**(7): p. 2447-2452.
16. Kalchman, J., et al., *A three-dimensional microfluidic tumor cell migration assay to screen the effect of anti-migratory drugs and interstitial flow.* Microfluidics and Nanofluidics, 2013. **14**(6): p. 969-981.
17. Shin, Y., et al., *Hydrogels: Extracellular Matrix Heterogeneity Regulates Three-Dimensional Morphologies of Breast Adenocarcinoma Cell Invasion (Adv. Healthcare Mater. 6/2013).* Advanced Healthcare Materials, 2013. **2**(6): p. 920-920.
18. Chung, S., et al., *Cell migration into scaffolds under co-culture conditions in a microfluidic platform.* Lab on a Chip, 2009. **9**(2): p. 269-275.
19. Kothapalli, C.R., et al., *A high-throughput microfluidic assay to study neurite response to growth factor gradients.* Lab on a Chip, 2011. **11**(3): p. 497-507.
20. Han, S., et al., *Three-dimensional extracellular matrix-mediated neural stem cell differentiation in a microfluidic device.* Lab on a Chip - Miniaturisation for Chemistry and Biology, 2012. **12**(13): p. 2305-2308.
21. Wan, C.R., S. Chung, and R.D. Kamm, *Differentiation of embryonic stem cells into cardiomyocytes in a compliant microfluidic system.* Annals of Biomedical Engineering, 2011. **39**(6): p. 1840-1847.