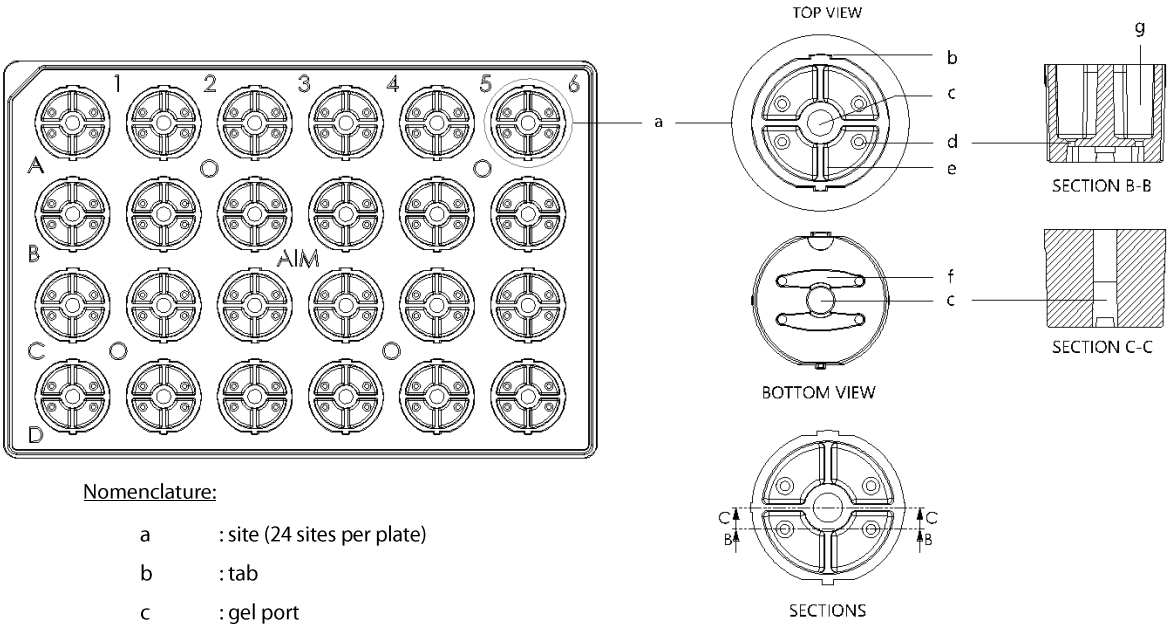
INTRODUCTION

The organiX Plate is a sterile, single-use, and stackable plate, with a throughput size of 24. This protocol covers the basic handling of the organiX Plate – the preparation and filling of fibrinogen gel, hydrating the media channels, changing medium, seeding monolayer cells on the media channel and immunofluorescence staining. The seeding of tumor spheroids and the formation of an endothelial vasculature network around the spheroid are used here as an illustration.

SCHEMATIC

The following schematic shows the 3D presentation of the organiX Plate. This nomenclature will be used extensively in this protocol.



Nomenclature:

a	: site (24 sites per plate)
b	: tab
c	: gel port
d	: media inlet
e	: divider
f	: media channel
g	: media reservoir

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PREPARING & FILLING FIBRINOGEN GEL ⏱TIMING 60 min

MATERIALS

Reagents

- Thrombin from bovine plasma (Sigma-Aldrich, Cat. No. T7513)
- Fibrinogen from bovine plasma Type I-S (Sigma-Aldrich, Cat. No. F8630-1G)
- Cell culture medium (Lonza, Cat. No. CC-3162)
- Cell culture medium (Life Technologies, Cat. No. 10569044)
- 1X PBS (Life Technologies, Cat. No. 70011044)
- Trypsin (Life Technologies, Cat. No. 25300054)

Others

- Human umbilical vein endothelial cells (HUVEC)
- Normal Human Lung Fibroblasts (NHLF)
- 1.5 ml microcentrifuge tube
- 0.2 ml PCR tubes
- Ice bucket or styrofoam box
- Ice
- organiX plate
- AIM Cooling Block

Calculations before Experiments

i. The following steps are calculated based on a thrombin stock solution at a concentration of 100 U/ml and a fibrinogen stock solution at a concentration of 6 mg/ml.

ii. The volume of resuspension medium needed for each insert is 25 µl, prepare 660 µl of resuspension medium to fill the whole plate. This volume is sufficient to fill a full organIx plate (24 inserts)

Reminder *Calculate and prepare at least 10 % more resuspension medium than needed.*

iii. The target seeding concentrations in the gel in this example is 4 M cells/ml for HUVECs and 1 M cells/ml for NHLF.

iv. Do a backward calculation to determine the concentration of cell suspension. As the cell suspension will be diluted with fibrinogen solution in a 1:1 ratio, a 2x more concentrated cell suspension as compared to the target seeding concentrations should be prepared. In this example, cell suspension with a mixture of HUVECs and NHLFs at 8 M cells/ml and 2 M cells/ml respectively is needed.

***Preparing fibrinogen gel* (with endothelial cells and fibroblast)** ⏱TIMING **20 min**

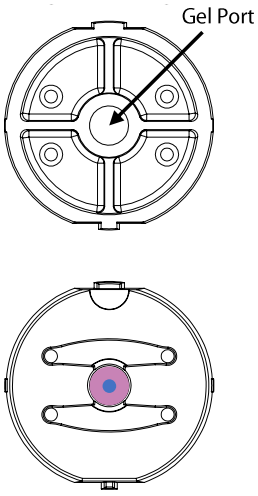
1. Prepare 24 aliquots of 6 mg/ml fibrinogen stock solution in PCR tubes, each has 25 µl. Keep them on ice.
2. Mix 6.6 µl of thrombin stock solution in 653.4 µl of cell culture medium to obtain 660 µl of resuspension medium at a concentration of 1 U/ml.

Reminder *Keep the resuspension medium and the fibrinogen stock solution on ice at all time.*

3. Trypsinise cells as per protocol. Briefly, remove medium from the cell culture flask/dish and wash the endothelial cells and the fibroblast 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 5 min in a CO_2 incubator.
4. Perform a visual inspection to make sure the cells have detached from the substrate.
5. Add medium with FBS, at least 5 times the volume of trypsin, into the culture flask/dish to neutralize the activity of trypsin.
6. Transfer the cell suspension to a 15 ml tube and perform a cell count.
7. Mix 5.28M HUVECs and 1.32M NHLF and pellet the cells by centrifuging at 250 x*g* for 5 min at RT.
8. Resuspend the cells in 660 µl of resuspension medium to get a cell suspension that comprises 8 M HUVEC/ml and 2 M NHLF/ml.
9. Transfer pre-formed spheroids to another 24 empty PCR tubes (1 spheroid per tube). Keep them on ice.

Filling fibrinogen gel (with spheroid) ⏱**TIMING 40 min**

10. Remove media from the PCR tubes containing spheroids and add 25 µl of the cell suspension to each of it, keep on ice.
11. Add 25 µl of fibrinogen stock solution to each of the PCR tube containing the spheroid that is in the cell suspension. Make sure the fibrin gel is well mixed and is always kept on ice.
12. Withdraw 50 µl of the fibrin gel using a micropipette and put the micropipette tip straight down into the gel port and position it slightly above the bottom laminate. Then, dispense the fibrin gel gently. The spheroid will sink and be close to the bottom of the port due to gravity. Repeat step 11. and step 12. for the rest of the sites.



13. Incubate the plate for 20 min at room temperature to allow the polymerization of the gel.

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

HYDRATING & COATING MEDIA CHANNELS ⏱TIMING 70 min

MATERIALS

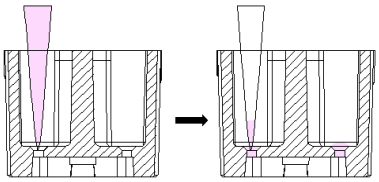
Reagents

- Fibronectin (Sigma-Aldrich, Cat. No. F0895) or any coating reagent for your specific application
- Cell culture medium (Lonza, Cat. No. CC-3162)

Others

- Gel-filled organiX plate

14. After incubation, insert a pipette tip into either inlet of the media channel and push gently until the tip fits. Inject 35 µl of coating solution (e.g., 50 µg/ml fibronectin solution diluted in culture medium or 1X PBS) into the channel. Repeat this step for the opposite channel. Use culture medium instead if coating is not required.



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

15. Incubate for 1 h in a 37 °C incubator.
16. Add 150 µl of medium into one side of the reservoir to flush out the coating solution. Repeat this for the other channel.
17. Remove the medium and add 300 µl of medium to one side of the reservoir. Repeat this for the other reservoir. If the coating solution must be removed completely, repeat this step twice.

    Optional: In this example, the application of interstitial flow helps the endothelial cells self-organise into perfusable vessels. To create interstitial flow, add 300 µl of medium in one of the media reservoirs and add 150 µl of medium in the opposite media reservoir. The additional 150 µl of medium creates a height difference between the two media reservoirs generating the interstitial flow across the gel.
18. Incubate the plate in a CO_2 incubator at 37 °C for 3 days, with medium exchange every two days.

CHANGING MEDIUM ⏱TIMING 10 min

MATERIALS

Reagents

- Cell culture medium (Lonza, Cat. No. CC3162)

Others

- organiX Plate

19. Remove medium from the 2 media reservoirs by carefully aspirating the medium away from the media inlets. Top up with 300 µl of medium per media reservoir.

    Optional: if interstitial is required, add 300 µl of medium into one reservoir and 150 µl of medium into the other reservoir.

20. Keep the plate in a CO_2 incubator at 37 °C.

SEEDING CELLS ⏱TIMING 30 min + 60 min incubation

MATERIALS

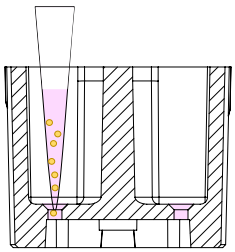
Reagents

- 1X PBS (Life Technologies, Cat. No. 70011044)
- 0.25% trypsin with EDTA (Lonza, Cat. No. CC5012)
- Cell culture medium (Lonza, Cat. No. CC3162)

Others

- organiX plate
- HUVECs
- Centrifuge

21. 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 5 min in a CO_2 incubator.
22. Perform a visual inspection to make sure the cells have detached from the substrate.
23. Add medium with FBS, at least 5 times the volume of trypsin, into the culture flask/dish to neutralize the activity of trypsin.
24. Transfer the cell suspension to a 15 ml tube and pellet the cells by centrifuging at 250 x*g* for 5 min at RT.
25. Resuspend the cells in culture medium with densities ranging from 1 M to 3 M cells/ml, depending on cell types and applications. In this example, 35 µl of HUVEC cell suspension at 1 M cells/ml is sufficient to form a confluent monolayer overnight.
26. Remove medium from the reservoirs by carefully aspirating the medium away from the media inlets.
27. Use a micropipette to withdraw 35 µl of the endothelial cell suspension (1 M cells/ml). Position the tip at the media inlet and inject the cell suspension slowly (e.g., over a duration of 3 s).



! Critical *Lay the plate on a flat surface while seeding cells into the organiX plates. Inclination of the plate affects the flow in the media channel, thus disturbing cell distribution.*

28. Visual inspection of the channel under a microscope is recommended.
29. Place the plate in the incubator and incubate for 30 min.

    Optional: Incline the plate at an angle between 30-45° for at least 30 min to promote cell attachment to the gel interface.
30. If cells need to be seeded in the other media channel, repeat steps 27. - 29. .

31. Add 300 µl of medium to both reservoirs.
32. Keep the plate in a 5 % CO_2 incubator at 37°C.

STAINING CELLS ⏱TIMING 2 days

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)
- Alexa Fluor® 488 Anti-CD31 antibody [JC/70A] (Abcam, Cat. No. ab215911)
- Rhodamine Phalloidin (Life Technologies, cat. No. R415)
- Hoechst (Life Technologies, Cat. No. H1399)

Cell Fixation ⏱TIMING **30 min**

33. Remove medium from the reservoirs by carefully aspirating the medium away from the media inlets.
34. Add 300 µl of 1X PBS into one media reservoir and then add 150 µl into the other media reservoir. The differential volumes in the two reservoirs create flow through the gel to wash the sample.
35. Remove 1X PBS from the media reservoirs by carefully aspirating the PBS away from the media inlets. Add 300 µl of 4% formaldehyde into one media reservoir and then add 150 µl into the other media reservoir.

Reminder *The 1X PBS within the media channels can be carefully removed using a micropipette before the addition of formaldehyde, if the dilution effect contributed by the 1X PBS is a concern. Replenish the media channels with 35 µl of 4% formaldehyde.*

36. Incubate for 20 min at RT.
37. Remove the 4% formaldehyde solution from the reservoirs with a micropipette. Wash the channels twice with 1X PBS as described in steps 33. and 34. .

! 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 **30 min**

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

38. Remove 1X PBS from the media reservoirs by carefully aspirating the PBS away from the media inlets. Add 300 µl of 0.1 % Triton X-100 into one media reservoir and 150 µl into the other media reservoir.

Reminder *The 1X PBS within the media channels can be carefully removed using a micropipette before the addition of Triton X-100, if the dilution effect contributed by the 1X PBS is a concern. Replenish the media channels with 35 µl of 0.1 % Triton X-100.*

39. Incubate for at least 30 min at RT.
40. Wash once with 1X PBS as described in steps 33. and 34. .

Blocking ⏱**TIMING 2 h**

(Optional: Blocking is only necessary for immunofluorescent staining)

41. Remove 1X PBS from the media reservoirs by carefully aspirating the PBS away from the media inlets. Add 300 µl of blocking buffer into one media reservoir and 150 µl into the other media reservoir.

Reminder *The 1X PBS within the media channels can be carefully removed using a micropipette before the addition of blocking buffer, if the dilution effect contributed by the 1X PBS is a concern. Replenish the media channels with 35 µl of blocking buffer.*

42. Incubate for 2 h at RT.

Primary/Conjugated Antibody Staining ⏱**TIMING 18 h**

43. Prepare antibodies according to the manufacturer's recommendations. In this example, the antibody is conjugated with GFP. Dilute the anti-CD31 antibody with 1X PBS in a 1:250 ratio.
44. Remove the blocking buffer from the media reservoirs by carefully aspirating the blocking buffer away from the media inlets. Add 300 µl of the antibody solution into one media reservoir and 150 µl into the other media reservoir.

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

45. Incubate overnight at 4°C.

Secondary Antibody Staining ⏱**TIMING 1.5 h**

(**Note**: Secondary antibody staining is only necessary if the primary antibody is not conjugated)

46. Remove the primary antibodies from the media reservoirs by carefully aspirating the antibody solution away from the media inlets. You may reuse the antibodies depending on the antigen and antibody.
47. Wash with 1X PBS 5 times, with a 15 min incubation between each wash, as described in steps 33. and 34..
48. Prepare the secondary antibodies according to the manufacturer's recommendations.
49. Add 300 µl of the secondary antibody solution into one media reservoir and 150 µl into the other media reservoir.

! Caution *Cover the plate with aluminium foil to minimize photobleaching.*

50. Incubate for 1 h at RT.
51. Remove the secondary antibodies from the media reservoirs by carefully aspirating the antibody solution away from the media inlets.
52. Wash with 1X PBS 5 times, with a 15 min incubation between each wash, as described in steps 33. and 34..

Fluorescent Staining ⏱**TIMING 1.5 h**

53. Prepare the staining solution containing Hoechst (10 µg/ml)/Rhodamine Phalloidin (3 U/ml) in 1X PBS.

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

54. Remove 1X PBS from the media reservoirs by carefully aspirating the PBS away from the media inlets. Add 300 µl of staining solution into one media reservoir and 150 µl into the other media reservoir.

55. Incubate for 1 h at RT.

56. Wash with 1X PBS 5 times, with a 15 min incubation between each wash, as described in steps 33. and 34. .

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

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

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 reservoirs does not dry up.*

HARVESTING SAMPLES ⏱TIMING 20 min

MATERIALS

Others

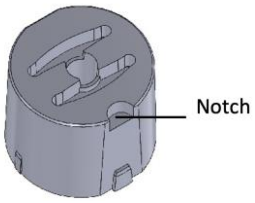
- organiX Extractor

Sample harvesting ⏱TIMING 20 min

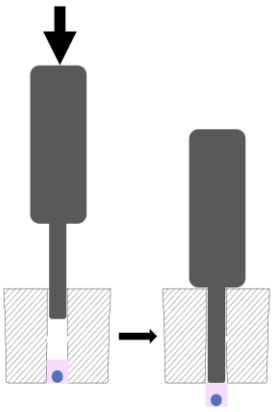
57. Remove the organiX insert from the organiX plate.

Reminder *The samples can be fixed or be frozen before the harvesting step. Greater force is needed to push frozen samples out from the organiX inserts as they expand in sizes.*

58. Grip the bottom laminate at the notch using a pair of tweezers and peel it off.



59. Use the organiX Extractor to push out the sample gently.



60. Collect the sample in a clean microcentrifuge tube or petri dish for further processing.

REAGENT SETUP

4% FORMALDEHYDE

Reagents

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

Others

- Hot plate with magnetic stirrer

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

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

62. 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.*

63. 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)

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

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