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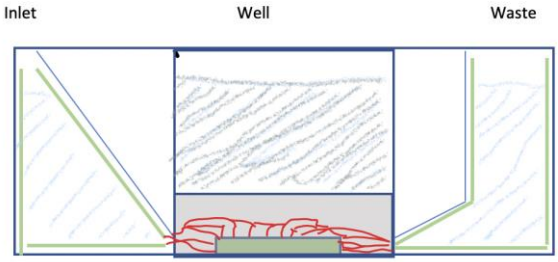
Hydrogel Preparation and 3D Cell Assay With No Flow Days 1-3, Then Flow for Days 4-10

Starting Reagents:

Fibrinogen: Make a 15mg/mL stock of fibrinogen in DPBS in a 37'C water bath. It will take about 1~2 hour to completely dissolve. [Fibrinogen doesn't dissolve in water.] DO NOT VORTEX. The solution will be hazy. Freeze in 1mL aliquots at -80'C. [Do not filter yet.]
Aprotinin: Dissolve 1mg of Aprotinin in 160uL of DPBS for a concentration of 6.25mg/mL. Dispense into 20uL aliquots and freeze at -80'C. [Do not filter yet.] Avoid freeze-thaws.
Thrombin: Reconstitute in DPBS at 100U/ml. Store in 100uL aliquots at -80'C.
rhVEGF: Reconstitute in 1% BSA at 50ug/mL.
rh Angiopoietin-1: Reconstitute in 1% BSA at 10ug/mL.
DPBS WITHOUT Ca and Mg
GFP-HBMEC's

Basic Outline:

GFP-HBMEC's (6E6 cells/mL) seeded in ~20uL bioink in center of device. [No VEGF.]
Create hydrogel with fibrin and thrombin. The hydrogel should cover the inlet and outlet pores.
Incubate to allow polymerization.
Coat inlet and outlet with QuickCoat, then remove after 5min.
Seed GFP-HBMECs into inlet and outlet. (Cell number ~5E5cells/mL?) Use media containing aprotinin and Thrombin (BUT NO VEGF!!).
Overlay hydrogel with media containing 85ng/mL VEGF, 100ng/mL Ang-1, 100ng/mL aprotinin, 1U/mL thrombin.
The next day, change media as follows:
Well: Media with 85ng/mL VEGF, 100ng/mL Ang-1, and 100ng/mL aprotinin.
Channels: Media with 100ng/mL aprotinin.
Thrombin is only used on day 1.
No flow for days 1-3, then flow for 15min/day for days 4-10.



Green box = cells in bioink (no VEGF)
Green lines = cells lining the inlet and waste channels
Gray = hydrogel with high VEGF
Gray shading = media with high VEGF
Blue shading = media without VEGF

Preparing Novogel-Fibrinogen-Aprotinin stock: This stock will last for 1week at 4 degrees.

1. Mix 2mL 15mg/mL Fibrinogen with 4mL DPBS in a 50mL conical tube. (6 mL total)
2. Add 24uL 6.25mg/mL aprotinin. Final concentrations: 5mg/mL fibrinogen and 0.025mg/mL aprotinin.
3. Filter sterilize using a syringe filter. (You will use most of this.)
4. Put 3mL of filter-sterilized DPBS/Fibrinogen/Aprotinin in a fresh 50mL tube.
5. Empty the Novogel tube contents into the 50mL tube and swirl gently.
6. Add another 2mL of filtered DPBS/Fibrinogen/Aprotinin to the original Novogel tube, and invert 2x to mix.
7. Place Novogel tube and 50mL tube in 37 degree water bath.
8. After 1-2h, recombine contents of both tubes in 50mL tube and place at 4 degrees until needed. It will solidify – requires 10-20m at 37 degrees to become liquid again.

Devices:

1. Devices were sterilized with EtO and 1hr UVO treatments.
2. Add QuickCoat to the wells and channels. Incubate 5min.
3. Aspirate and dry in the hood uncovered.

Preparing Cells:

Target: Bioink – $6x10^6$ endothelial cells/mL. 0.5 mL should be enough to seed many wells. So I'll need 3E6 cells.

Target: Seeding channels - use ~5E5 endothelial cells/mL.

1. A few days in advance, prepare 4 or more T75 flasks of GFP-HBMEC's. Each flask will give you ~2e6 cells – these cells like to take up as much space as possible.
2. For a T75 flask, gently add 2mL trypsin, cover cell surface by tilting, and remove within 20 sec. Watch the cells, and when loosened, rap flask to suspend cells, and then neutralize trypsin with at least 10mL of media (can combine multiple T75s in one tube)

3. Spin down cells in 50mL conical, 200g/5min.
4. Remove supernatant, add 1mL of media and then count cells with hemocytometer.
5. Prepare 0.5mL of 6e6 cells/mL for the bioink.
6. Prepare 4mL of ~5E5 cells/mL for seeding the channels.
7. If the channel cells are prepared in advance of when they'll be needed, put on a rotator until ready to use.

Bioink:

1. Spin down cells for bioink at 500 x g for 4 min and remove supernatant.
2. Add 1 mL (or appropriate amount scaled to cell #) Novogel-Fibrinogen-Aprotinin gel to cell pellet and mix by pipetting back and forth, minimizing bubbles. BioInk should be $6x10^6$ cells per mL (Use about 10-20 uL gel per well).
3. Transfer the bioink to a 15mL snap-top Falcon tube that can accommodate a 1-3mL syringe without needing to attach a needle.
4. Slowly draw the bioink into the syringe.
5. Invert and gently tap syringe to make bubbles move and gently push plunger to remove air before attaching 21-gauge dispensing needle (500um diameter).
6. After loading, place syringe in fridge to solidify for 10 mins.
7. Dispense 10-20uL of gel per well into the center of the well. The bioink will solidify at RT. For this experiment, we don't want the bioink near the pores leading into the channels.

Hydrogel Preparation:

1. Add 900uL of DPBS to an aliquot of thrombin to make 1mL of 10U/mL thrombin. At the end of the day, freeze the leftover 10U/mL thrombin, being sure to label the concentration clearly.
2. Put 30 uL of 10U thrombin in 1.5mL eppendorf tubes -- make $1/3^{rd}$ as many 30uL thrombin tubes as you have wells to cover. Prepare at least one more tube than you expect to use.
3. Add 0.51uL 50ug/mL VEGF to this. This gives a final VEGF concentration in the hydrogel of 85ng/mL.
4. Add 270 uL of Novogel-Fibrinogen-Aprotinin to one tube of thrombin, mix by pipetting up & down once without adding bubbles. Immediately pipet 100uL into each of three wells containing the dispensed BioInk. Work quickly because this will start to polymerize. (Always add Fibrinogen to Thrombin, not vice versa.)
5. Incubate for 15 min (or longer) at room temp for fibrin polymerization.

Adding Cells to the Inlet/Outlet Channels

1. Prepare a small volume, 1-3mLs, of 5E5 cells/mL of GFP-HBMECs in media with 100ng/mL aprotinin and 1U/mL Thrombin.
2. Add cell suspension to the precoated channels, and incubate at 37'C.
3. After 2 hours, remove media from channels, along with any cells that haven't attached.

4. Replace the media in the channels with fresh media containing 100ng/mL aprotinin and 1U/mL Thrombin, and return the device to the incubator. Make just enough media for the wells. [Tomorrow you won't need Thrombin.]

Filling Wells with Media (NOTE: Day 1 media contains 1U/mL Thrombin.)

1. Fill the wells with 200uL of media containing 85ng/mL VEGF, 100ng/mL Ang-1, 100ng/mL aprotinin, 1U/mL Thrombin. Make just enough for today.
2. Incubate the device at room temperature for 90-120 min to allow polymerization.
3. Store the device in a humidified box in the 37'C incubator overnight.

Days 2 and 3: Exchanging Media in the Wells and Channels

1. Prepare some media as follows:
   Well: Media with 85ng/mL VEGF, 100ng/mL Ang-1, and 100ng/mL aprotinin.
   Channels: Media with 100ng/mL aprotinin.
2. Carefully remove the media in the wells and channels. Replace the well media with High VEGF media, and the channels with the lo-VEGF media.
3. Take photos with the EVOS to monitor vasculogenesis and anastomosis.

Adding Flow (Days 4-10):

1. In no-flow control device, exchange the media manually every day. As before, use high VEGF media +100ng/mL Ang-1 + 100ng/mL aprotinin in the well, and low VEGF media + 100ng/mL aprotinin in the channels.
2. Insert black plugs into the wells.
3. Assemble some tubing into a blue ferrule. Add a brown screw and tighten to lock the tubing in place.
4. Connect the tubing to a syringe of media, and fill the tubing with media. Connect the tubing to the inlet channel.
5. Place the syringe into the syringe pump.
6. Remove media from the waste trough.
7. Run flow through the inlet at 0.5uL/sec for 15min per well.

Vasculogenesis will take about three days, and anastomosis will take about a week.

Media Components:

Complete VEGF media (stored in 50mL tubes in the fridge). [This is 5ng/mL VEGF, which is low concentration.]
6.25mg/mL Aprotinin: Stored at -20'C in E112.
Make a 1:10 dilution: **625ug/mL** Aprotinin, and store at -20'C.
100u/mL Thrombin: Stored at -20'C.
50ug/mL rh VEGF: Stored at -20'C.
10ug/mL rh Angiopoietin-1: Stored at -20'C.

High VEGF Media For Feeding Wells: Make 10mL.

10mL VEGF media
17uL 50ug/mL VEGF (final conc of 85ng/mL)

100uL 10ug/mL Ang-1 (final conc of 100ng/mL)
1.6uL **625ug/mL** Aprotinin (final conc of 100ng/mL)

Channel Media: Make 10mL.

10mL VEGF media
1.6uL **625ug/mL** Aprotinin (final conc of 100ng/mL)

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