GENERATION OF 3D MUSCLE TISSUES in PAPER SCAFFOLD
(96-well format)

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MATERIALS

S. No.	Materials Required	Ordering Information
1	Fibrinogen from bovine	Sigma #F8630
2	Geltrex	Thermo, #A1413202
3	Thrombin from human plasma at 100 U/ml (frozen vials can be kept in -20 freezer)	Sigma, #T6884
4	6-aminocaproic acid (ACA) sterile solution (50 mg/ml in ddH_2O)	Sigma, #A2504
5	Pluronic® F-127 powder, BioReagent, suitable for cell culture (5% w/v in ddH_2O)	Sigma, #P2443
6	0.9% (wt/vol) sterile NaCl solution	
7	Trypsin-EDTA (0.25%, 1X)	
8	96 well plates, ideally CellCarrier-96 Ultra Microplates, tissue culture treated, black, 96-well PhenoPlate with lid for optimal imaging	Revvity, #6055302
10	SB203580 – p38α/β MAPK	New England biolabs, #5633
11	Erlotinib Hydrochloride – EGFR	Santa Cruz Biotech., #sc-202154
12	DMSO	Sigma Aldrich, #D2650
	Wash medium SAT10 replete of bFGF After Seeding growth medium Myoblast differentiation medium Differentiation media supplemented with drugs +bFGF (Media formulations provided at the end of the protocol)	
13	Sterile PBS	
14	Paper Scaffolds	
15	Sterile Tweezers	
16	Cardiotoxin	Cedarlane Labs, #L8102

MYOTUBE TEMPLATE FABRICATION METHOD

Before making myotube templates

1) Cut out circular discs from a sheet of cellulose paper using a 5 mm biopsy punch. Put them all together in an aluminum pouch and then into an empty pipette tip container. Autoclave the box using the dry-cycle.

Note: This step can be done in advanced and sterile paper discs can be stored long term.

Day of myoblast seeding (Day -2)

A) Paper scaffold prep

1) Perform hydrophobic coating by adding 100 µL of 5% pluronic acid into each well of a 96 well plate that tissues will be placed in. Leave the plate in a 4 °C fridge for MINIMUM 1 HOUR (you can also coat the wells the day before and keep at 4 °C).

Note: Revvity PhenoPlates are suggested for optimal imaging

2) Aspirate the Pluronic acid out of the wells and leave the plate out in the biosafety hood to dry with the lid removed for 20 min (you should notice that the thin film of liquid is gone when it is dry).
3) Place one sterile paper disc (autoclaved) in each of the corresponding wells using sterile tweezers.
4) Make a 0.8 U/mL thrombin solution by diluting 0.4 µL of the 100 U/mL thrombin stock aliquot into 49.6 µL of PBS (This is for 50 µL total PBS + Thrombin, scale accordingly).
5) Dispense 4 µL of PBS + thrombin solution onto the center of each paper disc. The papers are now wet in their respective wells, leave them out to dry during the duration of the protocol.

Note: While adding PBS + thrombin solution to the circular disks make sure to bring the disk in the center of the well using your pipette tip.

B) Myoblast prep

1) Weigh required amount of fibrinogen in a 1.5 ml Eppendorf tube (~10 mg is more than enough).

Note: Fibrinogen powder aliquots can be maintained in -20 freezer for long-term storage.

2) Dissolve fibrinogen in 0.9% (wt/vol) solution of NaCl in water to arrive to a 10 mg/ml final concentration.

Note: Do not vortex vigorously. Add the NaCl solution, spin briefly and leave at 37 °C for 3-5 minutes. Spin again and tap with your fingers and it should be dissolved)

3) Filter the fibrinogen solution through a 0.22 µm filter using a syringe.

Note: Do not apply too much pressure. You can change the filter if blocked.

4) Put the fibrinogen solution on ice.
5) Thaw Geltrex slowly within densely packed ice.

While waiting for the reagents to thaw*:*

6) Collect cell culture plates (myoblast) from the incubator and check under microscope to ensure you have enough number of cells.
7) Wash cells with PBS and Trypsinize using 1X trypsin solution.
8) Halt trypsin activity using wash medium, and pellet cells by centrifuging at 300g for 10 mins.

Note: Use 15 ml conical tubes

9) Aspirate the media and resuspend your pellet in wash medium.
10) Count cells using hemocytometer and trypan blue (dilute your cell suspensions if required).
11) Transfer desired number of cells into a 0.5 or 1.5 mL Eppendorf tube and spin at 300g for 6 mins

Every tissue needs 100,000 primary human myoblast cells. If making 20 tissues, put 2,100,000 cells in your Eppendorf (*always have 1 extra tissue's worth*)
Immortalized myoblasts and primary mouse myoblasts require 25,000 cells/tissue.

While waiting for the cells to pellet:

12) Prepare ECM master mix for seeding tissues based on the following recipe and in the following order:
    - DMEM (40% vol)
    - 4 mg/ml fibrinogen (40% vol)
    - 20% Geltrex (20% vol)
13) Keep your master mix on ice until ready to use.

Each tissue requires 4 µL of ECM. If making 20 tissues you will need 84 µL of ECM (remember you have enough cells for 1 extra tissue, so you should make enough ECM for 21 tissues). Better to make 90-100 µL of ECM just in case you lose some during pipetting.

14) Make your after seeding growth media (200 µL for every tissue) and place it in the 37 °C water bath
15) Once your cell pellet is ready, aspirate as much of the supernatant as possible. Use a pipette to avoid accidentally aspirating your cells while you get as close to the pellet as possible.

CRITICAL STEP: It is important to aspirate as much media as you can to ensure not diluting your ECM master mix and cells in extra culture media.

16) Place the tube with the cell pellet on ice.
17) Transfer required amount of the ECM master mix on to your pellet (84 µL for 21 tissues) and resuspend thoroughly until you obtain a single cell suspension.
18) Place the cell suspension on ice.
19) Check to make sure your paper discs are now properly dried.
20) When you are ready, mix the ECM-cell suspension mixture with a P200 tip. Then use a P10 and dispense 4 µL of the mixture into the middle of each paper.

CRITICAL STEP: Care to not press too hard or accidentally slide the paper so that it touches the wall of the well.

21) Now that the ECM-cell mixture has diffused into each of your papers. Place the plate in the 37 °C incubator for 5 min.
22) Take the plate back out. Gently add 200 µL of the After Seeding Growth Media to your first tissue and check that the tissue has gelled properly under the microscope. If so, add media to the rest of your tissues

Note: It is important to dispense the media gently using a P200 to avoid the tissues from flipping over.

Note: The tissue has gelled properly if there is an obvious contrast at the paper's periphery.

23) Leave the plate in the incubator at 37 °C.

Note: Check the tissues the following day to make sure that the gel has properly detached from the bottom of the well. If some tissues are still partially stuck, gently pipette up and down with a P200 to detach the paper-gel scaffolds containing the cells.

Myoblast Differentiation (Day 0)

1) Prepare myoblast differentiation media.
2) Pre-warm the media in 37 °C water bath for 2-3 mins.
3) Do a FULL media change for each tissue

Note: Now you are at Day 0 differentiation time point. All the other time points (e.g Day 7, Day 10, Day 14, etc.) are measured from this origin.

**From this point on change half of the media every other day with fresh differentiation media

MuSC SEEDING ONTO MYOTUBE TEMPLATES (DAY 5) METHOD

1) Spray paper towel, the PDMS mold and Tupperware container with 70% ethanol and leave to dry in the BSC. Once dry, place the paper towel at the bottom of the Tupperware and put the PDMS mold on top. Add enough autoclaved water to just saturate the paper towel. Pre-warm the humidity chamber for at least 30 minutes before your resuspended MuSC solution is ready. (Alternatively, you can also use a sterile flat container and cover the base with parafilm to provide a soft hydrophobic surface)

2) Prepare SAT10 replete of bFGF.
3) Transfer desired number of MuSCs into a 0.5 or 1.5 mL Eppendorf tube and spin at 300g for 6mins.

   Every tissue will require 4 µL of MuSCs resuspended in SAT10 replete of bFGF. If making 20 tissues you will need 84 µL of SAT10 replete of bFGF (remember you have enough cells for 1 extra tissue, so you should make enough SAT10 replete of bFGF for 21 tissues). Better to make 90-100 µL of DM just in case you lose some during pipetting.

4) Once the cell pellet is ready, carefully aspirate the supernatant as much as you can.

CRITICAL STEP: It is important to aspirate as much media as you can to ensure not diluting your master mix and cells in extra culture media.

5) Resuspend the cell pellet in the required amount of SAT10 replete of bFGF and keep it on ice.
6) Bring the 96 well plate with the myotube templates from the incubator.
7) Using a sterile pair of tweezers, carefully take the templates (holding from the edge of the scaffold) and put them on the sterile PDMS mold with the bottom side facing up. (This is the side opposite of the one you seeded the myoblasts on)

8) Once all the tissues have been carefully placed on the PDMS mold, bring your Eppendorf containing the stem cells and mix thoroughly to reach a homogeneous single cell mixture.

Note: Ensure that the tissues do not dry out! If you notice they are beginning to dry, add a few uL of media from the myotube template wells.

9) Using a P10 pipette, dispense **4 µL of** MuSCs resuspended in SAT10 replete of bFGF onto the myotube template carefully.
10) Using a sterile cell spreader or the tip of a P10 pipette, make sure that the cells have been evenly distributed on the template.
11) Once you are ready, place the container with the tissues at 37 °C for 1 hour of incubation.
12) When the 1 hour long incubation is complete, bring the container and using a sterile pair of tweezers, carefully return the tissues into their wells in the same orientation (do not flip!).
13) Place the 96-well plate back into the incubator at 37 °C.

**From this point on, half media changes with fresh differentiation media are performed every other day until tissue harvest.

**Change full media every other day with fresh differentiation media supplemented with drugs (EGFRi/P38i/DMSO) + bFGF (if needed) for 5 days after which, change half of the media every other day with fresh differentiation media until you harvest the tissues.

MINI-MENDR MYOTUBE TEMPLATE INJURY (DAY 7) METHOD

1) Prepare the required amount of Injury media and place it in the water bath at 37 °C. Each tissue requires 100 µL of the injury media.
2) Remove the culture media from each well and dispense 100 µL of the injury media.
3) Place the 96 well plate to incubate on an orbital shaker within a 37 °C incubator for 4 hours.
4) When the 4 hour incubation is complete, gently remove the injury media from each well and wash the tissues in fresh DM.
5) Add DM supplemented with drugs and bFGF (if needed) and place the tissues back in the incubator at 37 °C.

**From this point on change full media every other day with fresh differentiation media supplemented with drugs (EGFRi/P38i/DMSO) + bFGF for 5 days after which, change half of the media every other day with fresh differentiation media until you harvest the tissues.

Media formulations:

SAT10 (replete of bFGF)

- DMEM/F12
- 1% P/S (penicillin/streptomycin)
- 20% FBS
- 10% HS
- 1% Glutamax (Gibco #35050061)
- 1% Insulin-Transferrin-Selenium (Gibco #41400045)
- 1% Non-essential amino acids (Gibco #11140050)
- 1% Sodium pyruvate (Gibco #11360070)

- β-mercaptoethanol

After Seeding Growth Media (to use for the first 2 days after seeding):

- SAT10 (replete of bFGF)
- 3% ACA

Myoblast Differentiation Media:

- DMEM
- 2% Horse serum
- 1% P/S (penicillin/streptomycin)
- 10 ug/ml bovine insulin (1:1000)
- 4% ACA

Wash Media:

- 89% DMEM
- 10%FBS
- 1% P/S (penicillin/streptomycin)

Primary Myoblast growth media for 2D culture:

- F-10
- 20% FBS
- 1% P/S (penicillin/streptomycin)
- bFGF 1:5000

Differentiation media supplemented with drugs +bFGF:

- DMEM
- 2% Horse serum
- 1% P/S (penicillin/streptomycin)
- 10 ug/ml bovine insulin (1:1000)
- 4% ACA
- Drugs: EGFRi/ P38i/ DMSO (1:1000)
- 5 ng/ml bFGF (1:5000)

Injury Media:

- DMEM
- 2% Horse serum
- 1% P/S (penicillin/streptomycin)
- 10 ug/ml bovine insulin (1:1000)
- 4% ACA
- Cardiotoxin (0.5 μM)