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Optimizing myoblast distribution within mini-MEndR tissues using C2C12 cells

Use this practice protocol if you notice that cells are unevenly distributed in your mini-MEndR tissues (concentrated in some areas and/or missing in other areas) in spite of well dissociated myoblasts as viewed during the cell counting step. This practice protocol helps hone technique related to: (a) resuspending cells into a monodisperse solution within a viscous hydrogel precursor solution and (b) evenly distributing the monodisperse solution into the paper scaffold

Tips that I found helpful for early users are outlined in blue text.

Seeding tissues

1. At least 1 hour prior to step 2, add 100 µL of a 5% Pluronic Acid solution to each well of a 96-well plate that will eventually contain tissues. Wrap the plate in Parafilm and store at 4˚C during coating.
2. Use a P100 or a vacuum aspirator to remove the Pluronic from all wells.
    - Gilbert lab specific note: Avoid using the vacuum aspirator pictured on the left for this step as the vacuum pressure is too strong and results in complete loss of the Pluronic coating. Use a P200 or the aspirator pictured on the right instead.


3. Allow the Pluronic remaining in the wells to evaporate in the BSC (i.e. plate lid off).
4. While waiting for Pluronic to evaporate, prepare the thrombin solution: Dilute the 100 U/mL stock to 0.8 U/mL using PBS. Prepare 4 µL diluted thrombin solution per tissue.
    - Prepare a volume of diluted thrombin solution for ~1-2 more tissues than planned to compensate for volume loss when pipetting.
    - Store the stock and diluted thrombin on ice in the BSC while working.
5. Also during the Pluronic evaporation, obtain fibrinogen powder and Geltrex solution (both pre-aliquotted into 1.5 mL centrifuge tubes). Store both on ice in the BSC.

- Bury the Geltrex under the ice to control the pace of thawing; rapid thawing of Geltrex will cause crosslinking, and crosslinked Geltrex will no longer behave like a liquid.

6. Once the Pluronic liquid film has evaporated from the coated wells (i.e. it should no longer be visible to the eye in the wells), use tweezers to place a circular piece of cellulose paper, pre-punched with a 5 mm biopsy punch, into each well. Do not place the lid on the plate after inserting the paper.
    - Ensure that a single sheet of cellulose paper is in each of the wells, rather than multiple sheets. Single sheets of paper should look slightly translucent, with light passing through small gaps in the paper.
    - When transferring the cellulose paper with tweezers, ensure that the tweezers only touch the edge of the paper, ideally as little of the edge as possible. This will prevent the paper from tearing.
7. Gently pipette 4 uL diluted thrombin solution onto the centre of each paper. Once the thrombin solution has been deposited, the paper should appear wet and more translucent than before. Note the appearance of the paper. Keep the lid off of the plate to dry the thrombin.
    - To ensure that the entire volume is being deposited onto the paper, suck up the solution with the P10 by pushing to the first stop, but deposit the solution by pushing to the second stop. Check the pipette tip for any volume left over.
    - Press the pipette into the center of the tissue, but only to the point where the pipette barely makes contact. This allows for accurate pipetting, and encourages the liquid to wick into the paper, but does not tear the paper.
8. Trypsinize a plate of C2C12 cells and centrifuge to pellet.
9. While C2C12 cells are pelleting, create fibrinogen solution: Reconstitute pre-weighed fibrinogen powder from a centrifuge tube into 0.9% (w/v) NaCl solution such that you achieve a final concentration of 10 mg/mL fibrinogen.
10. After adding the NaCl solution to the fibrinogen tube, warm the tube at 37˚C for 1-2 minutes, or until the fibrinogen powder is fully dissolved.
11. Filter the warmed fibrinogen solution through a 0.22 µm filter attached to a syringe, into another tube. Store this final filtered solution on ice in the BSC.
12. After C2C12 cells have pelleted, count the number of cells in the pellet using a hemacytometer.
13. Each tissue should be seeded with 25,000 cells. Transfer the appropriate number of cells for all of the tissues you will seed to a microcentrifuge tube. Centrifuge the tube with cells at 0.3 x g for 6 minutes in a fixed angle rotor.

- Transfer enough cells for two additional tissues than planned to compensate for volume loss when pipetting.
- Orient the tube in the centrifuge so the hinge faces toward the outside of the rotor; this ensures that the pellet is on the same side of the hinge.

14. While the cells are pelleting, prepare the hydrogel master mix: 40% DMEM, 40% filtered fibrinogen solution and 20% Geltrex. The final volume of master mix should correspond to the number of cells prepared in the previous step, with 4 uL being allocated to every 25,000 cells. Store this on ice.
15. After cells have pelleted in the microcentrifuge tube, aspirate the media in the microcentrifuge tube.
    - The media must be aspirated completely to ensure that the resuspension in the next step results in accurate cell numbers being seeded.
    - To avoid disturbing the pellet, do not touch the side of the tube that contains the pellet using the aspirating pipette. Instead, use a P100/P1000 which is gentler than a vacuum aspirator.
16. Critical step for cell distribution: Resuspend the cells within the master mix to a single cell suspension by pipette up and down thoroughly (about 20x). Be sure not to introduce bubbles. This can be achieved by avoiding the uppermost and lowermost limits of your pipette settings during trituration.
17. Check the paper in the 96-well plate to ensure that the thrombin has dried. Dry papers should appear less translucent than when they were initially seeded with thrombin solution.
18. Critical step for cell distribution: Pipette 4 µL of cell/master mix suspension into the centre of each paper scaffold.
    - To ensure that the entire volume of suspension is being deposited onto the paper, suck up the solution with the P10 by pushing to the first stop, but deposit the solution by pushing to the second stop. Move swiftly but gently. Check the pipette tip to ensure that no volume remains.
    - Although it is important to push to the second stop when seeding, do not push after you feel you have reached the second stop, because forceful pipetting will push the cells to the edge of the paper (and beyond) instead of evenly distributing them.
    - Press the pipette into the center of the paper scaffold, but only to the point where the pipette barely makes contact. This allows for efficient wicking and even distribution of cells but does not tear the paper scaffold.
    - Once the cells have been seeded, the paper should appear translucent and wet, and slightly pink in colour. The liquid should form an even layer

across the tissue. If at this point there are areas with uneven liquid coating, this suggests uneven cell distribution. Likewise, if there is liquid outside of the paper scaffold boundary, you can expect uneven cell distribution.

19. Place the lid back on the 96-well plate and incubate for 5 minutes at 37˚C to cell/master mix suspension to form a stable fibrin-based hydrogel.

Visualizing distribution of cells (nuclei) across tissues

1. Fix tissues for 15 min with 4% PFA immediately after crosslinking. Remove PFA with a p200/p1000 and transfer to PFA waste container. If you will store the samples for later analysis, add enough PBS to cover each tissue and store at 4C
2. Stain tissues with DRAQ5 nuclear stain (Remark: teabag paper displays blue autofluorescence which conflicts with DAPI/Hoechst signals).
3. Image each tissue at 4x using the confocal microscope, observing the distribution of nuclei across the entire tissue in the DRAQ5 channel. In each image, ensure that at least 80% of the tissue is visible, and that the center of the tissue is in the center of the image.
   All tissues should be immediately fixed after seeding and crosslinking, but multiple sets of tissues from multiple rounds of practice can be stained and imaged at the same time for convenience.

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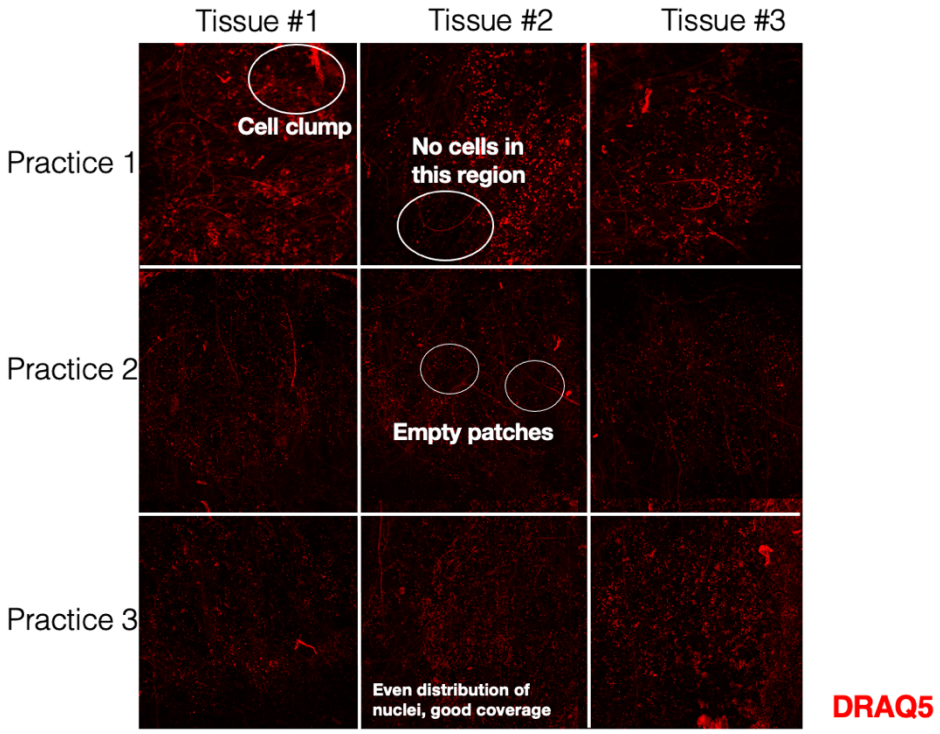
Example: mastery of cell seeding distribution across 3 rounds of C2C12 seeding



practice