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Title: A Multi-Cue Bioreactor to Evaluate the Inflammatory and Regenerative Capacity of Biomaterials Under Flow and Stretch

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or similar? **N**

2. **Software:** Does the part of your protocol being filmed demonstrate software usage? **N**

3. **Interview statements:** Considering the Covid-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group?

    ☒ Interviewees wear masks until the videographer steps away (≥6 ft/2 m) and begins filming. The interviewee then removes the mask for line delivery only. When the shot is acquired, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. **Filming location:** Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Protocol Length
Number of Steps: 31
Number of Shots: 54

Introduction

1. Introductory Interview Statements

1.1. **Anthal Smits**: Until now, identifying the cause-and-effect links between the body's hemodynamics and the outcome of vascular regeneration has been difficult because it is challenging to control individual mechanical loads **[1]**.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.2. **Anthal Smits**: This bioreactor allows us to mechanistically investigate individual and combined effects of shear stress and cyclic stretch on the regenerative capacity of a broad variety of tissue-engineered vascular grafts **[1]**.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B role: 3.2*

Introduction of Demonstrator on Camera

1.3. **Anthal Smits**: Demonstrating the procedure will be Suzanne Koch, a Ph.D. candidate from my laboratory. For the protocol steps in which 2 researchers are needed, Tamar Wissing, a Post-Doctoral researcher from my laboratory, will assist. **[1][2]**.

1.3.1. INTERVIEW: Author saying the above

1.3.2. The named demonstrator(s) looks up from the workbench or desk, or microscope and acknowledges the camera

Protocol

2. Bioreactor Setup

NOTE: steps need to be performed by 2 demonstrators

2.1. To mount the electrospun scaffold onto the silicone tubing, thread a 4-0 prolene suture into one end of a piece of silicone tubing and out of the other **[1-TXT]**.

2.1.1. WIDE: Talent A inserting suture into the tubing **TEXT: Work in a sterile laminar flow cabinet**

2.2. Make a small knot on both sides of the tube, leaving approximately 10 centimeters of wire on both knots **[1]**, and make a third knot on top of the tube. **[2]**.

2.2.1. The knot is being made at the end of the tube, with 10 cm of wire visible in the frame as possible

2.2.2. Third knot being made on top of the tube.

2.3. After removing the suture needle, trim the edges of the silicone tubing into a triangular shape **[1]**. And make a final not at the end of the two 10 cm wires.

2.3.1. Tubing being trimmed

2.3.2 Added shot: A knot is being made at the end of the two 10 cm wires

2.4. Dip the electrospun scaffold in 30% ethanol **[1]** and place the scaffold over one end of free suture wire **[2]**.

2.4.1. Scaffold being dipped into ethanol

2.4.2. Talent A placing scaffold over the wire, while Talent B holds the silicone tube

NOTE: Authors prefer Take 4 to be used for shot number 2.4.2.

2.5. Next, gently stretch both sides of the silicone tubing and the 10-centimeter suture knot **[1]** while a second researcher uses tweezers with a smooth inner tip to gently slide the electrospun scaffold over the tubing **[2]**. *Videographer: This step is important!*

2.5.1. Tubing and knot being stretched by Talent B

2.5.2. Scaffold being slid over tubing by Talent A

2.6. Slowly release the stretch on the silicone tubing while simultaneously smoothing the electrospun scaffold with the tweezers **[1-TXT]** and dip the scaffold and silicone tubing in ultrapure water two times **[2]**.

2.6.1. Stretch being released by Talent B/scaffold being smoothed by Talent A **TEXT: Some wrinkling may occur that will disappear during pre-stretch**

2.6.2. Scaffold being dipped

2.7. Construct the bottom compartment and make sure that the O-ring is properly placed.

Use a male Luer plug to close the flow outlet of the upper part of the bottom compartment **[1]** and push the pressure conduit with holes through the bottom compartment **[2]**.

2.7.1. Added shot: Bottom compartment is being constructed. Plug being inserted into the outlet.
2.7.2. Conduit being pushed through bottom compartment

2.8. Place a silicone O-ring around the lower end of the pressure conduit to prevent leakage **[1]** and screw the lower part of the bottom compartment to the upper part of the bottom compartment to secure the pressure conduit **[2]**.
2.8.1. O-ring being placed
2.8.2. Lower and upper parts of the compartment being attached

2.9. Make sure that the lower engraved groove of the pressure conduit is approximately 3-5 millimeters above the edge of the adapter bushing of the bottom compartment **[1]**.
2.9.1. Shot of groove 3-5 mm about adapter bushing *Video Editor: please emphasize distance and/or bushing when mentioned*

2.10. Pull the silicone tube with the electrospun scaffold over the pressure conduit **[1]** and make a knot with the suture wire at the lower end of the electrospun scaffold at the location of the engraved groove on the pressure conduit **[2]**. *Videographer: This step is difficult and important!*
2.10.1. Tube being pulled over conduit
2.10.2. Knot being made with suture wire by Talent A, while Talent B holds the silicone tube with electrospun scaffold into place
NOTE: Authors prefer Take 3 to be used for shot 2.10.2

2.11. Make a second knot at the opposite side to tightly secure the silicone tubing with the electrospun graft **[1]** and, placing a scissor clamp equipped with a rule at the upper end of the silicone tube **[2]**.
2.11.1. Knot being made by Talent A
NOTE: Use Take 1 for shot 2.11.1 up till 1:55 minutes
2.11.2. Clamp and ruler being placed by Talent B and A, respectively.
NOTE: Shots 2.11.1 and 2.11.2 are combined (Take 1) and also include footage for shots 2.12.1, 2.12.2 and 2.13.1.

2.12. Pull the scissor clamp upwards in a consistent manner **[1]** and gently pull on the electrospun scaffold to remove any wrinkles **[2]**. *Videographer: This step is important!*
2.12.1. Clamp being pulled upward by Talent A
2.12.2. Scaffold being pulled
NOTE: Shots 2.11.1 and 2.11.2 are combined (Take 1) and also include footage for shots

2.12.1, 2.12.2 and 2.13.1.

2.13. Use a suture wire to make two knots at both ends of the scaffold at the upper engraved groove of the pressure conduit **[1]**. *Videographer: This step is important!*

2.13.1. Knot being made by Talent B
NOTE: Shots 2.11.1 and 2.11.2 are combined (Take 1) and also include footage for shots 2.12.1, 2.12.2 and 2.13.1.

2.14. When both knots have been made, release the scissor clamp **[1]** and use a knife to remove any excess silicone tubing**[2]**.

2.14.1. Clamp being released by Talent A
2.14.2. Tubing being cut

2.15. Screw the nose cones on the screw thread of the pressure conduits with holes for the dynamic samples **[1]**

2.15.1. Nose cone being screwed onto screw thread

2.16. Dip the pressure conduit, tubing, and scaffold one time in 30% ethanol and two times in ultrapure water to pre-wet the setup **[1]**.
2.16.1. Setup being dipped in ethanol, with ethanol and water containers visible in frame

2.17. Place the glass tube over the pressure conduit **[1]** and push gently on the bottom compartment to secure the glass tube in place **[2]**.
2.17.1. Talent placing tube over conduit
2.17.2. Talent pressing compartment

2.18. Then place the flow straightener, a silicone O-ring, and adapter bushing in the top compartment **[1]** and secure the compartment over the open end of the glass tube **[2-TXT]**.
2.18.1. Talent placing straightener and ring and bushing in the top compartment
2.18.2. Talent securing compartment over tube **TEXT: Repeat construction for each flow culture chamber**

3. Cell Seeding

NOTE: steps need to be performed by 2 demonstrators

3.1. Remove the male Luer plug from the flow outlet **[1]**. Work in a sterile laminar flow cabinet

3.1.1. Talent A removing plug

3.2. Open the white Luer cap **[1]** and place an ethanol-soaked tissue in front of the flow outlet **[2]**. Deconstruct the flow culture chamber, by taking off the glass tube with the top compartment **[3]**.

3.2.1. Cap being opened/medium being removed

3.2.2. Talent A placing tissue in front of the outlet

3.2.3. Talent taking off glass tube + top compartment.

3.3. Place a vacuum Pasteur pipet onto the electrospun scaffold to remove as much medium as possible **[1]** and add fibrinogen solution at a 1:1 ratio with the thrombin-supplemented cell suspension **[2]**.

3.3.1. Talent A removing medium with a vacuum Pasteur pipet

3.3.2. Talent B adding fibrinogen to the cell suspension, with a fibrinogen container visible in the frame

NOTE: Shot 3.3.2. Take 1 also includes footage for 3.4.1.

3.4. Pipet the solution up and down one time **[1]** and immediately drip the solution over the full length of the scaffold **[2]**. *Videographer: This step is important!*

3.4.1. Solution being pipetted one time (Talent B)

NOTE: Shot 3.3.2. Take 1 also includes footage for 3.4.1.

3.4.2. Talent B dripping solution over the scaffold, while Talent A holds the bottom compartment+mounted electrospun graft.

NOTE: Shot 3.4.2. Take 1 also includes footage for 3.5.1.

3.5. When all of the cells have been delivered, slowly move the scaffold from left to right and up and down to achieve an even distribution of the cells **[1]**.

3.5.1. Scaffold being moved/cells being distributed by talent A

NOTE: Shot 3.4.2. Take 1 also includes footage for 3.5.1.

3.6. When both sides of the scaffold have been seeded, carefully reconstruct the flow culture chamber by putting the glass tube and top compartment back. **[1]** and place the flow culture chamber into the incubator **[2]**.

3.6.1. Scaffold being placed into flow chamber (glass tube + top compartment placed back on bottom compartment holding the seeded scaffold)

3.6.2. Talent A placing the flow culture chamber into the incubator

4. Bioreactor and Pump System Coupling

NOTE: all steps can be performed by 1 demonstrator

4.1. To couple the bioreactor to the pump system, place the flow culture chamber on one of the eight screw threads on the bioreactor base **[1],** and place a hose clip on the medium tubing **[2]**.

4.1.1. WIDE: Talent placing flow culture chamber onto the screw thread of bioreactor base

4.1.2. Talent placing clip onto medium tubing (of already prepared medium-ibidi-set-up)

4.2. Remove the white Luer cap covering the flow inlet of the top compartment of the flow culture chamber **[1]** and remove the female Luer coupler of the medium tubing **[2]**.

4.2.1. Talent removing cap

4.2.2. Talent removing coupler

4.3. Connect the medium tubing with one side of the flow inlet on the top compartment **[1]** and the other side with the flow outlet at the bottom compartment **[2]**.

4.3.1. Talent connecting medium tubing to flow inlet

4.3.2. Talent connecting medium tubing to flow outlet

4.4. Transfer the complete setup from the laminar flow cabinet to the incubator **[1]** and connect the fluidic units and the bioreactor base to the air pressure tubing and electric cable **[2]**.

4.4.1. Talent placing setup into the incubator

4.4.2. Talent connecting units to tubing and cable

4.5. Start the software **[1]** and initialize the pumps **[2]**. Start the medium flow for the samples one by one **[3]**.

4.5.1. Talent starting software

4.5.2. Talent initializing pump(s)

4.5.3. Talent starting medium flow

4.6. Then change the strain pump parameters to the desired settings **[1]** and start the strain. Monitor the stretch applied to the scaffold every other day **[2]**.

4.6.1. Talent setting strain pump settings, with monitor visible in the frame

4.6.2. Strain pump starting

Results

5. Results: Representative Biomaterial Inflammatory and Regenerative Capacity Evaluation Under Multi-Cue Bioreactor-Induced Flow and Stretch Conditions

5.1. The monitoring of stretch **[1]** and wall shear stress over long-term culture periods **[2]** shows that these values can be maintained at relatively constant levels over a period of up to 20 days **[3]**.
- 5.1.1. LAB MEDIA: Figures 3C and 3D *Video Editor: please emphasize Figure 3C*
- 5.1.2. LAB MEDIA: Figures 3C and 3D *Video Editor: please emphasize Figure 3D*
- 5.1.3. LAB MEDIA: Figures 3C and 3D *Video Editor: please emphasize red line in both graphs*

5.2. Three days after seeding, immunofluorescent staining **[1]** reveals a homogenous distribution of monocyte-derived macrophages **[2]** and myofibroblasts throughout the scaffold **[3]**.
- 5.2.1. LAB MEDIA: Figure 4B
- 5.2.2. LAB MEDIA: Figure 4B *Video Editor: please emphasize green signal*
- 5.2.3. LAB MEDIA: Figure 4B *Video Editor: please emphasize red signal*

5.3. After 20 days of co-culture **[1]**, cyclic stretch results in the deposition of more numerous and thicker collagen type one fibers **[2]**, while in the combined hemodynamic load group, the cyclic stretch effect is overruled by shear stress, resulting in a less pronounced collagen type one deposition **[3]**.
- 5.3.1. LAB MEDIA: Figure 4C
- 5.3.2. LAB MEDIA: Figure 4C *Video Editor: please emphasize cyclic stretch image*
- 5.3.3. LAB MEDIA: Figure 4C *Video Editor: please emphasize combined image*

5.4. After 8 days of macrophage monoculture, fiber erosion and fiber cleavage are observed in all of the hemodynamic loading regimes **[1]**, with the most pronounced resorption observed in the static group **[2]** and the least pronounced resorption observed in the shear stress group **[3]**.
- 5.4.1. LAB MEDIA: Figure 4D
- 5.4.2. LAB MEDIA: Figure 4D *Video Editor: please emphasize static image*
- 5.4.3. LAB MEDIA: Figure 4D *Video Editor: please emphasize shear stress image*

5.5. Both cyclic stretch **[1]** and shear stress impact the cytokine secretion profile of the co-culture setup **[2]**.
- 5.5.1. LAB MEDIA: Figure 5A *Video Editor: please emphasize cyclic stretch rows*
- 5.5.2. LAB MEDIA: Figure 5A *Video Editor: please emphasize shear stress rows*

5.6. Interestingly, the combined effects of both loads show either dominance of one of the two loads **[1]** or synergistic effects of both loads **[2]**.
   5.6.1. LAB MEDIA: Figure 5B *Video Editor: please emphasize CS+ SS data boxes in IL-6 and MCP-1 graphs*
   5.6.2. LAB MEDIA: Figure 5B *Video Editor: please emphasize CS + SS data box in IL-10 graph*

5.7. Co-culture experiments also show that the mechanical environment and resulting loading-dependent inflammatory environments modulate the phenotype of the myofibroblasts **[1]**.
   5.7.1. LAB MEDIA: Figure 6A *Video Editor: please add/emphasize brackets and asterisks*

5.8. Furthermore, the gene expression patterns of contractile marker alpha smooth muscle actin correlate with protein synthesis **[1]**.
   5.8.1. LAB MEDIA: Figure 6B

Conclusion

6. Conclusion Interview Statements

6.1. **Suzanne Koch**: The most important thing to remember when performing this protocol is that for applying stretch, it is critical that the set-up is leak-free **[1]**.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B role: 2.8, 2.9, 2.10, 2.13, 2.18 and 4.1*