FINAL SCRIPT: APPROVED FOR FILMING

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Scriptwriter Name: Shehnaz Lokhandwala
Supervisor Name: Anastasia Gomez
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Title: Megakaryocyte Culture in 3D Methylcellulose-based Hydrogel to Improve Cell Maturation and Study the Impact of Stiffness and Confinement

Authors and Affiliations:
Julie Boscher, Christian Gachet, François Lanza, Catherine Léon

Université de Strasbourg, INSERM, EFS Grand Est, BPPS UMR-S 1255, FMTS

Corresponding Authors:
Catherine Léon (catherine.leon@efs.sante.fr)

Email Addresses for All Authors:
julie.boscher@efs.sante.fr
christian.gachet@efs.sante.fr
francois.lanza@efs.sante.fr
catherine.leon@efs.sante.fr

Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

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 videographer steps away (≥6 ft/2 m) and begins filming, then the interviewee removes the mask for line delivery only. When take is captured, 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? **No**

Current Protocol Length

Number of Steps: 16
Number of Shots: 35

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Julie Boscher:** This protocol allows for *in vitro* evaluation of the impact of confinement and medium stiffness, to which native megakaryocytes are exposed in the bone marrow, on their behavior and maturation.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested b-roll: 3.1.2.*

1.2. **Julie Boscher:** The main advantage of this technique is a simplified model, which eliminates cell-cell and cell-matrix interactions and focuses on the mechanical aspects.

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

OPTIONAL:

1.3. **Julie Boscher:** Good laboratory practice and sterile working conditions are to be strictly observed. Indeed, even slight contamination of the hydrogel can have a dramatic impact on megakaryocyte differentiation.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested b-roll: 2.9.1.*

Ethics Title Card

1.4. Procedures involving animal subjects have been approved by the Review Board of the Etablissement Français du Sang.

Protocol

2. Cell Embedding in Methylcellulose Hydrogel

2.1. To obtain a single well of hydrogel cell culture, begin by thawing two 1-milliliter aliquots of 3% methylcellulose at room temperature **[1]**. Then, coat a 1-milliliter Luer lock syringe with the methylcellulose by first drawing 1-milliliter **[2-TXT]** and then expelling all of it **[3]**.

2.1.1. WIDE: Establishing shot of talent at the biosafety cabinet, thawed 1 mL aliquots of methylcellulose in view

2.1.2. Talent drawing 1 mL of the methylcellulose. **TEXT: Use an 18G needle**

2.1.3. Talent expelling the methylcellulose from the syringe.

2.2. Next, using the same syringe and needle, draw 333 microliters of methylcellulose from a fresh aliquot **[1]**.

2.2.1. Talent drawing methylcellulose.

2.3. After cautiously removing the needle **[1]**, use sterilized forceps to screw a Luer lock connector onto the end of the syringe **[2]**, then attach a second, non-coated, 1-milliliter Luer lock syringe to the connector **[3]**. *Videographer: This step is important!*

2.3.1. Talent removing the needle.

2.3.2. Talent screwing the Luer lock connector onto the end of the syringe.

2.3.3. Talent attaching a second syringe to the connector.

2.4. Equally distribute the methylcellulose between the two syringes and keep them aside **[1]**.

2.4.1. Talent distributing the methylcellulose between the two syringes and placing them on the side.

2.5. Next, resuspend the previously isolated mouse lineage negative hematopoietic stem and progenitor cells in the concentrated culture medium at a density of 1×10^6 cells per 167 microliters **[1-TXT]**.

2.5.1. Talent resuspending the cell pellet. **TEXT: See text for all cell and media preparation details**

2.6. Disconnect one of the syringes from the connector **[1]** and pipette 167 microliters of the cell suspension directly into the connector **[2-TXT]** while simultaneously drawing the syringe plunger slowly to make room for the cell suspension **[3]**. *Videographer: This step is important!*

2.6.1. Talent disconnecting one of the syringes.

2.6.2. ECU: Talent pipetting the cell suspension into the connector. **TEXT: Avoid air bubbles**

2.6.3. ECU: Talent slowly drawing the syringe plunger.

2.7. After adding all of the cell suspension into the connector, draw the plunger further to withdraw the suspension from the connector **[1]** and carefully reconnect the second syringe, taking care not to lose any suspension in the screw thread **[2]**. *Videographer: This step is important!*

2.7.1. ECU: Talent drawing the plunger to withdraw cell suspension from the connector.

2.7.2. ECU: Talent reconnecting the second syringe.

2.8. To homogenize the methylcellulose medium with the cell suspension, slowly move the plungers back and forth between the two syringes 10 times **[1]**. Then, draw the total volume into one syringe **[2]** and disconnect the two syringes, leaving the connector on the empty one **[3]**. *Videographer: This step is important!*

2.8.1. Talent moving the plungers back and forth.

2.8.2. Talent drawing total volume into one syringe.

2.8.3. Talent disconnecting the two syringes.

2.9. Empty the contents of the syringe into one well of a 4-well plate **[1]** and incubate the plate at 37 degrees Celsius under 5% carbon dioxide **[2]**.

2.9.1. Talent emptying the syringe into one well of a 4-well plate.

2.9.2. Talent placing the plate in the incubator.

3. Cell Resuspension for Proplatelet Analysis

3.1. To analyze proplatelets, on day 3 of culture, carefully transfer all cells from one well into a 15-milliliter tube containing 10 milliliters of DMEM with 1% PSG *(P-S-G)* **[1-TXT]**. Resuspend the cells by gentle pipetting to completely dilute the methylcellulose **[2]**,

then centrifuge the tube for 5 minutes at 300 × *g*, at room temperature **[3]**.
Videographer: This step is important!

3.1.1. WIDE: Talent transferring cells to the 15 mL tube. **TEXT: DMEM prewarmed to 37 °C; PSG: Penicillin-Streptomycin-Glutamin mix**

3.1.2. Talent gently resuspending the cells.

3.1.3. Talent placing the tube in the centrifuge.

3.2. After discarding the supernatant **[1]**, resuspend the cell pellet in 1-milliliter of complete culture medium **[2]** and reseed the cells at 500 microliters per well of a 4-well plate **[3]**. Incubate the plate at 37 degrees Celsius under 5% carbon dioxide **[4]**.

3.2.1. Talent discarding the supernatant.

3.2.2. Talent resuspending the cell pellet.

3.2.3. Talent adding cells to the 4-well plate.

3.2.4. Talent placing the plate in an incubator.

3.3. On the following day, using a bright field microscope with a 20x objective **[1]**, randomly acquire 10 images per well, making sure not to have too many cells in the field-of-view and capturing at least 5 megakaryocytes per field **[2]**.

3.3.1. Talent placing the plate under the microscope.

3.3.2. Talent at the computer, taking images, monitor visible in frame.

3.4. Using Image J, count the total number of megakaryocytes and those extending proplatelets in each image to calculate the proportion of megakaryocytes extending proplatelets **[1]**.

3.4.1. Talent at the computer opening Image J and beginning to count cells.

4. Cell Fixation and Retrieval for Future Analyses

4.1. To fix the cells for future analyses, add the fixative solution on top of the methylcellulose without disrupting the gel **[1-TXT]**.

4.1.1. Talent adding the fixative solution onto the gel. **TEXT: Fixative solution volume = Seeded volume**

4.2. After waiting for an appropriate time depending on the fixative used, use a P1000 *(P-one-thousand)* pipette to gently pipette the fixative and gel until the methylcellulose has been homogenously diluted **[1]**.

4.2.1. Talent pipetting the fixative and the gel.

4.3. Then, using the same pipette tip, transfer all the contents of the well into a 15-milliliter tube containing 10 milliliters of DPBS and homogenize by mixing **[1]**. Centrifuge the mixture **[2-TXT]**, discard the supernatant **[3]**, and resuspend the megakaryocyte pellet in a medium appropriate for the desired analysis **[4]**.

4.3.1. Talent transferring the well contents into a 15 mL tube and mixing.

4.3.2. Talent placing the tube in the centrifuge. **TEXT: 300 × *g*, 7 min, RT**

4.3.3. Talent discarding the supernatant.

4.3.4. Talent resuspending pellet in the medium.

Results

5. Results: Megakaryocyte Characteristics and Proplatelet Quantification According to Culture Conditions

5.1. By day 3 of culture **[1]**, megakaryocytes in the liquid medium have sedimented at the bottom of the well **[2]** and are in contact with the stiff plastic surface as well as other cells **[3]**.

5.1.1. LAB MEDIA: Figure 3A.

5.1.2. LAB MEDIA: Figure 3A. *Video Editor: Emphasize the left panel*

5.1.3. LAB MEDIA: Figure 3A. *Video Editor: Emphasize some examples of cells in contact with each other in the left panel*

5.2. In contrast, cells embedded in the methylcellulose hydrogel **[1]** are homogeneously distributed and isolated from the neighboring cells **[2]**.

5.2.1. LAB MEDIA: Figure 3A. *Video Editor: Emphasize the right panel*

5.2.2. LAB MEDIA: Figure 3A. *Video Editor: Emphasize the well distributed and isolated cells*

5.3. An analysis of the mean diameter of megakaryocytes under different culture conditions **[1]** shows that 2% methylcellulose slightly increases the mean megakaryocyte diameter **[2]** compared to the liquid culture **[3]**.

5.3.1. LAB MEDIA: Figure 3B

5.3.2. LAB MEDIA: Figure 3B. *Video Editor: Emphasize the mean diameter line (middle blue line) for 2% values*

5.3.3. LAB MEDIA: Figure 3B. *Video Editor: Emphasize the mean diameter line (middle blue line) for Liq values*

5.4. However, increasing the methylcellulose concentration by 0.5% impairs megakaryocyte differentiation, as indicated by a smaller mean diameter **[1]**.

5.4.1. LAB MEDIA: Figure 3B. *Video Editor: Emphasize the mean diameter line (middle blue line) for 2.5% values*

5.5. In representative transmission electron microscopy images **[1]**, the intracytoplasmic membranes in megakaryocytes differentiated *in vivo* within the bone marrow appear to be closely apposed with delineated cytoplasmic territories **[2]**.

5.5.1. LAB MEDIA: Figure 3C.

5.5.2. LAB MEDIA: Figure 3C. *Video Editor: Emphasize the middle and right-most panels in the upper row*

5.6. In liquid culture, the membranes mostly have a small round, oval appearance or elongated vesicles without delimitation of cytoplasmic territories **[1]**. In contrast, the 2% methylcellulose culture has closely apposed membranes with delimiting cytoplasmic territories **[2]**, resembling the *in situ* structure **[3]**.

5.6.1. LAB MEDIA: Figure 3C. *Video Editor: Emphasize the middle and right-most panels in the middle row*

5.6.2. LAB MEDIA: Figure 3C. *Video Editor: Emphasize the middle and right-most panels in the bottom row*

5.6.3. LAB MEDIA: Figure 3C. *Video Editor: Emphasize the middle and right-most panels in the upper and bottom row*

5.7. By day 4 of culture **[1]**, megakaryocytes previously cultured in hydrogel show increased proplatelet formation **[2]** compared to those cultured in liquid medium **[3]**.

5.7.1. LAB MEDIA: Figure 4A.

5.7.2. LAB MEDIA: Figure 4A. *Video Editor: Emphasize the proplatelets pointed to by black arrows in the right panel*

5.7.3. LAB MEDIA: Figure 4A. *Video Editor: Emphasize the proplatelet pointed to by the single black arrow in the left panel*

5.8. The mean proportion of megakaryocytes **[1]** extending proplatelets is 16% for the liquid pre-culture **[2]** compared to 39% for the methylcellulose hydrogel pre-culture **[3]**.

5.8.1. LAB MEDIA: Figure 4B.

5.8.2. LAB MEDIA: Figure 4B. *Video Editor: Emphasize the Liquid bar*

5.8.3. LAB MEDIA: Figure 4B. *Video Editor: Emphasize the Hydrogel bar*

Conclusion

6. Conclusion Interview Statements

6.1. **Julie Boscher:** When attempting this procedure, it is important to pipet the appropriate volumes very precisely as a minor change in the final methylcellulose concentration can significantly modify the media stiffness.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested b-roll: 2.2.1, 2.6.2.*

6.2. **Julie Boscher:** Following cell maturation in the hydrogel, megakaryocytes can be recovered for ploidy and cell marker analysis by flow cytometry or fixed for electron microscopy or immunostaining of proteins of interest.

6.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested b-roll: 3.1.1, 4.1.1.*