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TITLE:

Megakaryocyte culture in 3D methylcellulose-based hydrogel to improve cell maturation and study the impact of stiffness and confinement.

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KEYWORDS

Megakaryocyte culture, 3D culture, Methylcellulose, Thrombopoiesis, Proplatelet

SUMMARY:

It is now acknowledged that the three-dimensional environment of cells can play an important role in their behavior, maturation and/or differentiation. This protocol describes a three-dimensional cell culture model designed to study the impact of physical containment and mechanical constraints on megakaryocytes.

ABSTRACT:

The 3D environment leading to both confinement and mechanical constraints is increasingly recognized as an important determinant of cell behavior. 3D culture has thus been developed to better approach the *in vivo* situation. Megakaryocytes differentiate from hematopoietic stem and progenitor cells (HSPCs) in the bone marrow (BM). The BM is one of the softest tissues of the body, confined inside the bone. The bone being poorly extensible at the cell scale, megakaryocytes are concomitantly subjected to a weak stiffness and high confinement. This protocol presents a method for the recovery of mouse lineage negative (Lin-) HSPCs by immuno-magnetic sorting and their differentiation into mature megakaryocytes in a 3D medium composed of methylcellulose. Methylcellulose is non-reactive towards megakaryocytes and its stiffness may be adjusted to that of normal bone marrow or increased to mimic a pathological fibrotic marrow. The process to recover the megakaryocytes for further cell analyses is also detailed in the protocol. Although proplatelet extension is prevented within the 3D milieu, it is described below how to resuspend the megakaryocytes in liquid medium and to quantify their capacity to extend proplatelets. Megakaryocytes grown in 3D hydrogel have a higher capacity to form proplatelets compared to those grown in a liquid milieu. This 3D culture allows i) to differentiate progenitors towards megakaryocytes reaching a higher maturation state, ii) to recapitulate phenotypes that may be observed *in vivo* but go unnoticed in classical liquid cultures,

and iii) to study transduction pathways induced by the mechanical cues provided by a 3D environment.

INTRODUCTION:

Cells in the body experience a complex 3D microenvironment and are subjected to the interplay between chemical and mechanophysical cues including stiffness from the tissue and confinement due to neighboring cells and surrounding matrix [1–3]. The importance of stiffness and confinement for cell behavior has only been recognized in the last decades. In 2006, the seminal work from Engler et al. [4] highlighted the importance of the mechanical environment for cell differentiation. The authors demonstrated that variation in cell substrate stiffness resulted in the orientation of stem cells towards various differentiation lineages. Since then, the impact of mechanical cues on cell fate and behavior has become increasingly recognized and studied. Despite it being one of the softest tissues of the organism, the bone marrow has a 3D structural organization that is confined inside the bone. Marrow stiffness, although technically difficult to measure precisely, is estimated to lie between 15 and 300 Pa [5, 6]. Within the stroma, cells are tightly confined to one another. In addition, most of them are migrating toward the sinusoid vessels to enter the blood circulation. These conditions create additional mechanical constraints on adjacent cells, which have to adapt to these forces. Mechanical cues represent an important parameter whose consequences on megakaryocyte differentiation and proplatelet formation have just recently been explored. Although megakaryocytes can differentiate *in vitro* in traditional liquid culture, they do not reach the degree of maturation observed *in vivo*, in part due to the absence of the mechanical cues from the 3D environment [7]. Growing progenitors embedded in hydrogel brings 3D mechanical cues that are lacking in liquid milieu.

Hydrogels have been widely used for several decades in the hematological field, notably to grow cells in colony forming assays to quantify hematopoietic progenitors. However, such hydrogels have seldom been used to explore the biological impact of the 3D mechanical environment on maturation and differentiation of hematopoietic cells. Over the past few years our laboratory has developed a 3D culture model using a methylcellulose-based hydrogel [8]. This nonreactive physical gel is a useful tool to mimic the physical constraints of the native megakaryocyte environment. It is derived from cellulose by replacement of hydroxyl residues (-OH) by methoxide groups ($-OCH_3$). Both the degree of methyl substitution and the methylcellulose concentration determine the hydrogel stiffness once it has jellified. During the development stage of this technique, it was demonstrated that a Young's modulus in the range of 30 to 60 Pa is the optimal gel stiffness for megakaryocyte growth [9].

The following protocol describes a method to grow mouse megakaryocytic progenitors in a 3D methylcellulose hydrogel. It has been previously shown that compared with standard liquid culture, this hydrogel culture increases the degree of megakaryocyte polyploidization, improves the maturation and intracellular organization, and increases the capacity of megakaryocytes to extend proplatelets once resuspended in a liquid medium [9]. This manuscript describes in detail the protocol for the isolation of mouse bone marrow Lin− cells and their embedding in a methylcellulose hydrogel for 3D culture as well as the quantification of their capacity to produce proplatelets and the recovery of the cells for further analyses.

PROTOCOL:

All experiments should be performed in compliance with institutional guidelines for the care and use of laboratory animals. All protocols displayed in the video were carried out in strict accordance with the European law and the recommendations of the Review Board of the Etablissement Français du Sang (EFS). A first version of this protocol was originally published in 2018 in Methods in Molecular Biology [8].

NOTE: **Figure 1** presents a schematic view of the whole process. This process includes 1) bone dissection, marrow retrieval, and mechanical isolation of marrow cells, 2) magnetic sorting of lineage negative (Lin-) cells, 3) seeding in liquid or methylcellulose hydrogel, and 4) resuspension of megakaryocytes grown in 3D gel for examination of proplatelet formation in liquid medium.

1. Bone collection from adult mice

NOTE: In this section, it is important to minimize microbial contamination.

1.1. Prepare a 15 mL tube for bone collection with Dulbecco's Modified Eagle's Medium (DMEM) containing 1% of the total volume of penicillin-streptomycin-glutamin (PSG) antibiotic mix (penicillin 10000 U/mL, streptomycin 100000 µg/mL and L-glutamin 29.2 mg/mL).

1.1.1. If all the mice used have the same genotype, pool all bones in the same tube containing 1 mL of DMEM – PSG 1% per number of mice.

NOTE: Antibiotics are important to prevent possible bacteria proliferation during the time of bone sampling.

1.2. Fill a 50 mL tube with ethanol 70% for bone disinfection and another one for rinsing instruments during the procedure. Use sterilized dissection instruments.

1.3. Anesthetize the mice using isoflurane inhalation (4%) and rapidly proceed to cervical dislocation to euthanize the mice. Rapidly immerse the body in 70% ethanol to disinfect and avoid microbial contamination.

1.4. Rapidly dissect out the tibias and femurs.

1.5. Using a scalpel, cut away the epiphyses of the ankle side end for the tibia and of the hip side end for the femur.

1.6. Immerse the bones for one second in 70% ethanol before immersing them in DMEM medium containing 1% PSG.

2. Marrow dissociation and Lin- cells isolation

NOTE: This part of the protocol is performed under a laminar flow hood. For one culture, all the wells are part of the same experiment and cannot be considered as independent biological replicates. The cells from all mice are pooled together to ensure the homogeneity of all the wells and to be able to compare them to each other while eliminating possible inter-individual variability. For independent biological replicates, the culture must be repeated.

2.1. Place the bones in a Petri dish and rise them twice in sterile Dulbecco's phosphate-buffered saline (DPBS) to remove potential contaminants.

2.2. Prepare DMEM – 1% PSG in a 50 mL tube.

NOTE: Provide 2 mL of DMEM – 1% PSG per mice used for the experiment.

2.3. Fill a 5 mL syringe equipped with a 21-gauge needle with DMEM – 1% PSG.

2.4. Holding the bone with forceps, introduce only the bevel of the needle at the knee side end.

NOTE: The knee side epiphysis should remain intact from the dissection, leaving a small cavity in its center through which to insert the needle. The remaining epiphysis will maintain the bone attached to the needle during flushing. Be careful not to introduce more than the bevel in the bone as it might squash and damage the marrow.

2.5. Quickly press the syringe plunger to flush the marrow out into a 50 mL tube.

2.5.1. To avoid splashes and facilitates the marrow flush and liberation place the free end of the bone on the tube wall, immersed in DMEM – 1% PSG.

NOTE: In practice, a volume between 500 µL and 1 mL is generally sufficient to expel the marrow from the bone. When the marrow has been totally expelled, the bone has become white. In case the marrow has not been totally expelled from the diaphysis as judged by some remaining red color, it is possible to repeat the flush with fresh medium.

2.6. Repeat steps 2.4. and 2.5. for all bones, refilling the 5 mL syringe with DMEM – 1% PSG if necessary.

2.7. Use the same 5 mL syringe with the 21-gauge needle to transfer the total volume of medium containing flushed marrow into round bottomed 10 mL tubes.

NOTE: It is not absolutely necessary to switch to a round bottomed 10 mL tube but it makes it easier to proceed to the following dissociation steps. Do not hesitate to change syringe and/or needle if risk of contamination is suspected.

2.8. Proceed to cell dissociation by aspirating and expelling the medium and marrow cells successively two times through a 21-gauge needle, three times through a 23-gauge and once through a 25-gauge needle.

NOTE: Avoid air bubbles as it may be detrimental for the cells.

2.9. Transfer the suspension into 15 mL tubes.

2.10. Measure cell number and check the viability using an automated cell counter or a cell chamber for manual counting in the presence of trypan blue to exclude dead cells.

2.11. Centrifuge the 15 mL tubes for 7 min at 300 x *g*. Using a 1 mL transfer pipette, carefully pipette out and discard the supernatant.

2.12. Isolate stem and progenitor cells by negative immunomagnetic sorting using a mouse hematopoietic cell isolation kit.

NOTE: The aim of this cell sorting is to retrieve the cells that are negative for all the selection antibodies (CD5, CD11b, CD19, CD45R/B220, Ly6G/C(Gr-1), TER119, 7–4) and therefore to eliminate the cells that are already engaged in a differentiation lineage other than the megakaryocytic one.

2.13. Following the kit instructions, resuspend the cellular pellet in freshly prepared M medium (PBS with 2% of the final volume of fetal bovine serum (FBS), EDTA 1 mM) to a concentration of 1×10^8 cells/mL and distribute the suspension in round bottomed 5 mL polystyrene tubes to a maximum volume of 2 mL.

2.14. Add to the polystyrene tubes: normal rat serum at a concentration of 50 µL/mL as well as the biotinylated antibody mix (CD5, CD11b, CD19, CD45R/B220, Ly6G/C(Gr-1), TER119, 7–4) at a concentration of 50 µL of mix per mL and homogenize by gently flicking the tubes.

NOTE: These antibodies will bind to cells already engaged into a differentiation pathway except the megakaryocytic pathway.

2.15. Incubate the tubes on ice for 15 min.

2.16. Add streptavidin-coated magnetic beads at a concentration of 75 µL/mL and homogenize by gently flicking the tubes.

2.17. Incubate again on ice for 10 min.

2.18. If necessary, adjust to a final volume of 2.5 mL per tube with M medium.

2.19. Homogenize the suspension by gently flicking the tube just before placing them, without

their caps, inside a magnet and wait for three minutes.

NOTE: The cells already engaged into a differentiation pathway and coated with magnetic beads will be retained on the wall of the tube inside the magnet.

2.20. Invert magnet and tube to transfer the tube content into a new round-bottomed 5 mL polystyrene tube.

2.20.1. Do not take the tube out of the magnet for the transfer; it is done by inverting the magnet with the tube still in. Use a steady movement and do not shake the tube.

2.21. Discard the initial tube containing undesired magnetic-labeled cells and place the new one, without its cap, in the magnet for three more minutes.

2.22. Proceeding as in step 2.20, transfer the isolated Lin− cells into a new 15 mL tube.

2.22.1. If several 5 mL polystyrene tubes have been used for the previous steps, pool all the cells in the same 15 mL tube.

NOTE: The cells recovered after the cell sorting are hematopoietic stem cells and progenitors. The presence of thrombopoietin (TPO), the major physiological regulator of megakaryopoiesis [10], will direct the cell differentiation toward the megakaryocytic cell line.

2.23. Measure the Lin− cell number and viability as in step 2.10.

2.24. Calculate the required volume of cell suspension to centrifuge in order to have 1 x 10^6 viable cells x Well Number, Well Number being the number of wells to seed per condition.

2.25. Prepare one tube per condition with the appropriate volume of cell suspension and centrifuge at 300 x *g* for 7 min.

2.26. For liquid cultures, discard the supernatant and resuspend the cell pellet in complete culture medium (DMEM, PSG 1% of the final volume, FBS 10% of the final volume, hirudin 100 U/mL, TPO 50 ng/mL) to achieve the final concentration of 2 × 10^6 viable cells/mL (equal to 1 × 10^6 cells per 500 µL well). Incubate the cells at 37 °C under 5% CO2. (= day 0 of culture)

NOTE: See the next paragraph for methylcellulose cultures As an example, to prepare complete culture medium for one well, use 435 µL of DMEM, 50 µL of 100% FBS for 10% final, 5 µL of 100% PSG for 1% final, 5 µL of 10 000 U/mL for 100 U/mL final and 5 µL of 5 µg/mL TPO for 50 ng/mL final. 4-well or 24-well culture plates are typically used as their well diameter is a good fit for the 500 µL needed per well.

3. Cell embedding in methylcellulose hydrogel

NOTE: Please note that the following protocol describes the method to obtain a single well of hydrogel cell culture, adapt to the number of wells needed.

3.1. Thaw 1 mL aliquots of 3% methylcellulose stock solution at room temperature.

NOTE: At a concentration of 3%, methylcellulose remains liquid at room temperature (20-25 °C).

3.1.1. Prepare one separate extra aliquot of methylcellulose for syringe coating.

3.2. Coat a 1 mL Luer lock syringe equipped with an 18-gauge needle with methylcellulose by drawing 1 mL of methylcellulose from the extra aliquot. Totally expel the methylcellulose.

NOTE: This coating step ensures that the volume of methylcellulose collected in step 3.3 is exact.

3.3. With the same syringe and needle but using a new methylcellulose aliquot, draw the appropriate volume of methylcellulose (**Figure 2A**).

NOTE: To achieve a final concentration of 2% methylcellulose in a final volume of 500 µL per well, 333 µL of 3% methylcellulose is required.

3.4. Cautiously remove the needle. Using sterilized forceps, screw a Luer lock connector onto the end of the syringe (**Figure 2B-C**).

3.5. Attach a second, non-coated, 1 mL Luer lock syringe to the Luer lock connector in order to connect the two syringes together (**Figure 2D**).

NOTE: There is no need to coat this second syringe.

3.6. Equally distribute the methylcellulose volume between the two syringes (**Figure 2E**) and put them aside until step 3.11.

3.7. Prepare the concentrated DMEM culture medium so as to obtain in the final methylcellulose volume (step 3.11) a concentration identical to the one of the liquid culture medium for each compound (PSG 1% of the final volume, FBS 10% of the final volume, hirudin 100 U/mL, TPO 50 ng/mL).

3.7.1. Prepare 167 µL of concentrated culture medium per final well of 1×10^6 cells. This volume of medium is calculated so as to obtain a final methylcellulose concentration of 2%. The total volume in the well will be 500 µL (167 µL of cell suspension in concentrated culture medium + 333 µL of methylcellulose) and all the components will have a concentration identical to that in liquid wells.

3.7.2. As an example, to prepare complete culture medium for one well, use 102 µL of DMEM, 50 µL of 100% FBS for 10% final, 5 µL of 100% PSG for 1% final, 5 µL of 10 000 U/mL for 100 U/mL

final and 5 µL of 5 µg/mL TPO for 50 ng/mL final. It gives a volume of 167 µL used to resuspend the cells and with the addition of 333 µL of methylcellulose the final volume will be 500 µL.

3.8. After completing the centrifugation step 2.26, discard the supernatant and resuspend the cell pellet in the concentrated culture medium at a ratio of 1×10^6 cells per 167 µL.

3.9. Take back the syringes and disconnect one of them from the connector.

3.10. Pipette 167 µL of the cell suspension.

3.11. Add the cell suspension directly into the syringe connector (**Figure 2F**), making sure not to introduce air bubbles.

3.11.1. While adding the cell suspension, slowly draw the syringe plunger simultaneously to free some space for the cell suspension.

3.12. Carefully reconnect the two syringes (**Figure 2G**) without losing any suspension in the screw thread.

NOTE: Prior to the reconnection, draw the plunger in order to leave the connector half empty and let enough space for the second syringe to connect without the suspension overflowing.

3.13. Slowly homogenize the methylcellulose medium with the cell suspension with ten back-and-forth plunger movements between the two syringes (**Figure 2H**).

3.14. Draw the total volume into one syringe and disconnect the two syringes, leaving the connector on the empty one.

3.15. Empty the content of the syringe into a well of a 4-well plate (**Figure 2I**).

3.16. Incubate the cells at 37 °C under 5% CO_2 (= day 0 of culture).

3.16.1. It is possible to prepare two methylcellulose wells with one pair of syringes. Increase by two the volume of methylcellulose and the volume of cell suspension to have 2 $x10^6$ cells.

3.16.2. After completing step 3.13. distribute the volume equally between the two syringes to have 500 µL in each of them. Disconnect them and empty the one without the connector in a culture well.

3.16.3. Reconnect the syringes to transfer the volume from the one that kept the connector to the other. Disconnect the syringes, the one with the 500 µL should not have the connector attached, and seed the cells in a second culture well.

NOTE: The 3% methylcellulose is purchased as a stock solution in Iscove's Modified Dulbecco's

Medium (IMDM) while concentrated cells are suspended in DMEM. Comparative tests have been initially done to make sure that this mixed medium had no impact on the outcome of the experiment, especially compared to the liquid culture in 100% DMEM.

4. Cell Resuspension for Proplatelet Analysis

NOTE: Analysis of the capacity to form proplatelets has to be performed under comparable conditions between liquid and methylcellulose grown megakaryocytes. The physical constraints exerted by the methylcellulose hydrogel inhibit proplatelet extension. Therefore, methylcellulose-grown cells are resuspended in fresh liquid medium on day 3 of culture to allow them to extend proplatelets. Methylcellulose hydrogel is a physical hydrogel that is easily diluted upon liquid medium addition. Importantly, to avoid artifacts from resuspension and centrifugation, cells in the control liquid medium condition have to be treated simultaneously in the same way as methylcellulose-grown cells. Refer to the schematic representation of the experiment (**Figure 1**).

4.1. Prepare 10 mL of DMEM – 1% PSG preheated at 37 °C in a 15 mL tube for each well to resuspend.

4.2. Cautiously resuspend the cells from each well in the 10 mL of DMEM – 1% PSG.

NOTE: Gently do several up-and-down movements to dilute the methylcellulose completely. For the liquid wells make sure to collect all the cells deposited at the bottom of the well.

4.3. Centrifuge the tubes 5 min at 300 × *g*.

4.4. Meanwhile prepare complete culture medium (DMEM, PSG 1% of the final volume, FBS 10% of the final volume, hirudin 100 U/mL, TPO 50 ng/mL).

NOTE: At this step each well is retrieved to be diluted by half, therefore prepare 1 mL of complete culture medium per well.

4.5. Discard the supernatant and resuspend the cell pellet in 1 mL of culture medium for each tube.

4.6. Reseed 500 µL of cell suspension per well in a 4 or 24-well plate and incubate at 37 °C under 5% CO_2.

NOTE: From one initial well, obtain 2 wells for proplatelet visualization in duplicate. Please note that as these duplicates originate from the same sample they cannot be considered as independent replicates.

4.7. 24 hours after reseeding, on day 4 of culture, randomly acquire 10 images per well using bright field microscopy and the 20× objective.

NOTE: The cells tend to group at the center of the well, make sure not to have too many cells on the field as it may render proplatelet visualization and quantification difficult. Make sure to capture at least 5 megakaryocytes per field.

4.8. Count the total number of megakaryocytes and of megakaryocytes extending proplatelets in each image and calculate the proportion of megakaryocytes extending proplatelets.

4.8.1. The quantification is not automated; perform cell counting manually. Counting can be facilitated by the use of the cell counter plugin of ImageJ to click on the cells in order to mark them as they are counted. 10 fields acquired per well and wells in duplicate represent approximately 150–300 megakaryocytes per condition.

5. Cell fixation and retrieval for future analyses

CAUTION: This protocol uses fixatives which must be handled under a fume hood, wearing protective equipment.

NOTE: The aim is to maintain intact the gel constraints applied on the cells until they are fully fixed. Therefore, and regardless of the fixative used, it must be added in the well on top of the methylcellulose, without disturbing the gel. The same protocol is applied to liquid cultures.

5.1. Add a volume of fixative solution equal to the seeded volume (500 µL in this protocol), on top of the methylcellulose without disrupting the gel. Wait for the appropriate time according to the fixative used (at least 10 min).

NOTE: The fixative diffusion throughout the gel should be very rapid as revealed by a rapid change in the gel color (from pink to a yellow-orange shade). Paraformaldehyde (8% in DPBS, 500 µL per well) is usually used for immunolabeling, while glutaraldhehyde (5% in cacodylate buffer, 500 µL per well) is used for electron microscopy analysis.

5.2. Using a P1000 pipette gently do several up-and-down pipettings with the fixative and the gel so as to homogeneously dilute the methylcellulose.

5.3. Using the same pipette and tip, transfer all the volume from the well into a 15 mL tube containing 10 mL of DPBS and homogenize.

5.4. Centrifuge the mixture at 300 × *g* for 7 min.

NOTE: A second wash step might be needed to eliminate all the methylcellulose

5.5. Discard the supernatant and resuspend the megakaryocyte pellet in the appropriate medium according to the desired analysis (immunolabeling, flow cytometry[9], electron microscopy …) (for electron microscopy see also the paper method “In situ exploration of the

major steps of megakaryopoiesis using transmission electron microscopy" in this JoVE issue).

REPRESENTATIVE RESULTS:

Data obtained using this protocol were originally published in Blood in 2016[9].

According to the protocol, the cells were seeded in either liquid or methylcellulose hydrogel medium. Cells in liquid medium have all sedimented at the bottom of the well, in contact with the stiff plastic surface and sometime with other cells. In contrast, cells embedded in methylcellulose hydrogel are distributed homogeneously in the gel and are isolated from neighboring cells (**Figure 3A**). Methylcellulose gel at a final concentration of 2% very slightly increases the mean megakaryocyte diameter compared to the liquid culture (**Figure 3B**), in accordance with the higher reported ploidy[9]. By contrast, increasing methylcellulose concentration by 0.5% impairs megakaryocyte differentiation as shown by a smaller mean diameter (**Figure 3B**).

A noticeable difference in megakaryocyte ultrastructure is observed between megakaryocytes differentiated in liquid culture and those differentiated *in vivo* within the bone marrow. A characteristic feature of mature megakaryocytes is a complex intracytoplasmic membrane network, the DMS (Demarcation Membrane System) which serves as a reservoir for the membrane of the future platelets. In mature megakaryocytes the DMS organizes to form intertwined membrane sheets which occupy most of the cytoplasm. By transmission electron microscopy (TEM), they appear to be closely apposed and delineate cytoplasmic territories (**Figure 3C** upper panel) (for the TEM procedure, see the paper method "*In situ* exploration of the major steps of megakaryopoiesis using transmission electron microscopy" in this same JOVE Issue). In liquid culture, DMS membranes have mostly the appearance of small round, oval, or elongated vesicles without delimitation of cytoplasmic territories (**Figure 3C** middle panel). By contrast, 2% methylcellulose culture promotes the organization of the DMS in a majority of megakaryocytes, with membranes closely apposed and delimiting cytoplasmic territories, resembling the one *in situ* (**Figure 3C** lower panel). This result indicates that the 2% methylcellulose hydrogel culture allows for better megakaryocyte differentiation due to the mechanical constraints of the environmental medium.

After cell transfer into liquid medium at day 3, megakaryocytes begin to extend proplatelets after 4 h [9]. **Figure 4** shows the quantification of the proportion of megakaryocytes having extended proplatelets 24 h after resuspension in liquid milieu. Ten images were randomly acquired per well, using bright field microscopy and the 20× objective (**Figure 4A**). The quantification was performed blindly and manually using the cell counter plugin on Fiji (ImageJ) (**Figure 4B**). Because these are primary cell cultures, there is an inter-experiment variability but the protocol remains robust and offers a good reproducibility. In the liquid pre-culture condition, the proplatelet proportion should be between 10% and 20% whereas this proportion is doubled for the hydrogel pre-culture.

FIGURE AND TABLE LEGENDS:

Figure 1. Schematic representation of the whole process. Bones are dissected out, marrow is

flushed out and cells mechanically dissociated. Stem and progenitor cells of interest (Lin- cells) are isolated by an immunomagnetic negative sorting procedure and seeded in either liquid or hydrogel medium (day 0). At day 3 of culture (which in total represents a duration of 4 days), both conditions are resuspended in separate fresh liquid culture milieu. This second culturing step is carried out from day 3 until day 4 of culture. The proportion of MKs extending proplatelets is measured at day 4 of culture. For visual clarity, one cell is schematized per well. The blue circle is depicting a single cell with its nucleus in purple. In the final step, both MKs are represented with proplatelet. The proportion of MKs forming proplatelets varies depending on liquid or methylcellulose pre-culture.

Figure 2. Cell embedding in methylcellulose hydrogel. After pre coating the syringes wall, (**A**) draw the appropriate volume of methylcellulose; (**B**, **C**) disconnect the needle and screw a connector onto the syringe; (**D**) push the methylcellulose halfway through the connector and attach a second syringe; (**E**) distribute equally the methylcellulose between the two syringes and disconnect them; (**F**) add the cell suspension to the syringe bearing the connector; (**G**) reconnect the two syringes; (**H**) homogenize by pushing the whole volume from one syringe to the other a few times; (**I**) seed the cells by expelling the whole volume into a culture dish.

Figure 3. Megakaryocyte characteristics according to culture condition. (**A**) Representative images of megakaryocytes at day 3 of culture in liquid (left panel) or 2% methylcellulose hydrogel medium (right panel). Scale bar = 50 µm (**B**) Mean diameter of megakaryocytes grown in liquid medium, or in 2% or 2.5% methylcellulose hydrogel. Results are expressed as the mean ± SD in 3 independent cultures, with a total of at least 100 megakaryocytes examined. *, $P<0.05$, ***$P < 0.0001$, using 1-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test. (graph adapted from Aguilar et al. 2016) (**C**) Schematic view (left) and representative electronic microscopy images (middle) of murine megakaryocytes; right panels, close up views from the white squares (scale bar = 5 µm for the middle electronic microscopy images and 2 µm for close up views). Upper panels are *in situ* megakaryocyte, middle represents megakaryocytes grown *in vitro* in liquid culture and lower panels are megakaryocytes grown in 3D methylcellulose hydrogel. These data were originally published in Blood Journal, DOI10.1007/978-1-4939-8585-2_9[5].

Figure 4. Representative results of proplatelet quantification. (**A**) representative images of megakaryocytes at day 4 of culture. Cells were incubated three days in liquid (**left**) or 2% methylcellulose hydrogel medium (**right**) followed by one day of resuspension in liquid medium. Black arrows indicate megakaryocytes extending proplatelets. (Scale bar = 50 µm). (**B**) Representative quantification data of proplatelet formation. Proplatelet formation quantified at day 4 for megakaryocytes previously pre-cultured from day 0 to day 3 in liquid or 2% methylcellulose hydrogel medium. Results are expressed as the% of megakaryocytes extending proplatelets (mean ± SD) and are from 3 independent experiments, with a total number of megakaryocytes examined per condition >750 (t-test, p = 0.0023). The mean proportion of megakaryocytes extending proplatelets is 16% in liquid condition and 39% for the methylcellulose hydrogel pre-culture. This result corresponds to the previously demonstrated and published effect of hydrogel pre-culture that increases proplatelet formation compared to

liquid condition.

DISCUSSION:

In the previous decade, mechanobiology has raised more and more interest in many areas of biology. It is now commonly acknowledged that the mechanical environment surrounding the cells does play a role in their behavior, emphasizing the importance to study how megakaryocytes sense and respond to extracellular mechanical cues. It is challenging to accurately measure the stiffness of the bone marrow tissue *in situ* [11], especially if we consider the hematopoietic red marrow as it is located inside trabecular bones in large mammals while the more easily accessible marrow from the diaphysis is composed essentially of adipocytes (yellow marrow) [12]. In the case of an isolated marrow from mice, where diaphysis contains essentially red marrow, another issue is that, once extracted from the bone, the tissue does not remain cohesive. However, Shin and collaborators managed to measure mouse diaphysis marrow stiffness using atomic force microscopy and found a value of E_{marrow} = 0.3 ± 0.1 kPa, which places the marrow among the softest tissues [6].

The interest of the procedure described here is to compare megakaryocyte behavior in liquid medium to that in the hydrogel. In liquid milieu, cells have all sedimented at the bottom of the well, in contact with the stiff plastic surface and sometime with other cells. In contrast, cells embedded in methylcellulose hydrogel are distributed homogeneously in the gel and are fully isolated from the other cells (**Figure 3A**). Hence they are submitted essentially to mechanical cues provided by the confinement, excluding juxtacrine communication. Paracrine stimulation cannot be totally excluded. Nonetheless, the cells embedded in the methylcellulose hydrogel are distant from one another contrary to the situation in the bone marrow and we can thus assume that if secreted substances reach neighboring cells, they might be very diluted.

The method is easy to set up and does not require specific skills. Methylcellulose is a physical gel whose polymer chains form non-covalent cross-linkages. Being liquid at low temperature, it jellifies when increasing the temperature (please see the article from Aguilar et al. 2016 [9] for more information about the characterization of the mechanical properties of the gel). This gel state can be easily reversed following dilution in aqueous solution, which enables an easy recovery of the cells, whether fixed in gel or as live cells.

A critical factor here is the stiffness of the hydrogel. The appropriate methylcellulose volume should be very precisely dispensed as even a small change in the hydrogel concentration can have an important impact on the stiffness of the milieu and therefore on megakaryocyte maturation. For instance, it was previously shown that increasing methylcellulose concentration from 2 to 2.5% increased gel stiffness (Young's modulus) by 10 fold. One possible pitfall is that there is no easy quality control to verify precise rheological properties of the methylcellulose in each experimental well once it has been seeded with cells. Nonetheless, an essential criterion that will reassure about a correct gel concentration is the proper maturation of the megakaryocytes within the hydrogel, as reflected by their large size roughly similar to that in liquid medium. A decrease in their mean diameter could reflect a defective differentiation similar to what occurs when increasing stiffness with 2.5% methylcellulose (**Figure 3B**).

Another limitation of the method is that cell recovery from the hydrogel takes more time than in the classical culture as it is necessary to first dilute the gel before centrifugation. If methylcellulose needs to be totally removed, for instance to obtain cell lysate for further western blot or RNA isolation procedure, an additional washing step may be required, during which time modifications may occur in proteins or RNA (protein dephosphorylation, RNA degradation…).

A critical point to consider in the procedure is the cell count that has to be equal in each conditions. This is not that trivial since in the liquid culture, cells tend to sediment at the bottom of the well and some of them adhere on the plastic surface, which is not the case for cells in suspension in hydrogel. One pitfall is an incomplete collection of the cells in the liquid condition, resulting in a different cell content between "liquid" and "hydrogel" condition after suspension in liquid medium at day 3. Such a difference may lead to discrepancies in the final data. As a checkpoint, a cell numeration can be done at this stage before reseeding the cells. It is preferable to do it manually using a Nageotte hemocytometer as it is especially appropriate for larger cells such as megakaryocytes.

As for any primary cell culture, there is a possible risk of contamination. Contamination is the most probable explanation to an unusually low proplatelet proportion in methylcellulose pre-culture condition, as a small contamination appears more difficult to detect than in liquid medium. Therefore, it can go unnoticed until proplatelet quantification, leading to misleading results. Good laboratory practices must be strictly observed especially during methylcellulose cell encapsulation that requires numerous and precise manipulations of syringes and connectors. The megakaryocyte viability should also be checked with Trypan blue using a Nageotte cell chamber for manual counting before reseeding at day 3.

Overall, the protocol provided here describes an *in vitro* model for comparison between classical liquid culture and a 3D culture using methylcellulose hydrogel. Of note, this culture protocol is described for mouse primary Lin^- cells and has not yet been adapted to human cells. This 3D model is a useful tool to investigate the impact of the mechanical environment on megakaryocyte behavior and maturation [9]. It is also possible to add compounds in the culture (even on the gel) to study the influence of drugs on megakaryocyte behavior/maturation and proplatelet formation. Finally, by reproducing the mechanical constraints that cells may encounter in the bone marrow, this culture system allows for the investigation of abnormal phenotypes that could not be observed in classical liquid cultures as previously showed for *Myh9* knockout megakaryocytes[9, 13, 14].

ACKNOWLEDGMENTS:

The authors would like to thank Fabien Pertuy and Alicia Aguilar who initially developed this technique in the lab, as well as Dominique Collin (Institut Charles Sadron - Strasbourg) who characterized the viscoelastic properties of the methylcellulose hydrogel. This work was supported by ARMESA (Association de Recherche et Développement en Médecine et Santé Publique) and by an ARN grant (ANR-18-CE14-0037 PlatForMechanics). Julie Boscher is a recipient from the Fondation pour la Recherche Médicale (FRM grant number FDT202012010422).

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1. Schematic representation of the whole process.

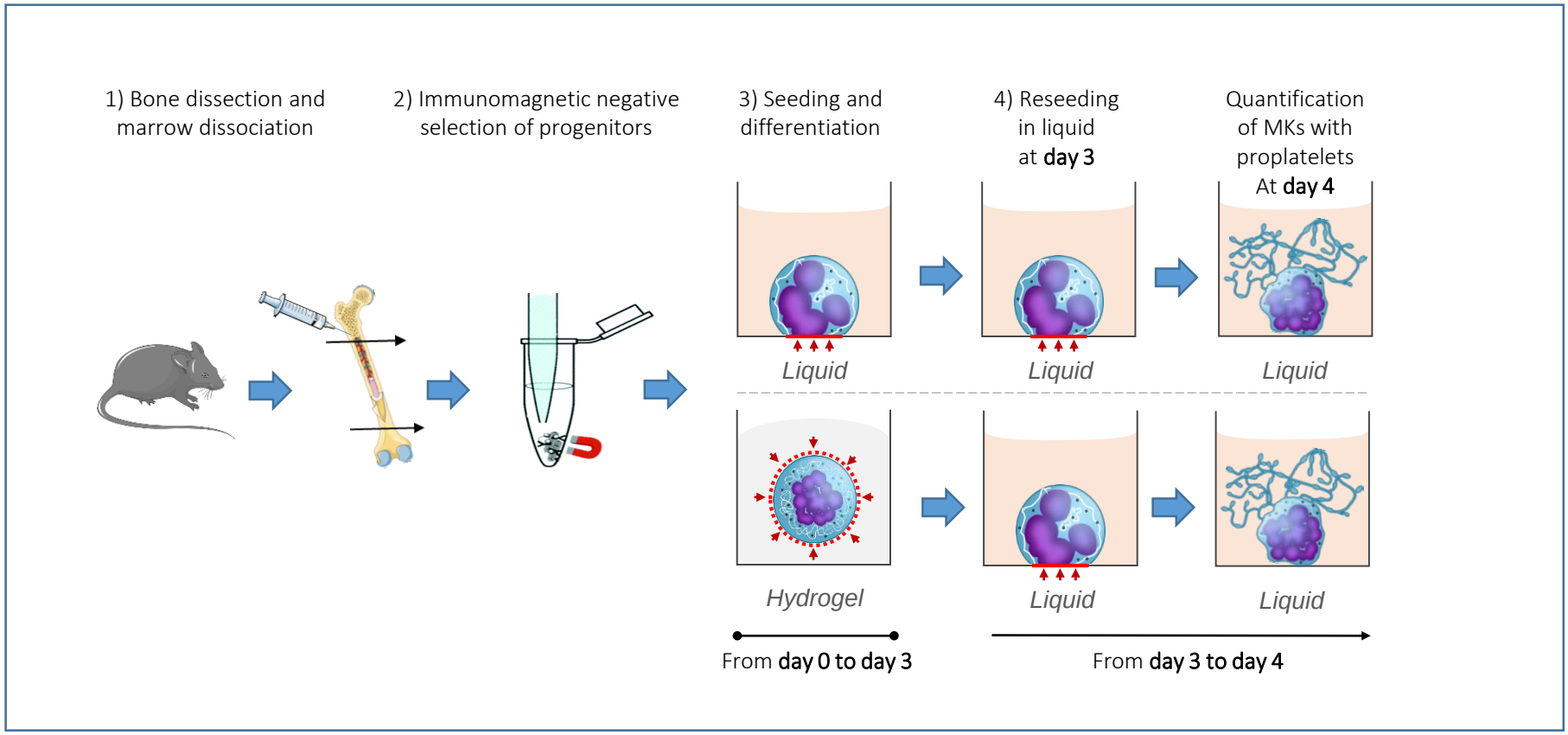


Figure 2. Cell embedding in methylcellulose hydrogel.

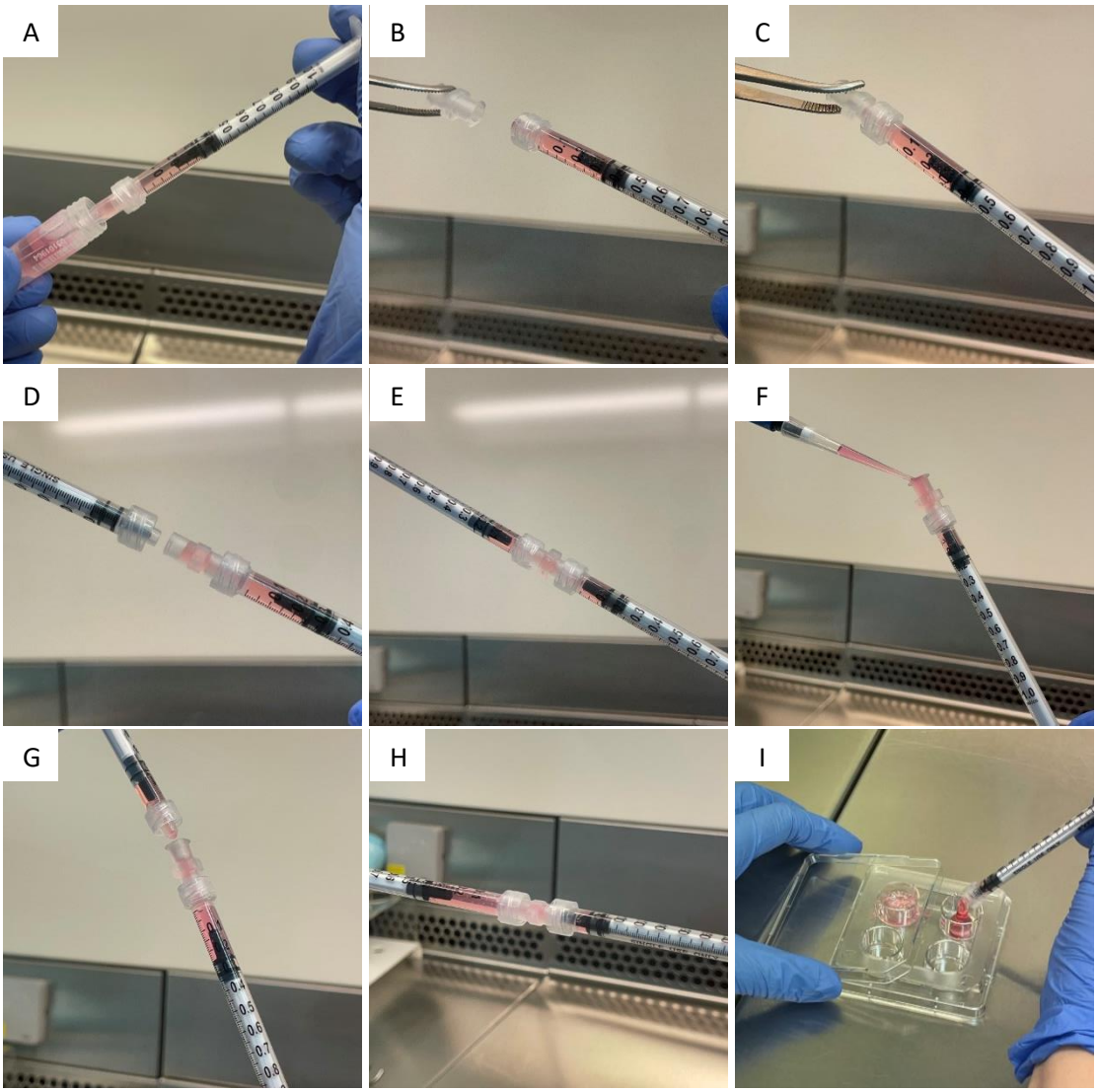


Figure 3. Megakaryocyte characteristics according to culture condition.

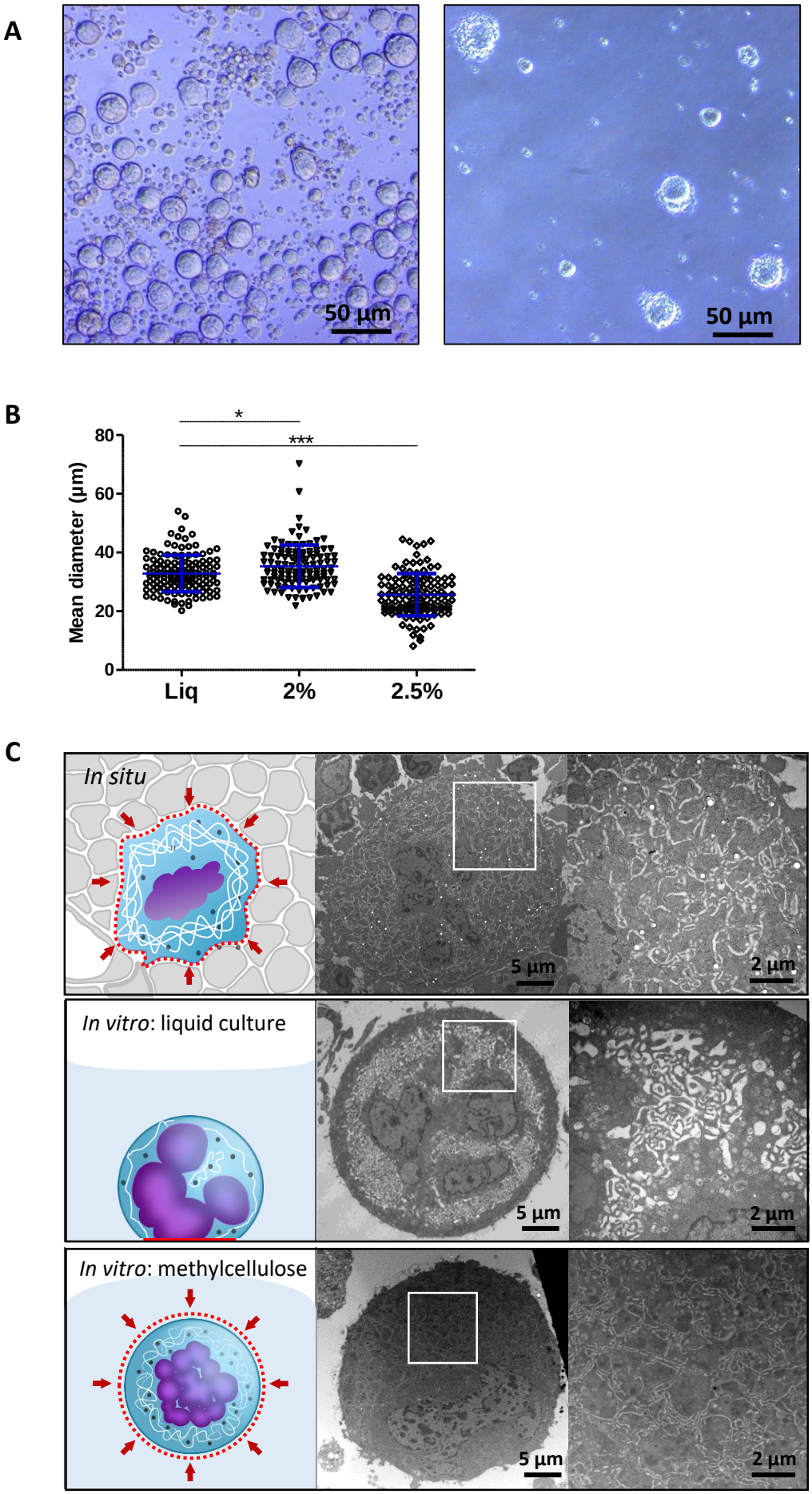
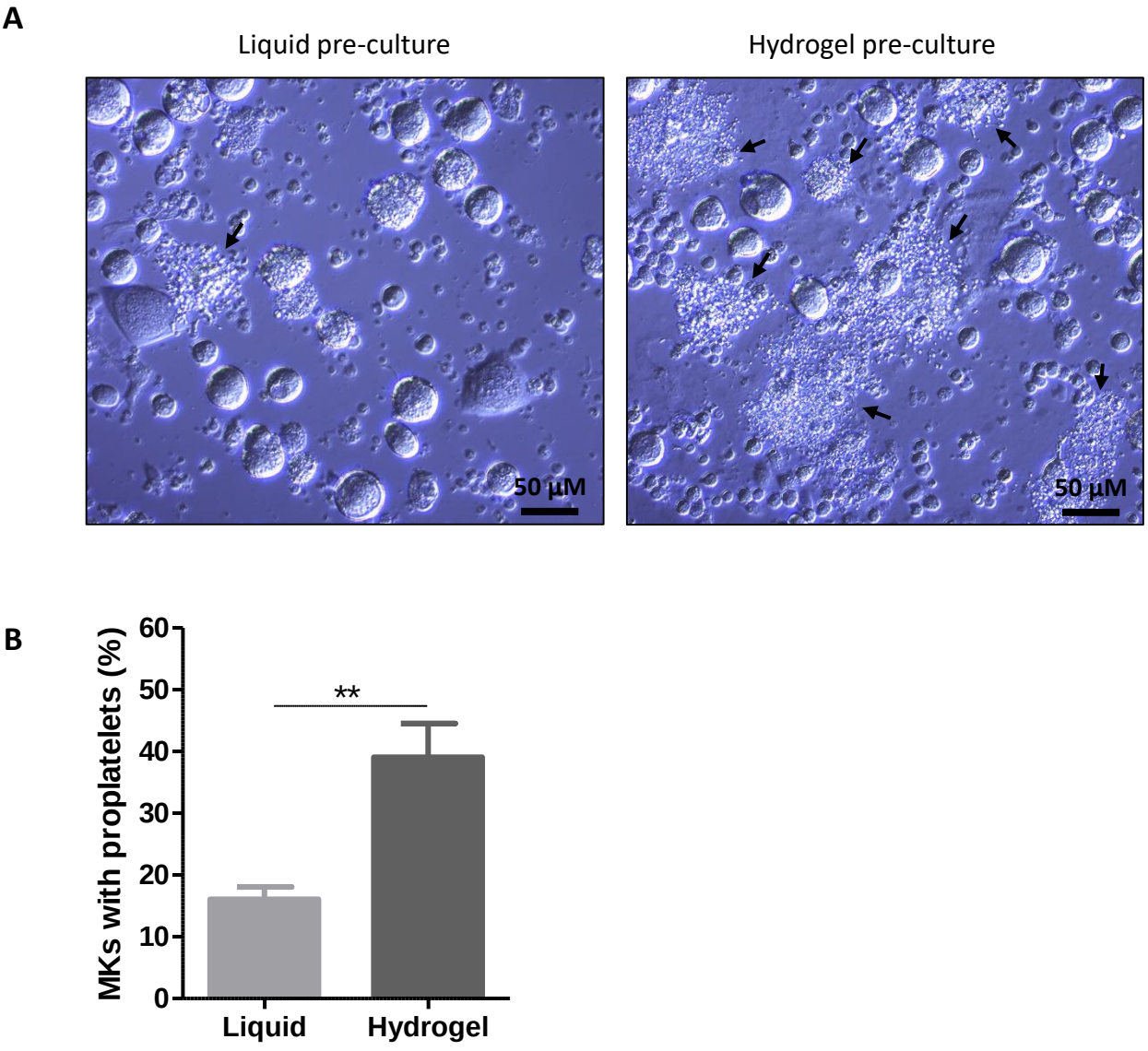


Figure 4. Representative results of proplatelet quantification.



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
18-gauge needles	Sigma-Aldrich	1001735825	
21-gauge needles	BD Microlance	301155	
23-gauge needles	Terumo	AN*2332R1	
25-gauge neeldes	BD Microlance	300400	
4-well culture dishes	Thermo Scientific	144444	
5 mL syringes	Terumo	SS+05S1	
Cytoclips	Microm Microtech	F/CLIPSH	
Cytofunnels equiped with filter cards	Microm Microtech	F/JC304	
Cytospin centrifuge	Thermo Scientific		Cytospin 4
Dakopen	Dako		
DMEM 1x	Gibco, Life Technologies	41 966-029	
DPBS	Life Technologies	14190-094	Sterile Dulbecco's phosphate-buffered saline
EasySep magnets	Stem Cell Technologies	18000	
EasySep Mouse Hematopoietic Progenitor Cell isolation Kit	Stem Cell Technologies	19856A	biotinylated antibodies (CD5,CD11b, CD19, CD45
EDTA	Invitrogen	15575-020	
Fetal Bovine Serum	Healthcare Life Science	SH30071.01	
Luer lock 1 mL syringes	Sigma-Aldrich	Z551546-100EA	or 309628 syringes from BD MEDICAL
Luer lock syringes connectors	Fisher Scientific	11891120	
MC 3%	R&D systems	HSC001	
Polylysin coated slides	Thermo Scientific	J2800AMNZ	
PSG 100x	Gibco, Life Technologies	1037-016	10,000 units/mL penicillin, 10,000 µg/mL streptc
Rat serum	Stem Cell Technologies	13551	
Recombinant hirudin	Transgène	rHV2-Lys47	
Recombinant human trombopoietin (rhTPO)	Stem Cell Technologies	2822	10,000 units/mL
Round bottomed 10 mL plastique tubes	Falcon	352054	

Round bottomed 5 mL
polystyrene tubes

ɜR/B220, Ly6G/C(Gr-1), TER119,7–4) and streptavidin-coated magnetic beads

ɔmycin and 29.2 mg/mL glutamine

Answers to the reviewers

We thank the editor and the referees for reviewing our manuscript entitled "**Megakaryocyte culture in 3D methylcellulose-based hydrogel to improve cell maturation and study the impact of stiffness and confinement.**". All points raised by the editor and the reviewers have been addressed and the changes appear in the text in red. Please find below the detailed point-by-point answers to the comments.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.
This has been carefully examined.

2. Please provide an email address for each author.
The institutional email addresses have been provided.

3. Please revise the following lines to avoid overlap with previously published work: 57-59
This specific part has been reworded.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript (text, figure legends, figures, tables) and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: EasyStep mouse hematopoietic cell isolation kit; EasyStep magnets etc
All the commercial languages have been removed from the manuscript.

5. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
This has been revised throughout the manuscript.

6. Please specify the euthanasia method without highlighting it.
This has been added to the protocol.

7. 2.11: By pastette, do you mean Pasteur pipette?
It is indeed a jargon mistake. The proper denomination is "transfer pipette". It has been corrected throughout the protocol.

8. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
As requested, the protocol has been further detailed to be as exhaustive as possible.

9. After including a one line space between each protocol step, highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. This will clarify what needs to be filmed.
As requested, the sections concerned by the video have been highlighted in yellow.

10. As we are a methods journal, please include any limitations of your method in the discussion.
Limitations of the methods have been addressed in the discussion.

11. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend.
Scale bars have been added to all the microscope images and have been specified in the figure legends.

12. Please include at least 10 previous publications as references.
More reference have been included to the manuscript.

_____

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The manuscript provides a detailed protocol on how hematopoietic stem and progenitor cells can be enriched (using commercial kits) and then differentiated in soft 2% methylcellulose gels. After gel dissolution, the protocol allows to assess pro-platelet production capacity, which is reportedly found to be higher in 3D methylcellulose culture than in corresponding liquid culture.

Major Concerns:
1) Given the absolutely central position of the methycellulose hydrogel, and also the difficulty of appyling exact volumes when filling the syringes, I find it difficult that there is no "quality control" of the wells. Young modulus measurement might be a bit difficult, but there is maybe a visual aspect to it either at room temperature or 37°C that helps to know whether it actually worked.
We agree with the reviewer and we had addressed this point in the discussion. It has been published that an increase of 0.5% in the methylcellulose concentration results in a 10-fold increase in the gel rigidity. It is therefore a critical point of the protocol that precise volume is used. The use of 1 mL syringe limits variations as the graduations are relatively precise, and from our experience it is better to multiply the 1 mL syringe rather than using 5 or even 10-mL syringe for which the risk of error in volume in higher. There is no easy quality control that may allow the verification of the rheological properties in each methylcellulose well. Nonetheless, an essential criterion to assume that the gel concentration is correct is the proper maturation of the MKs within the hydrogel. This can be appreciated by the mean diameter of the MKs as stiffer gels alter MK maturation which appear smaller. The paragraph concerning this limitation has been detailed in the discussion.

2) Liquid culture should be identical to methylcellulose culture except for the methylcellulose. The authors indicate that this should be calculated for the concentrated medium. It would be much better to explicit this calculation. I can see that this is more or less trivial for the additives - if one wants 10% FBS at the end, it needs to be 30% at the beginning since the volume of methylcellulose added makes up 2/3 of the final volume. But what about the inorganic salts, glucose, and generally the components of the DMEM? Unfortunately, I couldn't find the reference for the methylcellulose indicated (see minor comments below), but I assume it is HSC001 from R&D (rather than HS001 as indicated in the manuscript, Table of Materials). Now, HSC001 from R&D contains Iscove's DMEM (aka IMDM), not regular DMEM. The difference concerns additional amino-acids but also additional vitamins, some of which (B12 and biotin) are key in hematopoiesis. Please clarify the exact reference of the methylcellulose (or provide a direct link if it is really HS001) and please make sure that everything except for methylcellulose is the same between liquid and 3D culture. If indeed, due to the methylcellulose stock, the media composition is different between liquid and methylcellulose culture in such major points, I would strongly suggest correcting this, along with validation that the experiment still works as anticipated, and submit a major revision. If the differences are more minor

(not key vitamins) because I made a mistake when trying to compile these media differences, you can disregard much of this comment, but it might still be worth discussing matching between liquid and methylcellulose culture in the discussion section.
The 3% methylcellulose is purchased as a stock solution (indeed HSC001 and not HS001, this has been corrected) and as rightly pointed by the reviewer, this stock solution is prepared in Iscove's Modified Dulbecco's Medium (IMDM) and not in DMEM. For practical reasons of standardization in our lab where possible, we indeed use DMEM medium. The reviewer is absolutely right in pointing to the discrepancy between liquid and MC final medium composition. In fact, IMDM is especially suited for culture in absence of serum, a condition required for some hematopoietic cells including human megakaryocytes. However, the differentiation of mouse hematopoietic progenitors into megakaryocytes is performed here in the presence of 10% FBS, a concentration that bring excess nutrient. Comparative tests had indeed been done initially when we set up the culture to make sure that these small differences had no impact on the outcome of this experiment, especially by comparing MC vs. liquid culture, which was the case. This has been added as a note so that the reader is aware of these differences (lines 282-285).

3) A bit less critical, but more practical: temperature control is exquisitely important in working with methylcellulose. I assume the authors do not pre-heat the media, but this is clear from the text and should be specified (i.e. "thawing" is to room temperature? or 4°C? and if room temperature, would 35°C as it sometimes happens in summer still be OK or is there some upper limit to it?)
The methylcellulose stock solution is thawed at room temperature (in our lab the ambient temperature of the culture room is regulated at 23°C). It has now been specified to the protocol. Methylcellulose is a physical gel that liquefies at low temperature but remains liquid at room temperature, at least at a concentration of 3% or lower, unlike other gels such as matrigel for example. The jellification temperature and time depends on the polymer concentration.

Minor Concerns:
1) There are some troubles finding components needed. Firstly, the protocol heavily relies on the methylcellulose preparation. I don't find HS001 from R&D - I think it is probably HSC001 from R&D Systems, please update or correct, it is absolutely essential that this main component can be found with ease. Another component that's a bit of a mystery is SVF (Line 206). I could imagine this too should be updated. Maybe its generally worth again checking that everything is understandable and can be found straight away.
Thank you for pointing this out. There was indeed a spelling mistake in the methylcellulose reference. It is HSC001 from R&D systems. It has been corrected. As for SVF it is also a mistake, you were wright it is fetal bovine serum. This has also been rectified.

2) Really minor, but I guess when you write 10% addition, it means 10% of the total volume and so only 90% DMEM for example. One cannot be too clear about such things, again, it might make sense to include explicit volumes and not just final concentrations to reduce the probability of error and confusion
% of final volume have been specified throughout the protocol. As an example, exact volumes to prepare complete medium for one culture well have been added at step 2.25. and step 3.7. .

3) L. 146: EasySep, not EasyStep
As requested by the editor, all the commercial languages have been removed from the manuscript, but the spelling has been checked in the material table.

4) L. 155: The EasySep kit isolates hematopoietic stem and progenitor cells, if I'm not mistaken, but does not specifically enrich for megakaryocitic differentiation

The aim of this cell sorting is to retrieve the cells negative for all the selection antibodies (CD5, CD11b, CD19, CD45R/B220, Ly6G/C(Gr-1), TER119, 7–4) and therefore to eliminate the cells that are already engaged in a differentiation lineage other than the megakaryocytic one. Therefore, the cells recovered after the cell sorting are indeed hematopoietic stem cells and progenitors. It is the presence in the culture medium of thrombopoietin (TPO), the major physiological regulator of megakaryopoiesis (Hitchcock and Kaushansky, 2014), that will direct the cell differentiation toward the megakaryocytic cell line.
It is an important point that has been added to the manuscript.

5) L. 173: Lin- means negative for all the selection antibodies, maybe introduce this already in L. 151
The precision has been added to the manuscript in the form of a note, line 174.

Reviewer #2:
Manuscript Summary:
In 'Megakaryocyte culture in 3D methylcellulose-based hydrogel to improve cell maturation and study the impact of stiffness and confinement', Boscher et al. present a protocol to prepare 3D cell culture models using bone marrow derived hematopoietic stem and progenitor cells and methylcellulose hydrogels to explore the effects of the physical constraint and mechanical environment on their differentiation into and further behavior as megakaryocytes.

Major Concerns:
Major concerns include information on/characterization of the hydrogel materials and bone marrow in vivo with respect to mechanical and transport properties, if the protocol has substantially changed with respect to the previous publication of the methods chapter by the authors and how further analysis is performed, as well as some further explanation about the rationale for certain steps in the protocol and the use of replicate samples. More specifically:
1. In the abstract, regarding the statement "The BM is the softest tissue of the body", is this proven?
It has been corrected to “one of the softest” as it is said in the introduction. In fact, it is difficult to accurately measure the stiffness of a tissue *in situ,* and the values vary depending on the technique used. It is even more challenging concerning the bone marrow as it is located within the very stiff bone, and it is likely that the apparent stiffness varies between marrow contained in the trabeculae and hematopoietic marrow in the diaphysis. In the case of isolated marrow from the mouse femur diaphysis, for instance, another issue is that, once extracted from the bone, the tissue does not remain very cohesive. Nevertheless, the hematopoietic marrow stiffness has been estimated to range from 15 to 300 Pa (Choi et Harley 2012; Shin et al. 2014). The 300 Pa value was obtained by AFM measurement of hematopoietic marrow from femur diaphysis. In view of the reported stiffness for other tissues which are all above 1 kPa except for the brain which is around 1kPa, we can assume that bone marrow is one the softest tissue of the body (Discher et al. 2009).
A paragraph has been added to the discussion part to address this important point in more details.

2. Lines 37-38: What specific values for the stiffness are targeted/obtained?
We initially characterized the rheological properties of various concentrations of methylcellulose using a rheometer to select the concentration providing a stiffness that best matched that of the bone marrow and would allow progenitor differentiation into mature MKs. We found that a concentration of 1.3% and below presented a visquous liquid behavior, while 2% and 2.5% exhibited visco-elastic properties having a stiffness of $E_{2\%}$=30-60 Pa and $E_{2.5\%}$=300-600 Pa, respectively, both values being in the range of the predicted bone marrow stiffness. This has been published in Aguilar et al. 2016, which is cited in the method manuscript. Whereas the 2.5% concentration did not allow tproduction of mature MK, as also mentioned in the discussion, the 2% concentration promoted a

high degree of MK maturation even surpassing that in liquid culture (a higher ploidy and a better developed and organized demarcation membrane system).

3. Lines 90-91: Are there any substantial changes in the protocol compared to what was published in 2018?
This method has indeed already been published in 2018 in a classic publication format. There are no substantial changes in the protocol but we found it important to publish it in a video format because some parts of the protocol, especially the methylcellulose encapsulation, are much easier understood through video than through written protocols. It is a well detailed protocol adapted to the collection focused on "*in vitro* and *in vivo* models for the study of megakaryocytes".

4. Figure 1: From the caption vs. the figure, is the initial culturing step carried out for 3 or 4 days? In the figure itself, it is not clear if the blue circle is depicting a single cell. Are the purple regions nuclei? In step 4 and the proplatelet quantification, it is not obvious why these would be the same if they are different in step 3.
The caption was indeed misleading and has been reworded. The initial culturing step is carried from day 0 of culture (seeding day) until day 3 of culture (which in total represents a duration of 4 days). The second culturing step goes from day 3 until day 4 of culture. For reasons of visual clarity, it was decided to schematize only one cell per well. The blue circle is depicting a single cell with its nucleus in purple. Step 4 represents the fact that both conditions are passed into fresh liquid medium for 24h before proplatelet quantification. As only one cell is schematized we decided to represent it with proplatelets as we do observe them in both conditions but in different proportions. The legend has been expanded to clarify these important points.

5. Steps 3.2 and 3.5: What is the rationale for coating one syringe with methylcellulose but not coating the other syringe?
The coating has to be done to ensure that the volume of methylcellulose collected is exact. Therefore, only the syringe used to collect the appropriate volume of methylcellulose need to be coated. A note has been added to the protocol to clarify this point

6. General comment about replicates and independent samples: It would be interesting/relevant to include some commentary on the use of replicate samples and how best to achieve independent replicates. Is it better to work with a pool of cells from all mice or to keep cells from individual mice separate to obtain independent biological replicates?
For one culture, all the wells are part of the same experiment and cannot be considered as independent biological replicates. Cells from all the mice are pooled to ensure homogeneity of all the wells and to be able to compare them while eliminating possible inter-individual variability. Therefore, it is possible to test the effect of a pharmacological agent in a particular well and compare it to the control well from the same mix of cells.
For independent biological replicates, the culture has to be repeated. A note has been added to explain this aspect of the culture.

7. Lines 290-298: These steps seem to be a bit at odds. Care is taken to preserve the megakaryocyte structure while pipetting; however, the cells are then centrifuged, which would presumably disrupt the structure.
Thank you for pointing this out. It is a mistake, this is actually not a step of the protocol, the sentence has been removed. Cells are retrieved using a P1000 by gently pipetting up and down. Centrifugation might not seem ideal to ensure the preservation of MK structure but it is an unavoidable step to wash the fixative away and remove the residual methylcellulose. However, since the cells are fixed, we can assume that their structure will be mostly preserved despite the centrifugation.

8. Line 300: Can some references be added for these analysis methods?

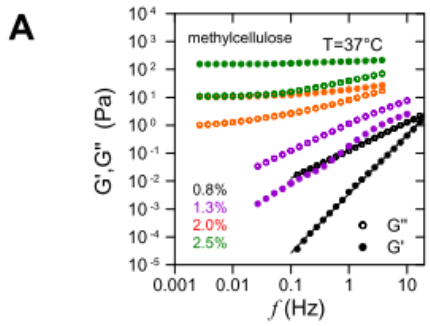
References have been added

9. Line 317: When referring to those differentiated in the bone marrow, does this refer to in vivo? Or to those cells cultured in the hydrogel with the optimized concentration?
It refers to the *in vivo*. This precision has been added to the text.

10. Lines 394-395: How are the cells suspended in the hydrogel excluded from paracrine stimulation? Can't paracrine factors diffuse through the hydrogel?
We indeed cannot totally exclude an effect of paracrine stimulation, nonetheless the cells embedded in the methylcellulose hydrogel are quite distant from one another and we can assume that if secreted substances reach neighboring cells they might be very diluted. This comment has been added to the discussion

11. Lines 397-400: Have the mechanical properties of the hydrogels been measured? Have they been shown by rheology to form hydrogels with storage modulus higher than the loss modulus?
The mechanical properties have been measured for concentration of methylcellulose ranging from 0.8 to 2.5% and presented in the paper of Aguilar et al. 2016; see the figure below, with G' = storage modulus (filled circles) and G'' = loss modulus (empty circles). A specific reference to this article has been added.

Figure 2: Viscoelastic properties of methylcellulose.



Minor Concerns:
These mostly concern correcting some typos, improving the figures, and clarifying some steps in the methodology. Specifically:
12. Line 34: review ◊ protocol?
This has been modified in the text

13. Line 56: Did reference 2 directly measure marrow stiffness?
In their paper, Choi and Harley (former reference 2, now reference 5) did not directly measure marrow stiffness. They refer to Discher et al. 2009 that mentioned that the marrow stiffness is under 0.1 Pa but did not directly measure it either. By contrast, reference 3 (Shin et al. Cell stem cell. 2014) used AFM to measure marrow stiffness in the hematopoietic marrow of the mouse femur diaphysis.

14. Lines 121-122: It's not clear what is meant by opening of the bone. From the earlier part of the protocol, the epiphyses on the knee sides would be intact.
The knee side epiphyses should indeed remain intact from the dissection, leaving a small depression in its center through which we can easily insert the needle so that it reaches the marrow cavity. More explanations have been added to the protocol to clarify this step.

15. Line 125: How much medium (approximately, per bone) should be injected to flush out the marrow?
There is no minimal or maximal volume for the flushing step. In practice, a volume between 500 µL and 1 mL is generally sufficient to expel the marrow from the bone. A note has been added to the protocol.

16. Lines 118-119 and 126-127: Does the DMEM - 1 % PSG also need to be prepared in a tube for the collection of the marrow? More description of this would be helpful.
More details have been added to the protocol to clarify this point.

17. Line 143: What is a pastette?
We apologize for this jargon mistake. The proper denomination is “transfer pipette”. It has been corrected throughout the protocol.

18. Line 172: In this step, are the cell suspensions from different 5 mL tubes combined into one 15 mL tube?
Yes, all the cells originating from mice of the same genotype are pooled in the same 15 mL tube. This information has been added to the protocol.

19. Lines 174-176: Is any extra added as a margin for error? Maybe also specify viable cells as in line 181.
I am not sure I understand the question correctly. In fact, at this step, the volume is calculated in order to have exactly 1 x 10^6 viable cells per well. “Viable cells” has been specified.

20. Line 180: Specify new abbreviations in the text.
This has been corrected.

21. Line 182: Is there something special about the 4-well culture plates or can this protocol be scaled to use other sizes of well plates?
The diameter of the wells from a 4-well culture plate (or 24-well plate, as it is the same diameter) are ideal for seeding the 500 µL needed for this culture, but the protocol can be adapted to smaller or larger volumes.

22. Line 189: For scaling up, is it better to use larger syringes or multiple 1 mL syringes?
For scaling up, larger luer lock syringes could be used, while also adapting the volume of medium and methylcellulose. However, one must ensure that the precision of the syringes remains superior or equal to the 1 mL ones, otherwise larger errors in final hydrogel stiffness may occur (see also answers to reviewer 1).

23. Line 190: How is the 3% methylcellulose stock solution prepared? Is this prepared in DMEM? Otherwise, how does one prepared a more concentrated DMEM in step 3.7?
The 3% methylcellulose is purchased as a stock solution in Iscove's Modified Dulbecco's Medium (IMDM). Please see the answer to comment 2 of the first reviewer who raised the same issue.

24. Lines 208-212: It would be helpful to include the specific concentrations of each compound in this example.
The final concentrations are the same as the ones for the liquid culture. There are specified at the step 2.25. and 3.7.

25. Line 252: Specify amount to prepare.
The information has been added to the protocol.

26. Lines 267-268: However, these duplicates are from the same original sample, so these are not really independent replicates.
A note has been added to insist on the fact that these cannot be considered as replicates.

27. Step 4.8: It would also be useful to include how this counting is performed using ImageJ, as mentioned in the results.
Counting is performed manually, there is no automated procedure for proplatelet quantification this is why we don't mention a specific technique. The cell counter plugin is only used to click on the cells to mark them as they are counted. A note has been added to explain this.

28. Figure 3: Mention size of scale bars in caption.
The information has been added.

29. Lines 379-382: Should this information be reported in the results section rather than the figure caption?
As these value are specific data from an experiment we preferred not to put them in the results section but in the figure as an example to illustrate the typical proportions that should be obtained. Nonetheless, the proportion ranges that should be expected for each culture conditions are specified in the results section.