Journal of Visualized Experiments

Proplatelet Formation Dynamics of Mouse Fresh Bone Marrow Explants

--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE62501R1
Full Title:	Proplatelet Formation Dynamics of Mouse Fresh Bone Marrow Explants
Corresponding Author:	Anita Eckly FRANCE
Corresponding Author's Institution:	
Corresponding Author E-Mail:	Anita.Michel@efs.sante.fr
Order of Authors:	Inès Guinard
	François Lanza
	Christian Gachet
	Catherine Léon
	Anita Eckly
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please specify the section of the submitted manuscript.	Biology
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Strasbourg, GEST, 67000, France
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please provide any comments to the journal here.	
Please indicate whether this article will be Standard Access or Open Access.	Standard Access ($1400)

TITLE:

Proplatelet Formation Dynamics of Mouse Fresh Bone Marrow Explants

AUTHORS AND AFFILIATIONS:

Inès Guinard[1], François Lanza[1], Christian Gachet[1], Catherine Léon[1], Anita Eckly[1]

[1]Université de Strasbourg, INSERM, EFS Grand Est, BPPS UMR-S 1255, FMTS, Strasbourg, France

Email Addresses of Co-Authors:

Inès Guinard (ines.guinard@efs.sante.fr)
François Lanza (francois.lanza@efs.sante.fr)
Christian Gachet (christian.gachet@efs.sante.fr)
Catherine Léon (catherine.leon@efs.sante.fr)
Anita Eckly (anita.michel@efs.sante.fr)

Email Address of Corresponding Author:

Anita Eckly (anita.michel@efs.sante.fr)

KEYWORDS:

bone marrow, explants, megakaryocytes, proplatelet formation

SUMMARY:

Here, we detail the bone marrow explant method, from sample preparation to microscopic slide analysis, to evaluate the ability of megakaryocytes which have differentiated in their physiological environment to form proplatelets.

ABSTRACT:

The last stage of megakaryopoiesis leads to cytoplasmic extensions from mature megakaryocytes, the so-called proplatelets. Much has been learned about the proplatelet formation using *in vitro*-differentiated megakaryocytes; however, there is an increasing evidence that conventional culture systems do not faithfully recapitulate the differentiation/maturation process that takes places inside the bone marrow. In this manuscript, we present an explant method initially described in 1956 by Thiéry and Bessis to visualize megakaryocytes which have matured in their native environment, thus circumventing potential artifacts and misinterpretations. Fresh bone marrows are collected by flushing the femurs of mice, sliced into 0.5 mm cross sections, and placed in an incubation chamber at 37 °C containing a physiological buffer. Megakaryocytes become gradually visible at the explant periphery and are observed up to 6 hours under an inverted microscope coupled to a video camera. Over time, megakaryocytes change their shape, with some cells having a spherical form and others developing thick extensions or extending many thin proplatelets with extensive branching. Both qualitative and quantitative investigations are carried out. This method has the advantage of being simple, reproducible, and fast as numerous megakaryocytes are present, and classically half of them form proplatelets in 6 hours compared to 4 days for cultured mouse megakaryocytes. In addition to the study of mutant mice, an interesting application of this method is the straightforward

evaluation of the pharmacological agents on the proplatelet extension process, without interfering with the differentiation process that may occur in cultures.

INTRODUCTION:

The bone marrow explant technique was first developed by Thiéry and Bessis in 1956 to describe the formation of rat megakaryocyte cytoplasmic extensions as an initial event in platelet formation[1]. Using phase contrast and cinematographic techniques, these authors characterized the transformation of mature round megakaryocytes into "squid-like" thrombocytogenic cells with cytoplasmic extensions showing dynamic movements of elongation and contraction. These arms become progressively thinner until they become filiform with small swellings along the arms and at the tips. These typical megakaryocyte elongations, obtained *in vitro* and in liquid media, have certain similarities with platelets observed in fixed bone marrow, where megakaryocytes protrude long extensions through the sinusoid walls into the blood circulation[2,3]. The discovery and cloning of TPO in 1994, allowed to differentiate megakaryocytes in culture able to form proplatelet extensions resembling those described in bone marrow explants[4-6]. However, megakaryocyte maturation is far less efficient in culture conditions, notably the extensive internal membrane network of bone marrow matured megakaryocytes is underdeveloped in cultured megakaryocytes, hampering studies on the mechanisms of platelet biogenesis[7,8].

We detail here the bone marrow explant model, based on Thiéry and Bessis, to follow in real-time proplatelet formation of mouse megakaryocytes, which have fully matured in their native environment, thus circumventing possible *in vitro* artifacts and misinterpretations. Results obtained in wild-type adult mice are presented to illustrate the ability of megakaryocyte to extend proplatelets, their morphology and the complexity of proplatelets. We also introduce a rapid quantifying strategy for quality validation to ensure data accuracy and robustness during the megakaryocyte recording process. The protocol presented here is the most recent version of the method published as a book chapter previously[9].

PROTOCOL

All animal experiments were performed in accordance with European standards 2010/63/EU and the CREMEAS Committee on the Ethics of Animal Experiments of the University of Strasbourg (Comité Régional d'Ethique en Matière d'Expérimentation Animale Strasbourg).

1. Preparation of reagents

1.1. Prepare reagents as described in **Table 1**.

1.1.1. For the Stock I, dissolve each powder separately. Ensure that the osmolarity of the preparation is higher than 295 mOsm/L. This solution can be stored at 4 °C for one year.

1.1.2. For the Tyrode's buffer preparation, make the solution as described in **Table 1**, adjust the volume to 100 mL with distilled water and add 0.1 g anhydrous D (+) sucrose. Adjust to pH 7.3 using 1 N HCl as needed and the osmolarity to 295 mOsm/L. To prevent bacterial growth, add penicillin G at 10 U/mL final concentration and streptomycin sulfate at 0.29 mg/mL final

concentration. Filter the final solution through 0.22 µm pores.

2. Preparation of the experimental set up

2.1. On the day of the experiment, warm the Tyrode's buffer at 37 °C, and turn on the heating chamber of the microscope to bring the temperature to 37 °C.

2.2. Prepare all the necessary tools, such as a timer, incubation chambers, 5 mL syringes, 21 G, forceps, razor blade, Pasteur pipettes, glass slides, and 15 mL centrifuge tube (**Figure 1A**).

3. Isolation of the mouse bone marrow

3.1. Euthanize C57BL/6 mice aged 8-12 weeks by CO_2 asphyxiation and cervical dislocation. This should be done quickly by a competent and qualified person.

3.2. To avoid microbial contamination, soak the mouse body in 70% (v/v) ethanol before removing the femurs. Use instruments disinfected in 70% ethanol for the dissection. Collect the two femurs and clean them by removing any adherent tissue. After rapid immersion in ethanol, place them in 15 mL centrifuge tube containing 2 mL Tyrode's buffer.

3.3. Cut away the epiphyses using a sharp razor blade and flush the bone marrow using a 5 mL syringe filled with 2 mL Tyrdode's buffer. To achieve this, introduce a 21 G needle into the opening of the femur (on the knee side) and slowly press the plunger to retrieve an intact marrow cylinder (**Figure 1B,C**). Keep the marrow collection session as brief as possible (under 10 min).

4. Bone marrow sectioning and placement into the incubation chamber

4.1. Use a 3 mL plastic pipette to carefully and gently transfer the intact bone marrow onto a glass slide. It is important that the samples are covered with buffer to prevent drying out (**Figure 1D**).

NOTE: Avoid flow-reflow to minimize shears that could dissociate the tissue.

4.2. Under a stereomicroscope (10x), cut off the ends of the marrow that may have been compressed at the time of the flush. Then cut transversal sections with a sharp razor blade. Sections should be thin enough to allow a detailed observation but ensure that megakaryocytes are not damaged by compression (usually thickness around 0.5 mm) (**Figure 1E,F**).

NOTE: The sections are made with a sharp razor blade and under a magnifier to adjust their thickness to about 0.5 mm. Only the sections of uniform thickness are selected. This procedure is not complicated but its standardization requires some experience.

4.3. Using a plastic pipette, collect 10 sections into 1 mL tube containing Tyrode's buffer (**Figure 1G**).

4.4. Carefully transfer the sections to an incubation chamber with a diameter of 13 mm (**Figure 1H**).

4.5. Aspirate the buffer and adjust the volume to 30 µL of Tyrode's buffer supplemented with 5 % mouse serum.

NOTE: It is at this stage that pharmacological agents can be added to evaluate their impact on proplatelet formation.

4.6. Position the sections at distance. Seal the self-adhesive chamber with a coverslip of the dimension 22 x 55 mm. Incline the coverslip while sticking to avoid the formation of air bubbles (**Figure 1I**).

4.7. Place the chamber in the heating chamber at 37 °C. From this moment, the chronometer is started (T= 0 h). The experiment runs for 6 h at 37 °C (T= 6 h).

5. Real-time observation of marrow explants

5.1. Use an inverted phase contrast microscope (40x lens for magnification) coupled to a video camera to observe the bone marrow explants. Let the cells incubate for 30 min before starting the observation. While observing, it is necessary to adjust the focus because megakaryocytes are moving.

NOTE: Other observation modes are possible (e.g., DIC, fluorescence using mice whose megakaryocytes express an endogenous fluorescent marker), but phase contrast microscopy is optimal to clearly visualize the long and thin megakaryocyte extensions, allowing precise quantification. After 30 min, the marrow cells gradually migrate to the periphery of the explant, forming a monolayer. After 1 h of incubation, megakaryocytes can be identified by their large size and polylobulated nuclei (**Figure 1J,K**). After 3 h of incubation, the number of megakaryocytes increases and some have long extensions.

5.2. Make videos to record the transformation of the megakaryocytes.

6. Quantification of the proplatelet-extending megakaryocytes

6.1. Draw a map to localize each section in the incubation chamber (**Figure 1K**).

6.2. After 1 h, identify the visible megakaryocytes (i.e., giant polylobulated cells) on each section's periphery and plot their positions on the drawing. Repeat this procedure after 3 and 6 h.

NOTE: On the basis of the drawing, each megakaryocyte can be easily located over time and the evolution of their morphology analyzed (e.g., size, deformation, proplatelet extension, etc.). Another possibility is the use of a specific navigation software.

REPRESENTATIVE RESULTS:

Qualitative results. At the beginning of the experiment, all cells are compacted in the bone marrow section. It takes 30 min for the cells to become clearly visible at the periphery of the explants. The megakaryocytes are then recognizable by their large size and their evolution can then be studied over time (size, shape, dynamic, proplatelet extension and platelet release) (**Figure 2A**). Small megakaryocytes have a diameter between 20 and 30 µm and their nuclei are polylobulated while mature round megakaryocytes are larger (> 30 µm in diameter) with an enlarged cytoplasm. A few dark megakaryocytes may be observed (Figure 2C). These represent dead cells whose proportion should not exceed 0.5%. A proportion higher than this value indicates a sample preparation problem. The morphology of the nucleus can be easily visualized by varying the focus.

Quantitative results. Megakaryocytes are counted manually as described in 6.2. and classified according to their morphology at 3 h and 6 h after sealing of the incubation chamber. **Figure 2A** summarizes the four essential megakaryocytes classes: (1) small MKs, (2) large MKs, (3) MKs with thick extensions, (4) MKs with thin, elongated, and ramified extensions. These later are the typical proplatelet-forming megakaryocytes, with the prominent characteristics of swellings along the proplatelets and the presence of refractive buds at their extremities. With the help of mapping (**Figure 1K**), their evolution can be followed over time. The results are expressed as a percentage of each class at each observation time. Classically, half of the megakaryocytes visible at the periphery extend proplatelets at 6 hours for wild-type C57BL/6 mouse bone marrow (**Figure 2B**).

It is possible to follow the fate of round MKs by capturing sequential images over time to image how they form proplatelets (**Video 1**). Interestingly, when MKs with thick extensions were monitored over a period of 3 h, it was observed that the thick extensions could either detach from the cell body and branch into proplatelets or retract to reform large round MKs.

FIGURE AND TABLE LEGENDS:

Table 1: Preparation of the Tyrode's buffer. Each stock solution is indicated in the first column of the table. The composition as well as the amount of reagent (given in grams) required for each stock solution is indicated per row. The catalog number and the company of each reagent are given in the table of essential supplies.

Figure 1: Photographic representations of the sample preparation method for the bone marrow explant. (**A**) Experimental setup required for the bone marrow preparation. (**B**) A 21-gauge syringe-mounted needle is inserted into the bone. (**C**) The bone marrow is flushed into a tube containing Tyrode's buffer. (**D**) The bone marrow is then gently deposited on a glass slide. (**E**) The extremities of the bone marrow are cut off. (**F**) The marrow cylinder is cut into ten 0.5 mm thick sections. (**G-H**) The ten sections are transferred to an incubation chamber and observed at 37 °C using an inverted microscope. (**I**) Representative photo of an incubation chamber containing the ten sections of bone marrow. (**J**) Peripheral cells migrate to form a layer in which

the megakaryocytes become visible. (**K**) Example of drawing illustrating the ten explant sections in the incubation chamber as well as the location of the megakaryocytes (X blue mark) that have migrated out of the tissue for each section. The arrow shows a megakaryocyte at the periphery.

Figure 2: Morphological classification of megakaryocytes in explants over time. (**A**) Megakaryocytes are classified as "small", "large", with "thick extension" or “proplatelet-extending". Bars: 50 µm (**B**) Proportion of megakaryocytes in each class was determined at 1 h, 3 h and 6 h for a total of 1,468 megakaryocytes, showing that the proportion of "small" and "spherical" megakaryocytes decreases with time while, in parallel, the proportion of megakaryocytes extending proplatelets increases (n=6 mice). Typically, in the explants of a WT mouse after 3 h, between 8.3 and 11.5 megakaryocytes are observed per section. The error bar corresponds to the standard error of the mean for each sample. (**C**) Representative image of a dark megakaryocyte. Bar: 50 µm (**D**) Representative image of a megakaryocyte with non-muscular myosin II-A labeled with a green fluorescent protein. Bar: 50 µm

Video 1: Time-lapse video showing a MK extending proplatelets.

DISCUSSION

Here we describe a simple and low-cost *in vitro* method to evaluate the efficiency of megakaryocytes to extend proplatelets which have grown in the bone marrow. The bone marrow explant model for mouse has four main advantages. First, there are no advanced technical skills required. Second, the time needed to obtain megakaryocyte-extending proplatelets is quite short, only 6 hours for the explant method, compared to a minimum of 4 days for a conventional culture method starting from mouse progenitors. Third, given that only a small amount of tissue is needed and that the results obtained are reproducible, it reduces the number of mice needed to a minimum (usually 6 mice per experimental condition), making these experiments economically and ethically efficient. Lastly, but importantly, the strength of this method lies in the use of megakaryocytes that have fully developed in their natural environment, which may prove invaluable in revealing phenotypes that could be masked *in vitro* by potential artifacts of the culture conditions. This has been previously documented in mice with megakaryocyte-restricted *MYH9* inactivation where opposing results have been found on proplatelet formation in *in vitro* (increased formation)[10] and *in vivo* (decreased formation) differentiated megakaryocytes[11]. These paradoxical results have been explained by the requirement of myosin IIA for normal megakaryocyte differentiation in a constraint environment, while myosin IIA is dispensable for megakaryocyte differentiation in liquid culture[7].

An interesting application of the bone marrow explant model is the possibility to study the impact of genetic mutations or deficiencies in transgenic mice and/or pharmacological agents exclusively on the platelet extension process, without interfering with the differentiation process as in the case of in vitro culture[12]. The ideal situation is to use the bone marrow of one femur as a treated sample and its counterpart as control. In addition, the use of transgenic mice allowing spontaneous fluorescence in the megakaryocytes facilitates the visualization of the platelet extension process. To visualize fluorescent megakaryocytes, one possibility could be to add fluorescence-labelled antibodies against specific megakaryocyte markers in the culture chamber.

Another possibility could be the use of genetically engineered mouse models expressing a fluorescent protein, either specifically in the megakaryocytic lineage such as mice with CD41-labelled YFP already reported in the literature[13], or in all cells such as mice where GFP is linked to the N-terminus of non-muscular myosin II-A[14] as illustrated in Figure 2D.

This explant method, therefore, provides both qualitative and quantitative information for a better understanding of platelet formation in their natural environment. Noteworthy, although this method is simple and fast it remains complementary to the studies performed using classical liquid culture. Each one brings separate knowledge according to the stages of proliferation, maturation, extension of the platelets and platelet release that one wishes to study. For example, where the explant method gives information on the capacity of extension of proplatelets by megakaryocytes that have grown in a physiological context, *in vitro* culture provides information on the importance of the bone marrow microenvironment such as the impact of cell ridigity[7] or extracellular matrix dependency[15]. Thus, *in vitro* megakaryocyte cultures make it possible to modulate the parameters of the microenvironment in terms of stiffness and adhesive proteins[7,16]. Please refer to the article "Megakaryocyte culture in three-dimensional methylcellulose-based hydrogel to improve cell maturation and study the impact of stiffness and confinement" by J. Boscher et al., presented in this issue for more information.

ACKNOWLEDGMENTS:

The authors wish to thank Jean-Yves Rinckel, Julie Boscher, Patricia Laeuffer, Monique Freund, Ketty Knez-Hippert for technical assistance. This work has been supported by ANR (Agence National de la Recherche) Grant ANR-17-CE14-0001-01 and ANR-18-CE14-0037.

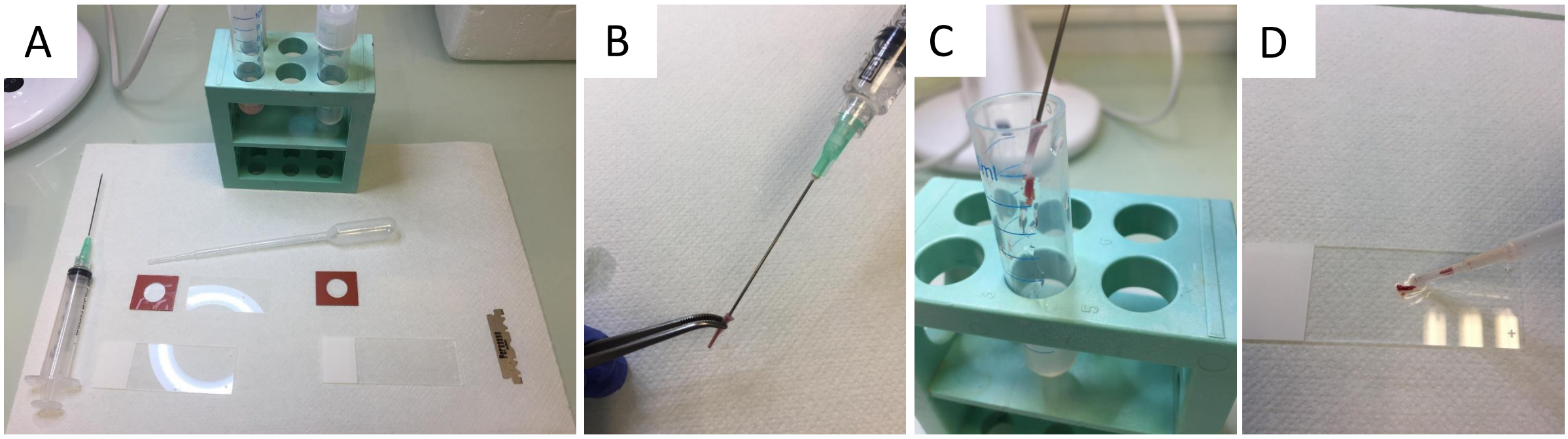
DISCLOSURES:

The authors declare no conflicts of interests.

REFERENCES:

1. Thiery, J. P., Bessis, M. Mechanism of platelet genesis; in vitro study by cinemicrophotography. *Reviews in Hematology.* **11** (2), 162-174 (1956).
2. Becker, R. P., De Bruyn, P. P. The transmural passage of blood cells into myeloid sinusoids and the entry of platelets into the sinusoidal circulation: A scanning electron microscopic investigation. *American Journal of Anatomy.* **145** (2), 183-205 (1976).
3. Muto, M. A scanning and transmission electron microscopic study on rat bone marrow sinuses and transmural migration of blood cells. *Archivum Histologicum Japonicum.* **39** (1), 51-66 (1976).
4. Cramer, E. M. et al. Ultrastructure of platelet formation by human megakaryocytes cultured with the Mpl ligand. *Blood.* **89** (7), 2336-2346 (1997).
5. Kaushansky, K. et al. Promotion of megakaryocyte progenitor expansion and differentiation by the c-Mpl ligand thrombopoietin. *Nature.* **369** (6481), 568-571 (1994).
6. Strassel, C. et al. Hirudin and heparin enable efficient megakaryocyte differentiation of mouse bone marrow progenitors. *Experimental Cell Research.* **318** (1), 25-32 (2012).
7. Aguilar, A. et al. Importance of environmental stiffness for megakaryocyte differentiation and proplatelet formation. *Blood.* **128** (16), 2022-2032 (2016).

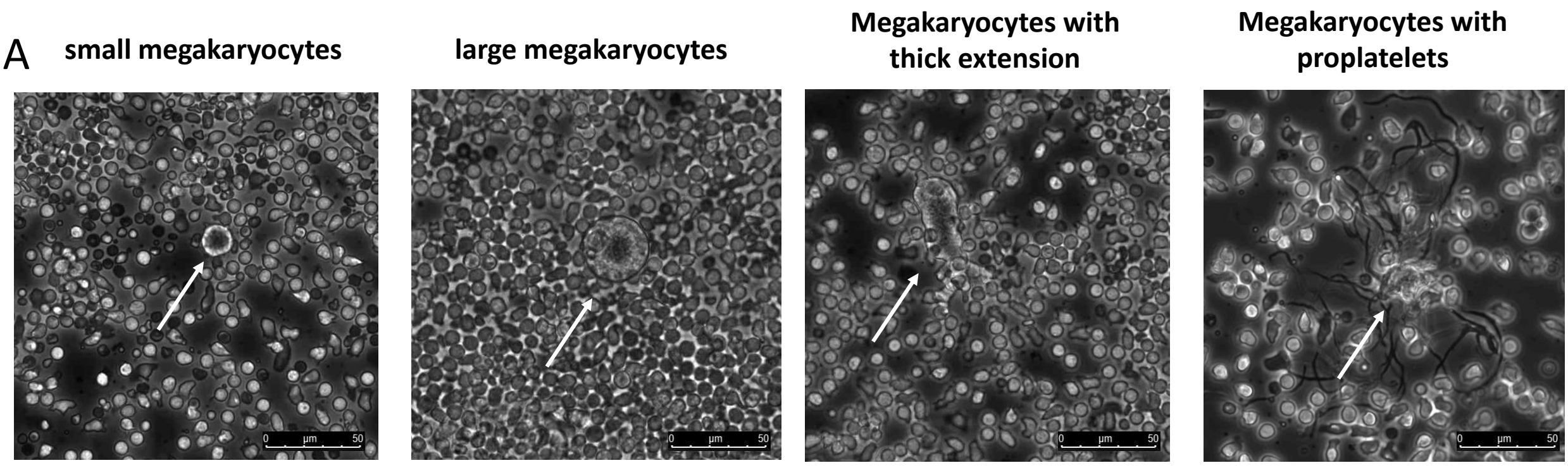
8. Scandola, C. et al. Use of electron microscopy to study megakaryocytes. *Platelets.* **31** (5), 589-598 (2020).
9. Eckly, A. et al. Characterization of megakaryocyte development in the native bone marrow environment. *Methods in Molecular Biology.* **788**, 175-192 (2012).
10. Eckly, A. et al. Abnormal megakaryocyte morphology and proplatelet formation in mice with megakaryocyte-restricted MYH9 inactivation. *Blood.* **113** (14), 3182-3189 (2009).
11. Eckly, A. et al. Proplatelet formation deficit and megakaryocyte death contribute to thrombocytopenia in Myh9 knockout mice. *Journal of Thrombosis and Haemostasis.* **8** (10), 2243-2251 (2010).
12. Ortiz-Rivero, S. et al. C3G, through its GEF activity, induces megakaryocytic differentiation and proplatelet formation. *Cell Communication and Signaling.* **16** (1), 101 (2018).
13. Junt, T. et al. Dynamic visualization of thrombopoiesis within bone marrow. *Science.* **317** (5845), 1767-1770 (2007).
14. Zhang, Y. et al. Mouse models of MYH9-related disease: mutations in nonmuscle myosin II-A. *Blood.* **119** (1), 238-250 (2012).
15. Malara, A. et al. Extracellular matrix structure and nano-mechanics determine megakaryocyte function. *Blood.* **118** (16), 4449-4453 (2011).
16. Balduini, A. et al. Adhesive receptors, extracellular proteins and myosin IIA orchestrate proplatelet formation by human megakaryocytes. *Journal of Thrombosis and Haemostasis.* **6** (11), 1900-1907 (2008).



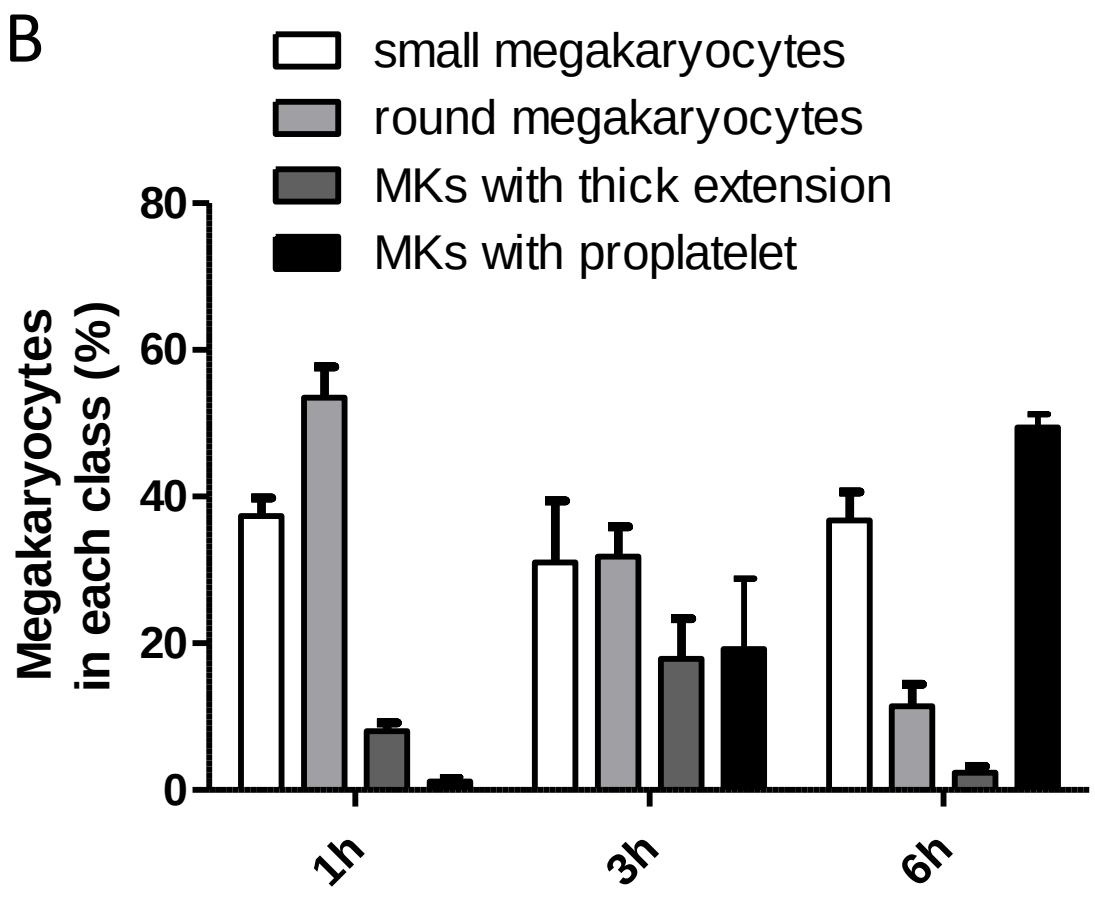
Map summarizing the positions of the explants and megakaryocytes

Figure 1

A

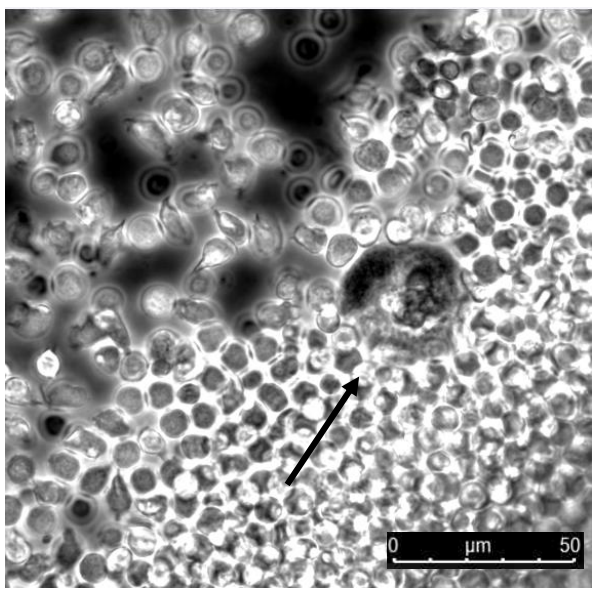


B

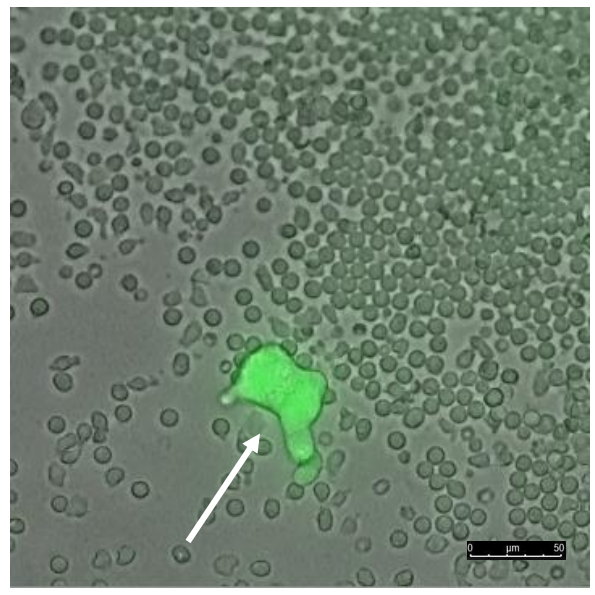


	T = 1 h 00	T = 3 h 00	T = 6 h 00
small megakaryocytes	37.3 ± 6.0%	31.0 ± 16.8%	36.8 ± 9.3%
large megakaryocytes	53.4 ± 10.4%	31.8 ± 8.1%	11.4 ± 7.3%
Megakaryocytes with large extremities	8.0 ± 2.8%	17.8 ± 10.9%	2.3 ± 2.0%
Megakaryocytes with proplatelets	1.1 ± 1.3%	19.2 ± 19.2%	49.4 ± 4.4%

C



D



Video or Animated Figure

Click here to access/download

Video or Animated Figure

video_WT_201210.avi

Table 1	Reag	
Stock I*	16 g NaCl (2.73 M)	0.4 g KCL (53.6 mM)
Stock II	2.033 g $MgCl_2.6H_2O$ (0.1 M)	
Stock III	2.19 g $CaCl_2.6H_2O$ (0.1 M)	
HEPES Stock**	119 g HEPES* (0.5M)	
Tyrode's Buffer***	5 mL Stock I	1 mL Stock II

ents		H_2O
2 g $NaHCO_3$ (238 mM)	0.116 g NaH_2PO_4, (8.6 mM)	to 100 mL
		to 100 mL
		to 1 L
2 mL Stock III	**1 mL HEPES Stock**	**1.8 mL albumin Stock**

Name of Material/ Equipment	Company	Catalog Number
5 mL syringes	Terumo	SS+05S1
21-gauge needles	BD Microlance	301155
$CaCl_2.6H_2O$	Sigma	21108
Coverwall Incubation Chambers	Electron Microscopy Sciences	70324-02
HEPES	Sigma	H-3375
Human serum albumin	VIALEBEX	authorized medication : n° 3400956446995
KCl	Sigma	P9333
$MgCl_2.6H_2O$	Sigma	BVBW8448
Micro Cover Glass	Electron Microscopy Sciences	72200-40
Microscope	Leica Microsystems SA, Westlar, Germany	DMI8 - 514341
microscope camera	Leica Microsystems SA, Westlar, Germany	K5 CMS GmbH -14401137
Mouse serum	BioWest	S2160-010
NaCl	Sigma	S7653
$NaH_2PO_4.H_2O$	Sigma	S9638
$NaHCO_3$	Sigma	S5761
PSG 100x	Gibco, Life Technologies	1037-016
Razor blade	Electron Microscopy Sciences	72000
Sucrose D (+)	Sigma	G8270

Comments/Description

Depth : 0,2 mm
pH adjusted to 7.5

20% (200mg/mL -100mL)

22 mm x 55 mm

air lens

image resolution : 4.2 megapixel

10,000 units/mL penicillin, 10,000 µg/mL streptomycin and 29.2 mg/mL glutamine

Answers to the reviewers

We thank the editor and the referees for reviewing our manuscript entitled “Dynamics of proplatelet formation by real-time observation of mouse fresh bone marrow explants”. 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:

Editorial Changes

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

This has been carefully examined.

2. Please provide an email address and affiliation for each author.

The institutional email addresses have been provided.

3. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s).

This has been modified throughout the manuscript.

4. Consider adding reagent preparation as a separate table, and reference it in the text.

This has been done.

5. Please specify the age, sex and breed of the mice used.

As requested by the editor, these specifications have been added in the protocol section (line 102).

6. Line 91: What temperature should the buffer be warmed to?

The buffer heating temperature of 37 °C has been added (now lines 93-94).

7. Line 104, 118: Which buffer is used?

We have used the Tyrode’s buffer as now mentioned lines 108 and 125.

8. Line 110: What care should be taken?

It is important that the samples are covered with buffer to prevent drying out, as explained line 115.

9. Include a single space between the quantity and its unit. E.g. “37 oC” instead of “37oC”, etc.

This has been modified throughout the manuscript.

10. 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 and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. E.g. Eppendorf, Falcon, Leica, etc.

As recommended, all the commercial languages have been removed and products are mentioned in a table referenced in the material and methods section.

11. Please include a single line space between successive protocol steps, and highlight up to 3 pages of the Protocol (including headings and spacing) that identify the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.
As requested by the editor, the sections concerned by the video have been highlighted in yellow.

12. Use decimal points instead of "," . E.g. see table in Figure 2.
This has been modified in the text and in Figure 2.

13. 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. E.g. Figure1 K (magnified region)
As requested, we have added a scale bar in all images, including the enlarged box. Please note that we also provide new representative images of the different classes of megakaryocytes. These pictures originate from a recently acquired microscope that will be used for the filming of the video.

14. Figure 2B. What do the error bars signify?
The error bars represent standard errors of the mean (SEM) and this has been clarified in the legend in Figure 2.

15. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Do not abbreviate journal names.Please include volume and issue numbers for all references.
This has been done.

16. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. E.g. Add details about the instruments used (microscopes etc).
This has been added to the essential products table.

Reviewers' comments:

Reviewer #1:

Comments:

-Are the steps listed in the procedure clearly explained?

* MAYBE THEY COULD EXPLAIN IN MORE DETAIL HOW TO CUT UNIFORM THICKNESS SECTIONS OF 0.5 MM FROM THE MARROW, SINCE THIS SEEMS TO BE A CRITICAL STEP.
The sections are made with a sharp razor blade and under a magnifier to adjust their thickness to ~ 0.5 mm. Only the sections of uniform thickness are then selected under the magnifier. This procedure is not complicated but its standardization requires some experience. This has now been better explained lines 122-124.

* PERHAPS FIGURE 1L CAN BE TAKEN SHOWING THE EDGE OF THE PLATE TO BETTER SEE THE INCLINATION.

We agree with the reviewer's suggestion and tried to take this picture. However, the result was not good enough to see the inclination of the transparent coverslip. As recommended by the reviewer, this part of the protocol will carefully be explained during the filming of the video (text highlighted in yellow).

* WHEN THE AUTHORS SUGGEST ANOTHER OBSERVATION MODE, E.G. FLUORESCENCE, PERHAPS THEY CAN EXPLAIN HOW TO VISUALIZED MEGAKARYOCYTES BY IMMUNOFLUORESCENCE (A METHOD THAT HELP TO IDENTIFY THEM). ONE SUGGESTION IS TO ADD A LABELED ANTIBODY AGAINST A MEGAKARYOCYTE MARKER (E.G. CD41, CD61) TO THE TYRODE´S BUFFER BEFORE PLACING THE EXPLANTS IN THE INCUBATION CHAMBER.
In the discussion section, we now explain how to obtain images of fluorescent megakaryocytes (lines 239-244). As suggested by the reviewer, one possibility could be to add fluorescently labelled antibodies against specific megakaryocyte markers in the culture chamber. Another possibility could be the use of genetically engineered mouse models expressing a fluorescent protein, either specifically in the MK lineage such as the mice with CD41-labelled YFP already reported in the literature (Junt *et al*, 2007), or in all cells such as the mice with GFP labelled at the N-terminus of non-muscular myosinII-A (Zhang *et al*., 2012) allowing an excellent visualization of the fluorescent MKs as now illustrated in the revised manuscript (new FIGURE 2C).

-Is there any additional information that would be useful to include? IT IS UNCLEAR WHAT THE AUTHORS MEAN BY "DARK MEGAKARYOCYTES". AN IMAGE SHOWING AN EXAMPLE WOULD BE USEFUL.
As recommended, we have added an image of the dark megakaryocytes (FIGURE 2D).

-Are any important references missing and are the included references useful? PERHAPS AUTHORS COULD CITE THE WORK BY ORTIZ-RIVERO ET AL, 2018, WHERE THIS METHOD WAS USED SUCCESSFULLY (DOI: 10.1186/S12964-018-0311-5).
We added the study of ORTIZ-RIVERO et al. to illustrate one of the applications of the explant model in the investigation of genetic mutations.(Discussion section Line 233-236).

Minor Concerns:
Line 54 " protrude long extensions through the sinusoid walls into the blood circulation" is repeated
This has been corrected.

Line 104: gauge instead gaude. As request This misspelling has been corrected.

Line 158: represent instead representing This has been corrected.

Reviewer #2:
Manuscript Summary:
This manuscript describes the explant method for observation of proplatelet formation from mature megakaryocytes. This seems to be an identical method to that previously published (PMID: 22130708) by the same group. While this paper is included in the list of references (number 9), it is not actually referred to anywhere in the manuscript. It is unclear to this reviewer why the method needs to be published for a second time.

The reviewer is correct. This method has been published in 2012 as a traditional text-based paper. As asked by the reviewer, it is now referred at the end of the introduction section (line 73-74). We decided

to publish it in a video format because 1) it will show the most recent version of the method, especially the visualization of fluorescent megakaryocytes and their automatic quantification using a navigation software, 2) some parts of the protocols, especially the flushing of the bone marrows, are much easier to understand through video than through written protocols and 3) it is a gold standard protocol perfectly adapted to the collection focused on "*in vitro* and *in vivo* models for the study of megakaryocytes".

Major Concerns:
None

Minor Concerns:
1. The title should make it clear that this is a method for mouse bone marrow.
As suggested, the title clarifies now that the method is based on mouse bone marrow.

2. Provide catalogue numbers and suppliers for specialist reagents (e.g. serum albumin)
This has been added to the essential products table.

3. in step 4.3 which buffer is used?
The buffer is Tyrode's buffer and this is now specified in the text (line 125).

4. The citation style used for the references is sub-optimal - the references are numbered in the reference list but not numbered in the text, and they are not in alphabetical order. I realise this may be a journal-specific format but it is not helpful for the reader.
We followed Jove's guidelines for the format of the references. This has been modified throughout the manuscript.

Reviewer #3:
Manuscript Summary:
The authors described a protocol preparing mouse bone marrow explants to observe proplatelet formation. The proportions of small megakaryocytes (MKs), large MKs, MKs with thick processes and proplatelet forming MKs over time were counted. The model may be used to quantify proplatelet formation under genetic and/or pharmacological manipulations.

Major Concerns:
1. The 'small megakaryocyte' numbers appear to be stable over time. How did the authors confirm that they were really megakaryocytes, not other blood cells?
The arguments that these cells represent small MKs are 1) their diameter between 20 and 30 µm, which is too large to correspond to other blood cells, 2) the multilobulated shape of their nuclei, which is a distinctive characteristic of MKs and 3) the fact that we observe only few cells with this size and aspect, which is consistent with the small proportion of immature MKs located in the bone marrow (and in contradiction with the large proportion of other blood cells). These points are now mentioned in representative results section, lines 163-167, 170.

2. Can the authors track the cells whether process-bearing MKs were derived from large MKs? Did the MKs with thick processes differentiate into MKs with thin proplatelet processes?
It is possible to follow over time the fate of large round MKs and thus visualize their ability to form proplatelets. Similarly, we tracked MKs with thick extensions over a period of 3 hours and noted that

the thick extensions could either detach from the cell body and branch into proplatelets, or retract to re-form large round MKs. This is now explained lines 181-184

Minor Concerns:

How many MKs can typically be derived from a marrow cylinder?

Typically, after 3 hours, between 8,3 and 11,5 megakaryocytes can be observed per section. This is now mentioned in Figure 2 and table legends sections (lines 209-211).