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In Situ Exploration of Murine Megakaryopoiesis using Transmission Electron Microscopy

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TITLE:

In Situ Exploration of Murine Megakaryopoiesis using Transmission Electron Microscopy

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KEYWORDS:

megakaryocyte, thrombopoiesis, bone marrow, transmission electron microscopy, in situ

SUMMARY:

Here, we present a protocol to analyze ultrastructure of the megakaryocytes *in situ* using transmission electron microscopy (TEM). Murine bone marrows are collected, fixed, embedded in epoxy resin and cut in ultrathin sections. After contrast staining, the bone marrow is observed under a TEM microscope at 120 kV.

ABSTRACT:

Differentiation and maturation of megakaryocytes occur in close association with the cellular and extracellular components of the bone marrow. These processes are characterized by the gradual appearance of essential structures in the megakaryocyte cytoplasm such as a polyploid and polylobulated nucleus, an internal membrane network called demarcation membrane system (DMS) and the dense and alpha granules that will be found in circulating platelets. In this article, we describe a standardized protocol for the *in situ* ultrastructural study of murine megakaryocytes using transmission electron microscopy (TEM), allowing for the identification of key characteristics defining their maturation stage and cellular density in the bone marrow. Bone marrows are flushed, fixed, dehydrated in ethanol, embedded in plastic resin, and mounted for generating cross-sections. Semi-thin and thin sections are prepared for histological and TEM observations, respectively. This method can be used for any bone marrow cell, in any EM facility and has the advantage of using small sample sizes allowing for the combination of several imaging approaches on the same mouse.

INTRODUCTION:

Megakaryocytes are specialized large polyploid cells, localized in the bone marrow, responsible for platelet production[1]. They originate from hematopoietic stem cells through an intricate

maturation process, during which megakaryocyte precursors progressively increase in size, while undergoing extensive concomitant morphologic changes in the cytoplasm and nucleus[2]. During maturation, megakaryocytes develop a number of distinguishable structural elements including: a polylobulated nucleus, invaginations of the surface membrane that form the demarcation membrane system (DMS), a peripheral zone devoid of organelles surrounded by the actin based cytoskeletal network, and numerous organelles including α-granules, dense granules, lysosomes, and multiple Golgi complexes. At the ultrastructural level, a major modification observed is the cytoplasmic compartmentalization into discrete regions delimited by the DMS[3]. This extensive supply of membranes will fuel the extension of long cytoplasmic processes in the initial phase of platelet production, which will then remodel into platelets inside the circulation. Any defect during megakaryocyte differentiation and maturation can affect platelet production in term of platelet count and/or platelet function.

Thin layer transmission electron microscopy (TEM) has been the imaging approach of choice for decades providing high-quality ultrastructure of megakaryocytes that have shaped our understanding of the physiology of thrombopoiesis[4,5]. This paper focuses on a standardized TEM method allowing to capture the process of platelet biogenesis occurring *in situ* within the native bone marrow microenvironment, which could also serve as a basis to analyze any bone marrow cell type. We provide ultrastructural examples of the development of megakaryocytes from immature to fully mature, which extend cytoplasmic processes into the microcirculation of sinusoids[6]. We also describe an easy procedure to quantify the different megakaryocyte maturation stages, instructing on the regeneration and platelet production capacity of the bone marrow.

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). The protocol is schematically shown in **Figure 1.**

1. Bone marrow collection and fixation (Figure 1A)

CAUTION: This procedure includes carcinogenic, mutagenic, and/or toxic substances and is performed under a chemical extraction hood. Wear appropriate protective equipment such as gloves and protections glasses.

1.1. Prepare the fixative solution consisting of 2.5% glutaraldehyde in cacodylate buffer (see **Supplementary File**).

1.2. Bone marrow collection

1.2.1. Use adult C57BL/6 mice of either sex 12-18 weeks of age. Euthanize the mice by CO_2 asphyxiation and cervical dislocation.

1.2.2. With a pair of thin scissors, cut the skin around the thigh and use tweezers to peel the skin off. Remove the extremity of the paw and then cut between the hip and thigh. Detach tibia from femur by cutting at the knee articulation and remove adherent tissue on tibias and femurs by using a scalpel.

1.2.3. Remove the epiphyses with a sharp razor blade. While holding the femur with tweezers, use a 5 mL syringe filled with cacodylate buffer with a 21 G needle to flush the bone marrow into a 15 mL tube filled with 2 mL cacodylate buffer. To do so, insert the bevel of the needle into the bone marrow opening and slowly press the plunger until the marrow is expelled.

1.3. Bone marrow fixation by rapid immersion into fixative.

1.3.1. Immediately after flushing, use a plastic pipette to transfer the bone marrow cylinder into 1 mL of fresh glutaraldehyde fixative solution (previously prepared in 1.1) for 60 min at room temperature.

NOTE: To preserve the tissue, ensure that the entire process, from bone dissection to the fixation step, is completed in less than 10 min. For the fixation, ensure that the fixative solution is at room temperature to avoid heat shock.

2. Embedding bone marrow in agarose

NOTE: Marrow tissue is not sufficiently cohesive to maintain its integrity during the different washing steps and material can be easily lost. To overcome this problem, the marrow is covered in a gel of agar before dehydration.

2.1. Prepare the agarose solution as described in the **Supplementary File**.

2.2. Wash the fixed marrow from section 1.3 in cacodylate buffer and transfer it carefully to a glass slide using a plastic pipette. Using a warm pipette, quickly apply a drop of 2% liquid agar to the bone marrow cylinder.

NOTE: The agar solidifies quickly while cooling. To ensure a homogenous covering of the bone marrow, the agar solution has to be kept warm until it is deposited onto the slide.

2.3. Quickly place the slide rapidly on ice until the agar solidifies (1-2 min).

2.4. Under a microscope, use a sharp razor blade to cut and discard the extremities of the bone marrow cylinder because of possible tissue compression in these areas. Transfer the marrow blocks in 1.5 mL microcentrifuge tubes containing 1 mL of cacodylate buffer.

3. Embedding bone marrow in resin

CAUTION: Resin components are toxic; some are carcinogenic and must be handled with care under a chemical extraction hood. Use appropriate protective equipment such as gloves and protection glasses. Osmium tetroxide is highly volatile at room temperature and its vapors are very harmful to the eyes, nose, and throat. Before being discarded, 2% osmium tetroxide must be neutralized by adding twice its volume of vegetable oil.

3.1. Prepare the epoxy resin as described in the **Supplementary File**.

3.2. Resin embedding

NOTE: Keep the samples in the same microcentrifuge tubes during incubations in successive baths of osmium, uranyl acetate and ethanol. Aspirate the supernatants with a Pasteur pipette. The volume of solution used for each bath must equal at least 10x the volume of the sample.

3.2.1. Post-fix the blocks with 1% osmium in cacodylate buffer for 1 h at 4 °C, wash once in cacodylate buffer and then once in distilled water.

3.2.2. Stain with 4% uranyl acetate in distilled water for 1 h, wash twice in distilled water.

3.2.3. Dehydrate through a graded series of ethanol in distilled water: 4 times in 75% ethanol for 5 min, followed by 3 times in 95% Ethanol for 20 min and then 3 times in 100% ethanol. At this step, take one syringe of epoxy resin out from the freezer.

NOTE: The protocol can be paused in 100% ethanol for 1 h.

3.2.4. To obtain uniform infiltration and polymerization of epoxy resin inside the marrow, incubate first the blocks in 2 successive baths of propylene oxide for 15 min.

3.2.5. Add a 1:1 mixture of 100% propylene oxide and epoxy resin and incubate for 1 h. Place the samples on a slow rotary shaker at room temperature.

3.2.6. Add 100% epoxy resin leave the sample for overnight incubation under agitation.

3.2.7. Add 100% epoxy resin for 2 h incubation, still under agitation.

3.2.8. Under a microscope, place the marrow blocks into flat silicone molds. Orientate samples to permit subsequent transversal sectioning of the entire bone marrow. Fill the molds with epoxy resin and place them at 60 °C for 48 h.

NOTE: All solutions (except ethanol and propylene oxide) are filtered through 0.22 µm filter to avoid samples contamination. To ensure adequate polymerization of the resin, avoid bubbles while filling the molds.

4. Ultrathin sectioning (Figure 1B)

NOTE: Transmission EM requires thin tissue sections through which electrons can pass generating a projection image of the interior of cells, structure, and organization of inner organelles (granules, endoplasmic reticulum, Golgi) and the arrangement of intracellular cell membranes.

4.1. Mount the sample block in an ultra-microtome support. Put it on the sample holder. Trim the samples at 45˚ in order to remove the excess of resin around the tissue with a rotating diamond or tungsten milling cutter.

4.2. Mount the samples on the ultramicrotome with a diamond knife blade equipped with a water tank. Cut transverse sections of 500 nm and 100 nm thickness for histological and TEM analyses, respectively. Collect floating sections on the water-surface with a loop.

4.3. Deposit the 500 nm thick section on a glass slide and 100 nm thick sections on 200 mesh thin-bar copper grids with a paper filter underneath. Prepare five grids for one condition: stain two grids first and keep the three remaining grids as a backup if necessary.

5. Toluidine blue staining for histology

NOTE: Staining sections for histology is important for three reasons: 1) to make sure that the tissue is actually cut and not the resin, 2) to check the quality of the inclusion, and 3) to rapidly evaluate the marrow sample. If this is not correct, cut deeper in the block.

5.1. Dry the semi-thin sections slide on a heat plate (60 °C).

5.2. Add filtered 1% toluidine blue/1 % sodium borate in distilled water on the slides and heat on a hot plate (60 °C) for 1-2 min. Wash the slides with distilled water and let it dry on the heat plate.

5.3. Mount sections on coverslips with a drop of Poly(butyl methacrylate-co-methyl methacrylate) mounting medium and examine under a light microscope.

6. Heavy metal staining for TEM observation (Figure 1C)

NOTE: For the contrast, the upper side of the grids are inverted on 100 µL drops of each successive bath with a loop. Prior to use, each solution is 0.22 µm filtered. Remove the excess of liquid between each bath by gently contact the grid side on a filter paper.

6.1. Stain with 4% uranyl acetate in distilled water for 5 min.

6.2. Wash 3 times in distilled water for 5 min.

6.3. Stain with lead citrate for 3 min.

6.4. Wash 3 times in distilled water for 5 min.

6.5. Deposit the grids by the lower side in contact with the filter paper to let them dry.

NOTE: Heavy metals react in the presence of carbon dioxide. To minimize the precipitates, avoid air displacement during the contrast, do not speak, keep the environment calm and turn off the air-conditioning.

7. TEM (Figure 1E)

NOTE: The sections are introduced in a TEM microscope and examined at 120 kV.

7.1. First examine the sections at low magnification (< 500x) to appreciate the general aspect of the preparation (absence of hole in the resin, folds/compression in the sections, precipitates due to staining).

7.2. Then examine the sections at higher magnification (~ 2000x allowing to distinguish the stage of maturation). Count manually the megakaryocytes from each stage of maturation over whole transversal sections (see Representative Results on how do visually identify each stage).

NOTE: Each square of the grids is defined as an area for examination (which equals 16000 µm2 for 200 mesh copper grids).

7.3. To assess the number of megakaryocytes, quantify only the squares that are fully covered with a section. To do so, use a model based on the screening of ranges. Observe a first range of squares from an extremity of the section to another, then another range in the same way, etc. Using this procedure, screen fully and systematically the whole marrow transversal section square by square.

7.4. For each square, score the number of Stage I, II or III megakaryocytes.

NOTE: Higher magnifications are required to analyze the granules, the DMS organization, the size of cytoplasmic territories and the polylobulated nucleus.

REPRESENTATIVE RESULTS

Bone marrow histology

Observation of the bone marrow toluidine blue histology under a light microscope is key to rapidly analyze the overall tissue architecture in terms of e.g., tissue compactness, microvessel continuity, and the size and shape of megakaryocytes **(Figure 1D).** It is performed before ultrathin sections to determine the need of cutting deeper in the bone marrow block. Due to their giant size and nuclear lobulation, the more mature megakaryocytes may be easily visualized with a 40x objective. This gives an excellent and rapid overview of the density of mature megakaryocytes in the tissue and their relative localization to the microvessels. Anomalies in megakaryocyte proliferation and maturation could already been detected in such semi-thin sections.

Bone marrow ultrastructure

On the basis of distinct ultrastructural characteristics, murine megakaryocytes are divided into 4 stages representing sequential stages in their maturation **(Figure 2A).** Stage I megakaryocytes are 10-15 µm in diameter with a large nucleus occupying most of the cell and containing abundant ribosomes and rough endoplasmic reticulum. The presence of the earliest detectable stage of the DMS, called pre-DMS, is also a key criterion for distinguishing stage I MKs in TEM analysis[3]. In the stage II of maturation, granule formation begins and the development of the DMS is initiated. Megakaryocytes increase in size, measuring 15-25 µm in diameter and develop nuclear lobulation. Mature stage III megakaryocytes are giant cells 25-50 µm in diameter. Their cytoplasm contains a well-developed DMS with clearly defined cytoplasmic territories and a peripheral zone devoid of organelles. At this stage, the nucleus is generally located eccentrically and appears very irregular with condensed chromatin located at the nuclear membrane. The last step is characterized by a naked nucleus, also called pyrenocyte, consisting of a large nucleus surrounded by a plasma membrane after the bulk of cytoplasm has been eliminated. In wild type C57BL/6 mice, the bone marrow comprises about 8% stage I, 20% stage II, 71% stage III megakaryocytes and < 1% pyrenocytes. The average number of megakaryocytes is between 1.7 and 2.2 cells per square. This arbitrary classification allows to conveniently monitor a continuous process of cell differentiation and detect its possible anomalies.

Beside these classical stages of maturation, observation of fixed megakaryocytes in the bone marrow allows to analyze the series of events occurring as megakaryocytes interact with the sinusoidal wall **(Figure 2B).** Megakaryocytes in contact with the endothelial cells are frequently observed in thin sections. On occasion one observes megakaryocytes forming short invasive protrusions penetrating the endothelium or extending large projection of its cytoplasm into the sinusoidal lumen[7,8]. Remarkably, these intravascular cytoplasmic processes display variable sizes, lengths, and diameters, illustrating the progressive platelet remodeling in the circulation. Platelets already present in the general circulation, having a discoid shape maintained by circumferential microtubule coils, are also visible in the lumen of the sinusoids. This typical morphology of the platelets is indicative of the correct fixation of the specimen.

Transmission electron microscopy has the level of resolution required to visualize ultrastructural details, such as nuclear lobulation, spatial organization of the DMS and granules in terms of size, shape, and distribution. **Figure 2C** is an example of the perinuclear region in a stage III megakaryocyte showing the presence of α granules, Golgi cisternae, mitochondria, and endoplasmic reticulum. Also noteworthy in **Figure 2C** is a multivesicular body, which represents an intermediate stage in the formation of alpha and dense granules, containing multiples exosomes measuring less than 200 Å in diameter[9,10]. Finally, transmission electron microscopy enables to visualize neutrophils and other hematopoietic cells present inside the megakaryocytes, **(Figure 2D)** following an uncommon process called emperipolesis whereby a cell penetrates another living cell[11]. This process, which concerns 4% of megakaryocytes in normal physiological condition, can be significantly increased in certain pathological conditions[12].

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic illustration of the experimental setup. (**A**) Bone marrow embedding

procedure. The bone marrow is flushed and fixed by rapid immersion in glutaraldehyde solution. The photograph illustrates the typical appearance of the bone marrow cylinder following flushing. After 1 h fixation at room temperature, the marrow is surrounded in agarose, post-fixed in osmium tetroxide and incubated in uranyl acetate. The tissues are then rinsed in buffer, dehydrated in a series of graded ethanol, incubated in propylene oxide and infiltrated with epoxy resin. (**B**) Bone marrows blocks sectioning. The embedded bone marrow is mounted on an ultramicrotome holder, trimmed at 45˚ and cut either in semi-thin (500 nm) or thin (100 nm) sections. For ultrastructural studies, the floating sections are picked up with a loop and deposited on grids with a paper filter underneath. (**C**) Contrast staining for TEM observations. Grids are inverted on uranyl acetate drops, washed on distilled water drops and incubated on lead citrate before another run of washings. After drying (upper side with the sections), the samples are ready to be examined under the TEM. (**D**) Histology of a mouse femoral marrow section stained with toluidine blue. The giant cells correspond to mature megakaryocytes (1), some being in contact with sinusoids (2). The sinusoids converge on a large central sinus vein (3). Bar: 20 µm. Inset: Normal appearance of a mature megakaryocyte at 40x magnification. (**E**) Representative TEM image of a bone marrow section at low magnification. Cells are tightly packed with little extracellular space. Each grid square of the section is observed from an extremity to another by following the schematic arrowed path (red arrows). Bar: 200 µm.

Figure 2: Representative *in situ* images of megakaryocytes ultrastructure. (**A**) Characteristic maturation stages of wild-type megakaryocytes. Megakaryocytes are classified in four maturation stages: Stage I, a cell 10–15 µm in diameter with a large nucleus; stage II, a cell 15–30 µm in diameter containing the DMS under development; stage III, a 30-50 µm cell containing a well-developed demarcation membrane system (DMS) defining cytoplasmic territories and having an organelle-free peripheral zone. A pyrenocyte corresponds to the naked polylobulated nucleus remaining in the bone marrow following full cytoplasmic extension. Bars: 10 µm (**B**) Megakaryocyte-endothelial cell interactions and intravascular cytoplasmic processes. (i) The peripheral zone (PZ) of a megakaryocyte is closely apposed to the abluminal surface of the sinusoidal endothelium. (ii) A megakaryocyte forming short invasive protrusions that penetrate deeply into the endothelial layer (arrowheads). (iii-v) The arrows indicate cytoplasmic processes of megakaryocytes with varying diameters, some of which are very large and have a peripheral zone that may represent fragments that have just entered the bloodstream. (vi) A typical discoid platelet (P) observed in the sinus lumen. In each micrograph, the red line indicates the luminal side of the endothelial barrier and the star indicates the sinusoid lumen. Bars: 2 µm. (**C**) Higher magnifications of the perinuclear region of a mature megakaryocyte. α, alpha granule; rer, reticulum endoplasmic reticulum; G, golgi; MVB, multivesicular body; m, mitochondria. Bar: 0.5 µm. (**D**) Example of a megakaryocyte showing emperipolesis. The engulfed neutrophil appears morphologically unaltered by the interaction with megakaryocytes. Bar: 2 µm.

Supplementary File: Preparation of the reagents.

DISCUSSION:

Direct examination of megakaryocytes in their native environment is essential to understand megakaryopoiesis and platelet formation. In this manuscript, we provide a transmission electron

353 microscopy method combining bone marrow flushing and fixation by immersion, allowing to
354 dissect *in situ* the morphology characteristics of the entire process of megakaryocyte
355 morphogenesis taking place in the bone marrow.
356
357 The flushing of the bone marrow is a critical step of this method, as the success of a high-quality
358 flushing depends on the practice and training of the operator. Although delicate, flushing the
359 bone marrow is the best way to avoid removal of the mineralized bone, which usually requires a
360 2-week EDTA treatment for complete decalcification associated with significant artifacts on the
361 megakaryocyte morphology. Additionally, a major advantage of collecting whole unfixed bone
362 marrows from tibia and femurs is the ability to combine several imaging approaches to the same
363 mouse. In practice, only a single bone marrow is required for the ultrastructural study, the other
364 three specimens being available for complementary analyses. The second bone marrow can then
365 be used for the preparation of fresh bone marrow explants, to study in real-time the dynamics
366 of proplatelet formation of native megakaryocytes[6]. The third sample is usually designed for
367 immunostaining studies on thick sections, providing 3D imaging and distribution of
368 megakaryocytes within their natural environment. The last sample can be frozen and stored for
369 further studies by immunogold electron microscopy, where the subcellular localization of
370 proteins is investigated at high resolution[4]. These combined imaging methods, together with the
371 availability of the targeted deletion/mutation of genes in a mouse, provide an important means
372 of delineating *in situ* the biological role of a given protein in thrombopoiesis. However, it should
373 be pointed out here that one limitation of this method is the withdrawal of the epiphyses needed
374 to flush the marrow. Epiphyses are known to be important areas for hematopoiesis, and their
375 removal therefore hinders any possibility of analyzing hematopoietic stem cells and the initial
376 phases of engagement[13]. Another limitation is that progenitors of megakaryocytes before the
377 immature stage I cannot be identified because these cells do not have specific ultrastructural
378 features. To overcome this limitation, an EM immunogold approach could be used.
379
380 The second important step of the method is the bone marrow fixation by immersion. When
381 performed under the conditions described here, i.e., fixation immediately after flushing out the
382 compact bone marrow cylinder, it has the following advantages: (i) it is quick and easy to perform,
383 (ii) it preserves an ultrastructure close to that observed following fixation by perfusion[6], and (iii)
384 it maintains free megakaryocyte processes and platelets in the sinusoid bloodstream which are
385 otherwise flushed out/lost after perfusion. With this technique it is possible to investigate the
386 entry of megakaryocytes into the sinusoidal circulation and to characterize all the intermediate
387 forms of cytoplasmic processes from which platelets arise[8]. In line with this, it has recently been
388 reported that the large protrusions intravasating from megakaryocytes *in vivo* are structurally
389 distinct from the thin extensions formed by megakaryocytes *in vitro*, with notably a different
390 arrangement of the microtubules[7]. Similarly, we have recently shown that the mechanism
391 governing platelets formation *in vivo* differs from that identified *in vitro*[14].
392
393 Important ultrastructural differences are increasingly recognized between *in vitro* cultured and
394 *in vivo* generated native megakaryocytes, underscoring the need for the bone marrow
395 microenvironment for a full megakaryocyte differentiation/maturation. Following combination
396 of bone marrow flushing and fixation by immersion described in this article, conventional

transmission electron microscopy still remains an invaluable tool to study megakaryocyte biology and platelet formation, under physiological and pathological conditions.

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DISCLOSURES:

The authors have no conflicts of interests to declare.

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Figure 1

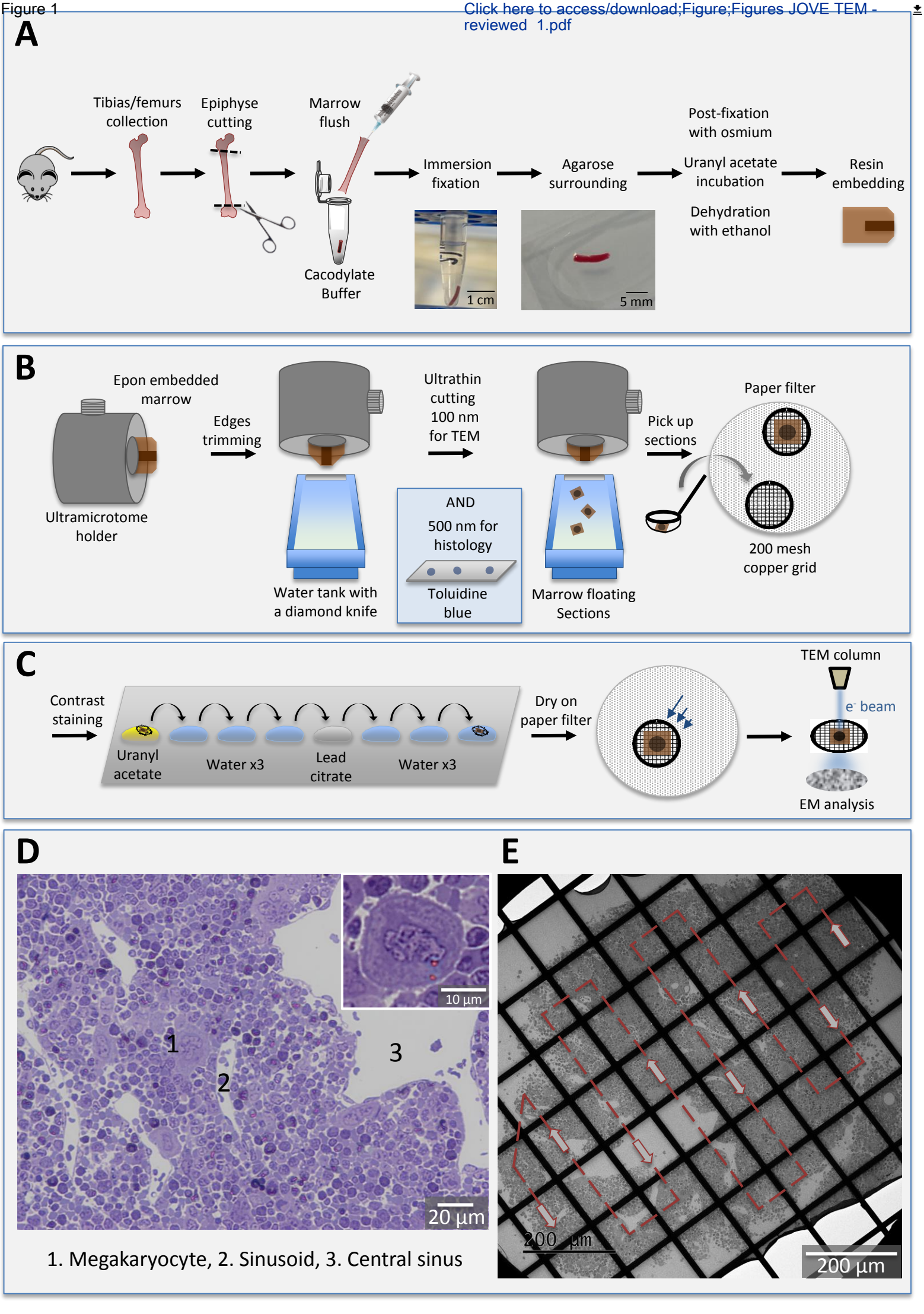
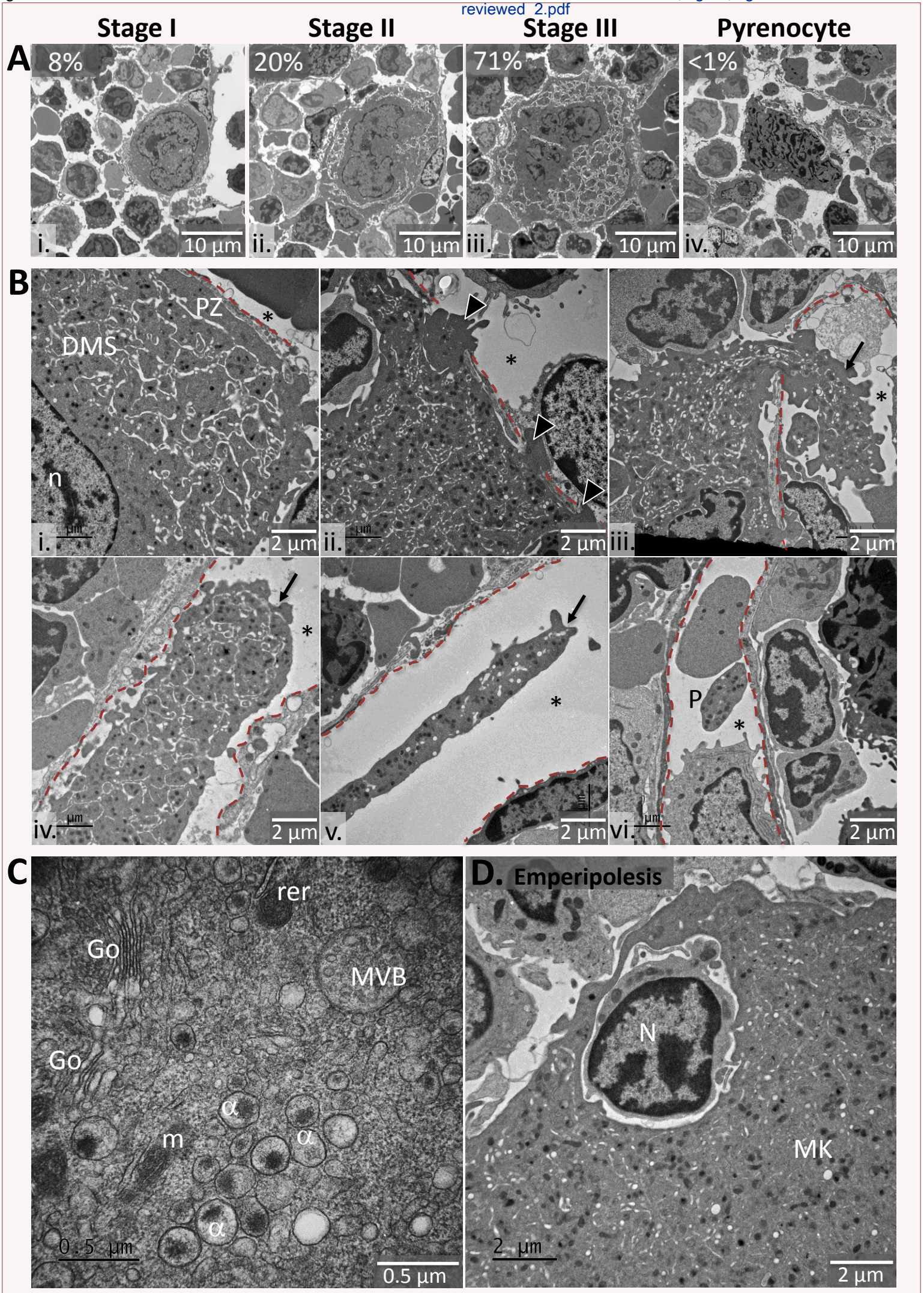


Figure 2



Name of Material/ Equipment	Company
2,4,6-Tri(dimethylaminomethyl)phenol (DMP-30)	Ladd Research Industries, USA
Agarose type LM-3 Low Melting Point Agar	Electron Microscopy Sciences, USA
CaCl2 Calcium chloride hexahydrate	Merck, Germany
Copper grids 200 mesh thin-bar	Oxford Instrument, Agar Scientifics, England
Dimethylarsinic acid sodium salt trihydrate	Merck, Germany
Dodecenyl Succinic Anhydride (DDSA)	Ladd Research Industries, USA
Double Edge Stainless Razor blade	Electron Microscopy Sciences-EMS, USA
Ethanol absolut	VWR International, France
Filter paper, 90 mm diameter	Whatman, England
Flat embedding silicone mould	Oxford Instrument, Agar Scientific, England
Glutaraldehyde 25%	Electron Microscopy Sciences-EMS, USA
Heat plate Leica EMMP	Leica Microsystems GmbH, Austria
Histo Diamond Knife 45°	Diatome, Switzerland
JEOL 2100 Plus TEM microscope	JEOL, Japan
Lead citrate - Ultrostain 2	Leica Microsystems GmbH, Austria
LX-112 resin	Ladd Research Industries, USA
MgCl2 Magnesium chloride hexahydrate	Sigma, France
Mounting medium - Poly(butyl methacrylate-co-methyl methacrylate)	Electron Microscopy Sciences-EMS, USA
Nadic Methyl Anhydride (NMA)	Ladd Research Industries, USA
Osmium tetroxide 2%	Merck, Germany
Propylene oxide (1.2-epoxypropane)	Sigma, France
Saline injectable solution 0.9% NaCl	C.D.M Lavoisier, France
Scalpel Surgical steel blade	Swann-Morton, England
Sodium tetraborate - Borax	Sigma, France
Sucrose	Merck, Germany
Syringe filter 0.2µm	Pall Corporation, USA
Toluidine blue	Ladd Research Industries, USA
Trimmer EM TRIM2	Leica Microsystems GmbH, Austria
Ultramicrotome Ultracut UCT	Leica Microsystems GmbH, Austria
Uranyl acetate	Ladd Research Industries, USA

Catalog Number	Comments/Description
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T200-CU	
8.20670.0250	
21340	
EM-72000	
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16210	
705402	
1044797	
EM-21001BU	
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21310	
M2393-100g	
15320	
21350	
19172	
82320-250ML	
MA 575 420 6	
.0508	
B-9876	
84100-1KG	
514-4126	
N10-70975	
702801	
656201	
23620	

Answers to the reviewers

We thank the editor and the referees for reviewing our manuscript entitled "*In situ* exploration of the major steps of murine megakaryopoiesis using transmission electron microscopy". 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.
This has been carefully examined.

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

3. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"
The summary has been rephrased.

4. Use 12-pt, black, Calibri font throughout the text. Maintain a 0-inch left indent throughout the text and indicate new paragraphs using single-line spacing. Do not underline section/sub-section headings or parts of the text.
The text has been modified as requested.

5. Provide details on the age, sex, strain etc. of the mice used.
These informations have been added in the protocol, line 105.

6. Consider adding the steps for reagent, resin preparation as a separate table, and referencing it in the protocol.
A separate section for reagents has now been added at the beginning of the protocol.

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." E.g. "Examine sections…" instead of "sections are examined..", etc.
This has been done.

8. Line 164: Which mounting medium is used? Please specify.
The mounting medium has been specified as Poly(butyl methacrylate-co-methyl methacrylate) mounting medium in the text and in the material table (now line 200).

9. Line 183: 16000 µm2.
This has been modified (now line 225).

10. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
The protocol has been revised to exclude personal pronouns.

11. 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, Epon etc.
The terms “Epon” have been replaced by “epoxy resin” and “Eppendorf” by “microcentrifuge tube” in all the manuscript.

12. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies 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.
This has been done. The essential steps of the protocol for the video are highlighted in yellow.

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Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript describes an efficient method to prepare mouse bone marrow samples for electron microscopy investigations which allow to avoid the de-calcification step which may severely damage the sample. The method is represented by flushing the bone marrow from the cavity of long bones aver removal of the two diaphysis zones. As an example of the potential applications of the method, the manuscript present images of megakaryoctes at different stages of maturation. Although not particularly original, the method is clearly described and supported by electron microscope images of extreme high quality.

Major Concerns:

The limitations of the method described are insufficiently discussed. The authors should at least mention that, since hematopoietic stem cells and the initial phases of commitment occurs in the trabecular bone, removal of the diaphysis bias the investigation toward the medulla, which is becoming a zone of relatively low interest for hematopoiesis.
We would like to thank the reviewer for raising this point. Indeed, it has been reported that epiphyses are an important area for hematopoiesis (Ellis et al., 2011), and that their removal impedes hematopoietic stem cell analysis and initial phases of engagement. This point has now been addressed in the discussion (line 351) and the reference of Ellis *et al* added. One possibility to overcome this limitation is decalcification of whole bone. However, we have observed that treatment with EDTA causes the formation of calcium inclusion artifacts that impair the quality of EM images, as already discussed in line 338.

There is emerging information indicating that some of the megakaryocytes with the morphology of immature cells are not immature at all but are instead committed toward immunological and niche function. The authors should also discuss the limitation of defining megakaryocytes as immature based only on morphological investigation.
At the EM level, we use several ultrastructural features to define stage I megakaryocytes, namely their size (10-15 µm), their nuclear shape and the presence of extensive ribosomes and rough endoplasmic reticulum. In addition, the appearance of the earliest detectable stage of the DMS, called pre-DMS, is also a key criterion for distinguishing stage I MKs in TEM analysis (Eckly et al., 2014). This structure is not present in cells committed toward immunological and niche function. Finally, we know from our immunoelectron microscopy experiments that these cells express CD41 at their plasma membrane which demonstrates that these cells represent immature megakaryocytes. These points are now mentioned in the results section (line 251).

Minor Concerns:
None. The manuscript is a delight to read.
We would like to thank the reviewer for this positive comment.

Reviewer #2:
Manuscript Summary:
The manuscript titled, "In situ exploration of the major steps of megakaryopoiesis using transmission electron microscopy" by Scandola et al, has provided a detail method of transmission electron microscopy combining bone marrow flushing and fixation by immersion. This further provides in situ morphology characteristics of megakaryocyte morphogenesis taking place in the bone marrow. The protocol is highly instrumental for people interested in the hematopoietic stem cell science, specifically, phenotypes pertaining to megakaryocytic formation, development and it's entry into circulation.

Minor Concerns:
1. No scale bar in Figure 1D.
This has been done.

2. The scale bar Font, Size and Color should be uniform for all the images in Figure 2.
The scale bars have been updated uniformly on the right corner for all the images. In all images, the bars are now in white.

3. Figure 2B iii has inverted scale bar and needs correction.
This has been corrected.

4. Can authors provide a section for troubleshooting, which can include problems with steps involved in Fixation, polymerization, staining etc.
As suggested, we now added three troubleshooting notes concerning the fixation (ensure that the fixative is at room temperature, line 119), polymerization (avoid bubbles, line 173) and staining (avoid air displacement, line 213) steps.

Reviewer #3:

Manuscript Summary:

This manuscript aims to describe the process of collection of bone marrow from mouse bones (principally femur), fixation, resin embedding, sectioning and staining for visualization by transmission electron microscopy. This is a valuable addition to the JOVE collection, because of the importance of understanding the details of how platelets are made from their precursor cells, in vivo.

Major Concerns:

1. The title and summary need to make it clear that the focus on the mouse. Although it is indicated in the abstract, it is not very clear that this article deals specifically with mouse megakaryopoiesis.

As requested by the reviewer, the title and the abstract clarify now the focus on the mouse.

2. Although the authors have written this from a perspective of observing megakaryocytes in bone marrow, it is actually applicable to the visualization of any bone marrow cell. This broader applicability of the techniques described could be mentioned.

We thank the reviewer for this suggestion. We now added two statements about this.

Line 41 in the abstract: “This method can be used for any bone marrow cell, in any EM facility”

Line 65 in the introduction: “This chapter focuses on a standardized TEM method allowing to capture the process of platelet biogenesis occurring *in situ* within the native bone marrow microenvironment, which could also serve as a basis to analyze any bone marrow cell type.”

3. There is a description also of staining sections with toluidine blue for histology. The need for histological sections, in a manuscript about TEM, needs to be explained to the reader.

The reviewer is correct. Staining sections for histology is important for three reasons: 1) to make sure that the tissue is actually cut and not the resin, 2) to check the quality of the inclusion, and 3) to determine that the architecture of the marrow is correctly preserved. This has been clarified lines 193-195 and line 241.

Minor Concerns:

1. There are spelling errors in the text at various places, and so a spell check is required.

This has been carefully examined.

2. Line 217 states 'megakaryocytes per square is between 1.7 and 2.2'. A word is missing, to state 'per square …..'.

This has been corrected. “The average number of megakaryocytes is between 1.7 and 2.2 cells per square”

3. Line 302: states that 'proteins is investigated at nanometer resolution4'. This suggests localization down to 1 nm, which is not quite possible with TEM.

The reviewer is correct. The sentence has been modified to avoid suggesting such nonsense: “localization of proteins is investigated at high resolution”

PREPARATION OF THE REAGENTS

Cacodylate buffer. Dissolve 21.4 g dimethylarsinic acid sodium salt trihydrate and 20 g sucrose in 1 L H_2O. Then add 1 mL of 1 M $CaCl_2$ and 1 mL of 1 M $MgCl_2$ under constant agitation. Ajust the pH to 7.3 by addition of 1 M HCl or 1 M NaOH and the osmolarity to 306 mOsm/L with an equal amount (13 mL) of 1 M $MgCl_2$ and 1 M $CaCl_2$. Once filtrated (0.2 µm filter) the cadodylate buffer is clear and stable at + 4°C for three months.

Agarose. Dissolve agar powder in boiling cacodylate buffer to prepare a 2% (w/v) solution. NOTE: The agar solution remains as a liquid at 45 °C, keep the agar solution in a water bath during this step.

Epoxy resin. Mix the components under constant agitation in the following order: LX-112 resin 217.4 g, Dodecenyl Succinic Anhydride (DDSA) 104.4 g, Nadic Methyl Anhydride (NMA) 102.8 g and 2,4,6-Tri(dimethylaminomethyl)phenol (DMP-30) 7.9 g. Drawn the resin into 10 mL plastic syringes and stored at -20 °C.