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Title: In Situ Exploration of Murine Megakaryopoiesis Using Transmission Electron Microscopy

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Author Questionnaire

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

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

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

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

4. **Filming location:** Will the filming need to take place in multiple locations? **No**

Current Protocol Length
Number of Steps: 19
Number of Shots: 42

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Cyril Scandola:** The overall goal of this procedure is to observe the ultrastructure of megakaryocytes located within the mouse bone marrow and to quantify the different stages of their maturation using high resolution transmission electron microscopy **[1]**.

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

1.2. **Cyril Scandola:** The main advantage is the direct examination of MKs in their native environment, which differs from in vitro cultured MKs that, as we know, do not reach the complete level of maturation. **[1]**.

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

Ethics Title Card

1.3. All procedures involving 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).

Protocol

2. Bone Marrow Collection and Fixation

2.1. After harvesting the tibia and femurs from 12-to-18-week-old C57BL/6 (C-fifty-seven black six) mice according to standard protocols **[1]**, use a sharp razor blade to remove the epiphyses from each bone **[2]**.
- 2.1.1. WIDE: Establishing shot of Talent placing bones into collection container
- 2.1.2. Epiphysis being removed from end of bone

2.2. Holding each bone with tweezers **[1]**, insert a 21-gauge needle attached to a 5-milliliter syringe filled with cacodylate buffer into one end of the bone **[2]** and flush the bone marrow into a 15-milliliter collection tube containing 2 milliliters of fresh cacodylate buffer **[3]**.
- 2.2.1. Bone being picked up tweezers
- 2.2.2. Needle being inserted into bone *Videographer: This step is important*
- 2.2.3. Bone marrow being flushed into tube

2.3. Immediately after flushing, use a plastic pipette to transfer the bone marrow cylinders into 1-milliliter of fresh glutaraldehyde fixative solution for a 60-minutes incubation at room temperature **[1-TXT]**.
- 2.3.1. Shot of bone marrow fixation/Talent transferring the bone marrow to a fixative solution **TEXT: Dissect and fix bone marrow within 10 min of collection**

3. Embedding Bone Marrow in Agarose

3.1. For embedding of the bone marrow in agarose, wash the fixed samples in fresh cacodylate buffer **[1]** and use a plastic pipette to carefully transfer the bone marrow to a glass slide **[2]**. Using a warm pipette, quickly apply a drop of 2% liquid agar to the bone marrow cylinders **[3]** and immediately place the slide on ice for 1-2 minutes **[4]**.
- 3.1.1. WIDE: Talent giving buffer wash
- 3.1.2. Talent transferring the bone marrow to glass slide
- 3.1.3. Talent applying liquid agar to bone marrow cylinder
- 3.1.4. Talent placing the slide on ice

3.2. When the agar has solidified, use a stereomicroscope **[1]** and a sharp razor blade to discard the extremities of each bone marrow cylinder **[2]** and transfer the trimmed marrow blocks to a 1.5-milliliter microcentrifuge tube containing 1-milliliter of cacodylate buffer **[3-TXT]**.
- 3.2.1. Talent placing slide under microscope
- 3.2.2. SCOPE: Extremity being cut *Videographer: This step is important*

3.2.3. Talent transferring the marrow block to tubes **TEXT: Repeat for each bone marrow cylinder**

4. Embedding Bone Marrow in Resin

4.1. For resin embedding, fix the blocks with 1% osmium tetroxide in cacodylate buffer in a chemical hood for 1 hour at 4 degree Celsius **[1]** before washing the blocks one time with cacodylate buffer **[2]** and one time with distilled water **[3]**.
- 4.1.1. WIDE: Talent placing the blocks for fixation
- 4.1.2. Talent giving buffer wash
- 4.1.3. Talent giving water wash

4.2. After the water wash, stain the blocks with 4% uranyl acetate in distilled water for 1 hour **[1]** followed by two washes in distilled water as demonstrated **[2]**. After the last wash, dehydrate the blocks through an ascending series of ethanol immersions in distilled water as indicated **[3-TXT]**.
- 4.2.1. Talent placing the block for staining
- 4.2.2. Talent giving water wash
- 4.2.3. Talent placing block in ethanol, with other ethanol concentrations visible in frame as possible **TEXT: 75% EtOH 5 min x4 -> 95% EtOH 20 min x3 -> 100% EtOH 20 min x3**

4.3. To obtain a uniform infiltration and polymerization of the epoxy resin inside the marrow, incubate the blocks in two successive baths of propylene oxide for 15 minutes **[1]** before incubating the samples in a 1:1 mixture of 100% propylene oxide and epoxy resin for 1 hour on a slow rotary shaker at room temperature **[2]**.
- 4.3.1. Talent placing the blocks in propylene oxide bath
- 4.3.2. Shot of marrow rotating on rotator

4.4. At the end of the incubation, add 100% epoxy resin to the marrow blocks for a 2-hour incubation under the same conditions **[1]**.
- 4.4.1. Talent adding resin solution to the blocks

4.5. At the end of the incubation, use a microscope to orient the marrow blocks in flat silicone molds to permit their subsequent transverse sectioning **[1]** and fill the molds with epoxy resin **[2]** before placing them at 60 degrees Celsius for 48 hours **[3]**.
- 4.5.1. SCOPE: Block being oriented in mold *Videographer: This step is important*
- 4.5.2. Talent filling the molds with epoxy resins *Videographer: This step is important*
- 4.5.3. Talent placing mold(s) at 60 °C

5. Ultrathin Sectioning

5.1. For ultrathin sectioning, mount the sample block onto an ultramicrotome support **[1]** and mount the support onto the sample holder **[2]**.
  5.1.1. WIDE: Talent mounting the sample block in microtome support
  5.1.2. Talent mounting support onto holder

5.2. Use a diamond milling cutter to trim the samples at a 45-degree angle to remove the excess resin around the tissue **[1]** before using a diamond knife blade equipped with a water tank to cut transverse 500- and 100-nanometer-thick sections for histological and TEM (T-E-M) analysis, respectively **[2]**.
  5.2.1. Resin being trimmed
  5.2.2. Section(s) being acquired

5.3. Then use a loop to transfer the 500-nanometer-thick sections floating on the water-surface **[1]** onto a glass slide **[2]** and to deposit the 100-nanometer thick sections onto 200 mesh thin-bar copper grids with a paper filter underneath **[3]**.
  5.3.1. Section being collected from water surface *Videographer: This step is important*
  5.3.2. Talent depositing 500-nm section on a glass slide *Videographer: This step is important*
  5.3.3. Talent depositing 200-nm section on copper grids *Videographer: This step is important*

6. Toluidine Blue Staining

6.1. For toluidine blue staining, after drying the 500-nanometer thick sections on a 60-degree hot plate **[1]**, add filtered 1% toluidine blue in distilled water to the sections for a 1-2-minute incubation **[2]**.
  6.1.1. WIDE: Talent placing slide(s) onto hot plate
  6.1.2. Talent adding stain onto the slides on hot plate

6.2. At the end of the incubation, wash the samples with distilled water **[1]**. When the slides have dried, use mounting medium to mount the samples with a coverslip **[2-TXT]** and view the samples by light microscopy **[3]**.
  6.2.1. Talent giving water wash
  6.2.2. Talent adding mounting medium **TEXT: e.g., poly(butyl methacrylate-co-methyl methacrylate)**
  6.2.3. LAB MEDIA: Figure 1D

7. Heavy Metal Staining

7.1. For contrast staining, label the 100-nanometer sections with 4% uranyl acetate for 5 minutes **[1]** followed by three, 5-minute washes with distilled water **[2]**. After the last wash, stain the sections with lead citrate for 3 minutes **[3]** followed by three, 5-minute washes in stilled water as demonstrated **[4]**.

7.1.1. WIDE: Talent applying uranyl acetate to the sections *Videographer: This step is important*
7.1.2. Talent giving water wash
7.1.3. Talent applying lead citrate *Videographer: This step is important*
7.1.4. Talent washing slide(s)

7.2. After the last wash, place the lower side of each grid in contact with a piece of filter paper first to allow the grids to be placed onto the filter paper to dry **[1]**.
7.2.1. Lower slide being placed onto filter paper, then grid being placed onto paper *Videographer: This step is important*

8. Transmission Electron Microscopy (TEM)

8.1. To exam the sections by TEM, when the grids have dried, select a low magnification **[1]** to assess the general quality of the preparations **[2]**.
8.1.1. WIDE: Talent selecting magnification
8.1.2. LAB MEDIA: Figure 1E

8.2. To determine the number megakaryocytes from each stage of maturation in each transverse section, select a higher magnification **[1]** and quantify the number of stage one, two or three megakaryocytes only in squares that are fully covered by tissue **[2]**.
8.2.1. Talent selecting higher magnification
8.2.2. LAB MEDIA: Figure 2A *Video Editor: please emphasize big cell in right side of Stage I, Stage II, and Stage III images when mentioned*

Results

9. Results: *In Situ* Ultrastructure Exploration of Murine Megakaryopoiesis

9.1. In this representative histological analysis **[1]**, the compactness, microvessel continuity, and size and shape of the megakaryocytes can be clearly observed **[2]**.
- 9.1.1. LAB MEDIA: Figure 1D
- 9.1.2. LAB MEDIA: Figure 1D *Video Editor: please emphasize big cell near "1" in main image and/or cell in magnified inset*

9.2. Murine megakaryocytes are divided into 4 stages of maturation **[1]**.
- 9.2.1. LAB MEDIA: Figure 2A

9.3. Stage one megakaryocytes have a large nucleus **[1]**. The presence of the earliest detectable stage of the demarcation membrane system is also a key criterion of this stage **[2]**.
- 9.3.1. LAB MEDIA: Figure 2A Stage I image *Video Editor: please emphasize nucleus in center of big cell on right side of image*
- 9.3.2. LAB MEDIA: Figure 2A Stage I image *Video Editor: please emphasize circular membrane network between lobes of nucleus*

9.4. In stage two **[1]**, granule formation begins **[2]** and the development of the demarcation membrane system is initiated **[3]**.
- 9.4.1. LAB MEDIA: Figure 2A Stage II image
- 9.4.2. LAB MEDIA: Figure 2A Stage II image *Video Editor: please emphasize granules/black dots in cytoplasm*
- 9.4.3. LAB MEDIA: Figure 2A Stage II image *Video Editor: please emphasize white, intertwined tubular membrane network around nucleus*

9.5. Stage three megakaryocytes are giant cells **[1]** with well-developed demarcation membrane systems **[2]**, clearly defined cytoplasmic territories and peripheral zones devoid of organelles **[3]**, and eccentrically located nuclei **[4]**. The pyrenocyte stage of megakaryocyte maturation is characterized by a naked nucleus **[5]**.
- 9.5.1. LAB MEDIA: Figure 2A Stage III image
- 9.5.2. LAB MEDIA: Figure 2A Stage III image *Video Editor: please emphasize white network around nucleus*
- 9.5.3. LAB MEDIA: Figure 2A Stage III image *Video Editor: please emphasize grey empty area between white structures in dark outlined nucleus in center of cell*
- 9.5.4. LAB MEDIA: Figure 2A Stage III image *Video Editor: please emphasize dark outlined nucleus in center of cell*

9.5.5. LAB MEDIA: Figure 2A Pyrenocyte image *Video Editor: please emphasize dark black marbled nucleus in center of image*

9.6. As illustrated, megakaryocytes are frequently observed in contact with endothelial cells **[1]**. On occasion, the megakaryocytes form short invasive protrusions that penetrate the endothelium **[2]** or extend into the sinusoidal lumen **[3]**.

9.6.1. LAB MEDIA: Figure 2B i *Video Editor: please emphasize red dashed line*

9.6.2. LAB MEDIA: Figures 2B ii and v *Video Editor: please emphasize black arrowheads in Figure 2B ii*

9.6.3. LAB MEDIA: Figures 2B ii and v *Video Editor: please emphasize black arrowhead in Figure 2B v*

9.7. TEM also permits the visualization of ultrastructural details **[1]** as well as the presence of hematopoietic cells that have been engulfed by the megakaryocytes **[2]**.

9.7.1. LAB MEDIA: Figures 2C and 2D *Video Editor: please emphasize white text/structures labeled by white text in Figure 2C*

9.7.2. LAB MEDIA: Figures 2C and 2D *Video Editor: please emphasize on letter 'N' in Figure 2D*

Conclusion

10. Conclusion Interview Statements

10.1. **Cyril Scandola:** Bone marrow flushing has to be performed carefully and immediately after bone dissection. To maximize tissue integrity, the marrow is surrounded with a gel of agar before dehydration **[1]**.

10.1.1. INTERVIEW: JoVE talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B role: 2.2, 2.3*

10.2. **Cyril Scandola:** Following this procedure, the bone marrow blocks can be imaged at different magnifications or can be used for other 3D electron microscopy analyses, such as Focused-Ion Beam Scanning Electron Microscopy **[1]**.

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