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Title: Real-Time Imaging of CCL5-Induced Migration of Periosteal Skeletal Stem Cells in Mice

Authors and Affiliations: Laura Ortinau[1,2], Kevin Lei[1], Youngjae Jeong[1], and Dongsu Park[1,2,3]

[1]Department of Molecular & Human Genetics, Baylor College of Medicine
[2]Center for Skeletal Biology, Baylor College of Medicine
[3]Department of Pathology & Immunology, Baylor College of Medicine

Corresponding Author:
Dongsu Park
dongsu.park@bcm.edu

Co-authors:
laura.ortinau@bcm.edu
kevin.lei@bcm.edu
youngjae.jeong@bcm.edu

Author Questionnaire

1. Microscopy: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **Y**

If **Yes**, can you record movies/images using your own microscope camera?

Y

2. Software: Does the part of your protocol being filmed demonstrate software usage? **Y**

If **Yes**, we will need you to record using screen recording software to capture the steps. If you use a Mac, QuickTime X also has the ability to record the steps. Please upload all screen captured video files to your project page as soon as reasonable possible.

Videographer: please film for reference

3. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Protocol Length
Number of Shots: **37**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Laura Ortinau**: This standardized protocol can be used to assess endogenous cell migration and can be applied to several cell types and skeletal phenotypes **[1]**.

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

1.2. **Laura Ortinau**: The advantage of this technique is that it allows in vivo cell migration to be tracked for individual cells, rather than a population of cells, in response to injury and/or cytokine treatment **[1]**.

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

Ethics Title Card

1.3. Procedures involving animal subjects have been approved by the Institutional Animal Care and Use Committee (IACUC) at Baylor College of Medicine.

Protocol

2. Surgical Procedure

2.1. Before beginning the procedure, confirm a lack of response to toe pinch in the anesthetized mouse **[1]** and apply ointment to the animal's eyes **[2]**.

2.1.1. WIDE: Talent pinching toe *Videographer: More Talent than mouse in shot*

2.1.2. ECU: Ointment being applied NOTE: Macro

2.2. Next, make a transverse less-than-1-centimeter incision from immediately medial to the right ear toward the left ear **[1]** and make a second incision from the initial incision approximately 2-3 millimeters past the right eye toward the nose **[2]**. NOTE: Combined macro shot of 2.2.1 and 2.2.2 as 2.2.1

2.2.1. Incision being made from right ear toward left ear *Videographer: Important step*

2.2.2. Incision being made toward nose *Videographer: Important step*

2.3. Use forceps to separate the skin from the periosteum **[1]** and use scissors to gently cut through the connective tissue to separate the skin from the calvaria periosteum **[2]**. NOTE: Combined macro shot of 2.3.1 and 2.3.2 as 2.3.1

2.3.1. Tissues being separated *Videographer: Important step*

2.3.2. Tissue being cut *Videographer: Important step*

2.4. Using a sterile cotton swab, apply ophthalmic ointment to the top of the skin flap **[1]** and gently fold the flap over the left eye **[2]**. The intersection of the sagittal and coronal sutures should be clearly exposed **[3]**. NOTE: Combined shot of 2.4.1 and 2.4.2 as 2.4.1

2.4.1. Ointment being applied

2.4.2. Flap being folded

2.4.3. ECU: Shot of sutures

2.5. Flush the open surface with sterile PBS to clean the area of any blood and residual hair **[1]** and place the tip of a bevel one to two needle widths toward the nose from the coronal suture and one to two needle widths to the right of the sagittal suture **[2]**.

2.5.1. Skull being flushed NOTE: Macro

2.5.2. SCOPE: Bevel being placed NOTE: Combined shot of 2.5.2 with 2.6.1 and 2.6.2

2.6. Using a very small amount of downward pressure, carefully rotate the syringe clockwise one time **[1]** and counterclockwise several times **[2-TXT]**.

2.6.1. Syringe being rotated clockwise *Videographer: Important/difficult step*

2.6.2. Syringe being rotated counterclockwise *Videographer: Important/difficult step* **TEXT: Caution: Do not penetrate brain**

2.7. Then use a 27-gauge needle to widen the microfracture to approximately 40 micrometers **[1]**.

2.7.1. SCOPE: Fracture being widened *Videographer: Important step*

2.8. If bone fragments remain, flush the microfracture with PBS **[1]**. If bone fragments still remain, use a 29-gauge needle to gently scoop the fragments out **[2]**.

2.8.1. Bone being flushed NOTE: Macro

2.8.2. SCOPE: Fragments being scooped

3. CC Chemokine Ligand 5 (CCL5) Treatment

3.1. For CCL5 (C-C-L-five) treatment, use a 100 nanogram/milliliter stock CCL5 solution **[1-TXT]** and basement membrane matrix to obtain a 10 nanogram/milliliter working chemoattractant solution **[2]**.

3.1.1. WIDE: Talent adding stock to working solution container, with stock container visible in frame **TEXT: CCL5: RANTES**

3.1.2. Talent adding basement membrane matrix to container, with basement membrane matrix container visible in frame

3.2. To treat the injury, load a 2-20-microliter pipette with 5 microliters of the solution **[1]** and apply 2 microliters of the chemokine to the suture **[2]**.

3.2.1. Talent loading pipette *Videographer: Important step*

3.2.2. SCOPE: Solution being applied *Videographer: Important step*

3.3. Then allow the matrix to solidify **[1]** and gently cover the calvaria with the skin flap to ensure that the tissue does not become dehydrated during the 1-hour chemokine treatment **[2]**.

3.3.1. SCOPE: Shot of solidified matrix

3.3.2. SCOPE: Skin flap being replaced

4. Intravital Imaging Preparation

4.1. For intravital imaging of periosteal stem cell migration in real-time, before moving the mouse to the microscope **[1]**, apply gentle pressure to the back of the mouse's head to check the visual plane of the calvaria **[2]**. NOTE: 4.1.1, 4.1.2 and 4.2.1 shots are combined

4.1.1. WIDE: Talent applying pressure *Videographer: More Talent than mouse in shot*

4.1.2. Pressure being applied

4.2. If the plane is not level, gently rotate the mouse restraint to the left or the right to adjust the position **[1]**.

4.2.1. Restraint being rotated

4.3. Next, cover the entire calvaria and skin flap with sterile 2% methylcellulose in water **[1]** and place the mouse on the XYZ-axis motorized microscope stage **[2]**.

4.3.1. Bone and flap being covered with methycellulose

4.3.2. Talent placing mouse onto stage *Videographer: More Talent than mouse in shot*

4.4. Using the epifluorescent light, align the mouse so that it is facing the microscope **[1]** and that the intersection of the coronal and sagittal sutures is the centered reference point of the stage **[2]**. NOTE: Shots 4.4.1, 4.4.2, 4.5.1 and 4.5.2 are combined

4.4.1. Talent aligning mouse *Videographer: More Talent than mouse in shot*

4.4.2. Suture being centered

4.5. Then place a glass cover on the imaging area **[1]** and double-check the alignment **[2]**.

4.5.1. Cover glass being placed

4.5.2. Talent checking alignment

5. Time-Lapse Imaging

5.1. For time-lapse imaging of the migration into and out of the suture, select a low magnification, water immersion lens to scan the calvaria **[1]**.

5.1.1. SCREEN: ~~WIDE~~: Talent selecting objective NOTE: Videographer submitted the screen capture video along with the rest of the footage

5.2. Acquire a reference image of the sagittal and coronal suture intersection and, using the SHG (S-H-G) and fluorescent signals from the cells, locate each injury site and record the XYZ coordinates as well as the distances from the sagittal and coronal sutures **[1-TXT]**.

5.2.1. SCREEN: Reference image being acquired, injury being located *Video Editor: please emphasize coordinates and distances when mentioned* **TEXT: SHG: second harmonic generation** NOTE: Videographer submitted the screen capture video along with the rest of the footage

5.3. Select a location on either the coronal or sagittal sutures for long-term imaging and obtain a detailed Z-stack image of this area from reference during imaging **[1-TXT]**.

5.3.1. SCREEN: Location being selected, image being acquired **TEXT: P-SSC will migrate from sutures in response to calvaria injury** NOTE: Videographer submitted the screen capture video along with the rest of the footage

5.4. Use the time-lapse software settings to record an image every 1 minute for at least 1 hour **[1]**, comparing the current field of view with the initial field of view in the time between snapshots **[2]**.

5.4.1. SCREEN: Image being acquired NOTE: Videographer submitted the screen capture videos along with the rest of the footage

5.4.2. SCREEN: FOVs being compared NOTE: Videographer submitted the screen capture videos along with the rest of the footage

5.5. If the fields of view are different, use the Z-stack image to determine in which direction the field of view needs to be adjusted **[1-TXT]**.

5.5.1. SCREEN: Z-stack image being used to determine in which direction FOV needs to be adjusted NOTE: Videographer submitted the screen capture videos along with the rest of the footage

Results

6. Results: Representative Real-Time In Vivo *Mx1*$^{+}$*alpha-SMA*$^{+}$ Periosteal Skeletal Stem Cells (P-SSC) Migration Imaging

6.1. Recently, Mx1-Tomato-alphaSMA-GFP (m-x-one-tomato-alpha-S-M-A-G-F-P) reporter mice were generated in which periosteal skeletal stem cells are marked by *Mx1-positive alpha-SMA-positive* dual labeling **[1]**.

6.1.1. LAB MEDIA: Figure 2B *Video Editor: please yellow signal* **TEXT: SMA: spinal muscular atrophy**

6.2. Little to no *Mx1-positive alpha-SMA-positive* periosteal skeletal stem cell migration is observed over a 1-hour period of imaging 24 hours after suture placement **[1]**.

6.2.1. LAB MEDIA: Figure 2C images *Video Editor: please sequentially emphasize yellow signal in images from 0:0 to 0:40*

6.3. The CCL5 chemokine treatment of a calvaria defect 24 hours post-injury induces directionally distinct migration away from the coronal suture and toward the injury sight **[1]**.

6.3.1. LAB MEDIA: Figure 2D images *Video Editor: please sequentially emphasize yellow signal in images from 0:00 to 1:00*

6.4. In this representative analysis, within 1 hour of imaging, one of the *Mx1-positive alpha-SMA-positive* periosteal skeletal stem cells migrated approximately 50 micrometers toward the injury **[1]**.

6.4.1. LAB MEDIA: Figure 2E *Video Editor: please sequentially emphasize yellow cell near white asterisk from 0:00 to 1:100 image*

Conclusion

7. Conclusion Interview Statements

7.1. **Laura Ortinau**: The most important aspect of this protocol to remember is to limit the CCL5 treatment to the injury site in order to stimulate cell specific migration **[1]**.

7.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (3.2., 3.3.)

7.2. **Laura Ortinau**: Once this procedure is complete, localized treatment can be continued for one week and µCT can be used to assess calvaria injury healing and mineral deposition **[1]**.

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