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# Title: Skeletal Phenotype Analysis of a Conditional Stat3 Deletion Mouse Model

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# Author Questionnaire

1. **Microscopy**: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes**

**Videographer: Please film SCREEN shot 3.5.2.**

3. **Filming location:** Will the filming need to take place in multiple locations? **Yes**

   If **Yes**, how far apart are the locations? **7 km apart**

**Current Protocol Length**

Number of Steps: 18
Number of Shots: 53

# Introduction

**1. Introductory Interview Statements**

**REQUIRED:**

1.1. **Yiling Yang:** Genetically engineered mouse models are powerful tools for studying the mechanisms of human disease in vivo. Analyzing the skeletal phenotype of mice is the basis of skeletal research.

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

1.2. **Yiling Yang:** This protocol describes some typical techniques for analyzing the skeletal phenotype, which may be interesting for those who are new to skeletal tissue research.

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

**OPTIONAL:**

1.3. **Yiling Yang:** When attempting this protocol, keep in mind that animal experiments take time. Collect as many high-quality samples as possible during each experiment, even if you don't need them in the short term.

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

**Introduction of Demonstrator on Camera**

1.4. **Yiling Yang:** I will demonstrate this procedure. NOTE: Skip this

1.4.1. INTERVIEW: Author saying the above.

1.4.2. The named demonstrator(s) looks up from workbench or desk or microscope and acknowledges the camera.

**Ethics Title Card**

1.5. Procedures involving animal subjects have been approved by the Institutional Animal Care and Use Committee (IACUC) at Shanghai Jiaotong University School of Medicine.

# Protocol

## 2. Specimen Collection

2.1. Begin by placing the euthanized mouse in a supine position **[1]** and gently dislocating the bilateral hip joints by hand **[2]**. Use ophthalmic scissors to vertically cut off the skin from the distal tibia and then remove all skin from the hind limb **[3]**.

2.1.1. WIDE: Establishing shot of talent positioning the mouse.

2.1.2. Talent dislocating the hip joints.

2.1.3. Talent cutting off the skin.

2.2. Cut off the articular ligament of the right hip joint and knee joint with scissors to separate the hind limb **[1]**, then cut the trochanter and the junction of the fibula **[2]**. Immerse the hind limb in 4% PFA, keeping the right hind limbs for paraffin sectioning **[3]**. Cut the bone at both ends to fully immerse the bone marrow in 4% PFA **[4]**. *Videographer: This step is important!*

2.2.1. Talent cutting off the articular ligament.

2.2.2. Talent cutting the trochanter and fibula junction.

2.2.3. Talent putting the hind limb in PFA.

2.2.4. Talent cutting the ends of the bones.

2.3. Cut the articular ligaments of the left hip and knee joints with scissors **[1]** and gently remove the soft tissue **[2]**. Separate the tibia and femur **[3-TXT]** and immerse them separately in 75% ethanol. Keep the femora for micro-CT scanning and the tibiae for calcein and alizarin red double labeling **[4]**. *Videographer: This step is important!*

2.3.1. Talent cutting articular ligament.

2.3.2. Talent removing the soft tissue.

2.3.3. Talent separating the tibia and femur. **TEXT: Keep the trochanter intact**

2.3.4. Talent immersing the bones in separate containers of ethanol.

## 3. Paraffin Section Preparation

3.1. To prepare paraffin sections, gently wash the fixed right hind limb 3 times with PBS for 10 minutes per wash **[1]**, then decalcify it in 15% EDTA with an ultrasonic decalcifier for 3 to 4 weeks until the bones can be bent, replacing the decalcifying fluid every other day **[2]**. *Videographer: This step is important!*

3.1.1. Talent washing specimen in PBS.

3.1.2. Talent putting the sample in the ultrasonic decalcifier.

3.2. After decalcification, wash the specimens 3 times with PBS **[1]** and immerse them in 75% ethanol at 4 degrees Celsius overnight **[2]**. On the second day, sequentially immerse specimens in 95% ethanol, 100% ethanol, and xylene, for 1 hour each **[3-TXT]**.

3.2.1. Talent washing decalcified specimen in PBS.

3.2.2. Talent immersing the specimen in ethanol.

3.2.3. Talent moving the specimen from one solution to another, with all solutions labeled. **TEXT: Perform dehydration 2 X**

3.3. Immerse the specimens in half xylene and half paraffin for 30 minutes **[1]**, then in paraffin at 65 degrees Celsius overnight **[2]**.

3.3.1. Talent immersing the specimen in xylene and paraffin, with the container labeled.

3.3.2. Talent immersing the specimen in paraffin, with the container labeled.

3.4. To embed the specimen, submerge it in paraffin, placing the femur and tibia at a 90-degree angle. When the paraffin has fully cooled, remove the specimens from the embedding tank **[2]**. Number and store them at -20 degrees Celsius overnight **[3]**.

~~3.4.1. Talent positioning the specimens in paraffin.~~

3.4.2. Talent removing the specimens from the embedding tank.

3.4.3. Talent putting the numbered specimens in the freezer and closing the door.

3.5. After scanning the left femora with a micro-CT scanner, reconstruct 3D images of the cortical bone and trabecular bone using the scanner's supporting software **[1]**. The regions of interest are in a total 1-millimeter width of trabecular bone near the distal growth plate and in a 1-millimeter width of cortical bone in the middle of the femora **[2]**.

3.5.1. Talent at the computer.

3.5.2. SCREEN: ROIs selected in the software. *Videographer: Please film the screen, authors were not able to record SC video.*

**4. TRAP Staining**

4.1. Bake the paraffin sections at 65 degrees Celsius for 30 minutes **[1]**, then dewax them by immersing them in xylene for 10 minutes. Immerse the sections 3 times, with fresh xylene each time **[2]**.

4.1.1. Talent putting the sections in the oven.

4.1.2. Talent immersing the sections in xylene, with the container labeled.

4.2. Rehydrate the sections by immersing them sequentially in 100% ethanol, 95% ethanol, 70% ethanol, and distilled water for 5 minutes each **[1-TXT]**. Prepare the staining solution using the TRAP staining kit and warm it to 37 degrees Celsius **[2]**.

4.2.1. Talent moving the specimen from one solution to another, with all solutions labeled. **TEXT: Perform rehydration 2 X**

4.2.2. Talent warming up the prepared TRAP staining solution.

4.3. Add 50 to 100 microliters of staining solution to each section **[1]** and incubate them in a 37 degrees Celsius humid chamber for 20 to 30 minutes **[2]**. Check the staining status of the osteoclasts under a light microscope every 5 minutes until red multinucleated osteoclasts can be seen **[3]**, then end the reaction with water **[4]**.

4.3.1. Talent adding staining solution to a section.

4.3.2. Talent putting the sections in the humid chamber.

4.3.3. Talent checking the staining under a microscope.

4.3.4. Talent adding water to end the reaction.

4.4. Counterstain the sections in hematoxylin solution for 30 seconds **[1]** and create a stable blue color by immersing them in 1% ammonia solution for 1 minute **[2]**. Then, rinse them in slowly running tap water **[3]**. Mount the sections using coverslips with neutral balsam and dry them overnight **[4]**.

4.4.1. Talent staining the sections in hematoxylin.

4.4.2. Talent immersing the sections in ammonia solution.

4.4.3. Talent rinsing sections under running water.

4.4.4. Talent mounting the sections.

~~4.5. **[1-TXT] [2], [4]**.~~

~~4.5.1. Talent cutting sections. **TEXT: Cut 20–40 sections**~~

~~4.5.2. Talent spreading the sections on water.~~

~~4.5.3. Sections on microscope slides.~~

~~4.5.4. Talent putting the slides in the oven.~~

4.6. Capture 3 to 5 fields of view with a microscope and analyze the trabecular perimeter with Image J **[1]**.

4.6.1. Talent using the microscope.

4.7. Use the **straight line** tool to measure the length of the scale bar as L1, then use the **segmented line** tool to measure the length of trabecular perimeter as L2. Calculate the physical length and count the number of TRAP-positive cells with more than three nuclei **[1-TXT]**.

4.7.1. SCREEN: TRAP.mov. 0:04 – end. *Video Editor: Speed up from 0:25 – 1:00 as necessary.* **TEXT:** $L_p = \frac{L_s \times L_2}{L_1}$

## 5. Calcein and Alizarin Red Double Labeling

5.1. After fixation, gently wash the tibiae 3 times with PBS **[1]** and sequentially immerse the specimens in 95% ethanol, 100% ethanol, and xylene for 5 minutes each **[2-TXT]**. Immerse the specimens in acetone for 12 hours **[3]**, in half acetone and half resin for 2 hours **[4]**, and in pure resin in a drying oven overnight **[5]**.

5.1.1. Talent washing the tibia in PBS.

5.1.2. Talent moving the specimen from one solution to another, with all solutions labeled. **TEXT: Perform dehydration 2 X** NOTE: Not filmed, use 3.2.3 here

5.1.3. Talent immersing the specimen in acetone, with the container labeled.

5.1.4. Talent immersing the specimen in acetone and resin mix, with the container labeled.

5.1.5. Talent putting the specimen in resin in the drying oven.

5.2. Add pure resin into a suitable silica gel embedding tank and gently place the specimens in the tank, avoiding bubbles **[1]**. Polymerize the resin in a drying oven at 60 degrees Celsius for 48 hours **[2]**.

5.2.1. Talent placing specimens in the embedding tank.

5.2.2. Talent putting the tank in the oven and closing the door.

5.3. Cut the specimens into 5-micrometer thick sections continuously with a rotary microtome **[1]** and store the rest of the samples with desiccant at room temperature **[2]**.

5.3.1. Talent cutting the specimens.

5.3.2. Samples stored with desiccant.

5.4. Adhere the sections with tweezers in a drop of 75% alcohol **[1]** and mount them with coverslips using neutral balsam **[2]**. Capture the red and green fluorescence labeling with a fluorescence microscope **[3]**.

5.4.1. Talent adhering the sections.

5.4.2. Talent mounting the sections.

5.4.3. Talent using the microscope.

# Results

## 6. Results: Effects of *Stat3* deletion on Osteoclast Differentiation

6.1. Osteoclast specific *Stat3* deletion mice were generated to study the influence of STAT3 deletion on osteoclast differentiation **[1]**. Femora reconstruction and quantitative analysis by micro-CT indicated that the bone mass of the *Stat3*$^{Ctsk}$ *(pronounce 'stat-3-C-T-S-K')* mice was increased compared to wild type mice **[2]**.

6.1.1. LAB MEDIA: Figure 2 A and B.

6.1.2. LAB MEDIA: Figure 2 C – H.

6.2. Histomorphology of the femora from wild type and *Stat3*$^{Ctsk}$ mice was examined via H and E staining **[1]**.

6.2.1. LAB MEDIA: Figure 3.

6.3. Osteoclastogenic activity was detected using TRAP staining. Osteoclasts are large, TRAP-positive cells with multiple nuclei **[1]**. The number of TRAP-postive osteoclasts was lower in *Stat3*$^{Ctsk}$ mice compared with wild type mice, indicating that STAT3 deficiency impaired osteoclast formation **[2]**.

6.3.1. LAB MEDIA: Figure 4 A.

6.3.2. LAB MEDIA: Figure 4 B.

6.4. Osteogenesis was measured with calcein and alizarin red double labeling **[1]**. The area between the calcein **[2]** and alizarin red fluorescence **[3]** represents newly formed bone **[4]**. The deleted STAT3 in osteoclasts did not influence bone anabolism **[5]**.

6.4.1. LAB MEDIA: Figure 5 A and C.

6.4.2. LAB MEDIA: Figure 5 A and C. *Video Editor: Emphasize the green.*

6.4.3. LAB MEDIA: Figure 5 A and C. *Video Editor: Emphasize the red.*

6.4.4. LAB MEDIA: Figure 5 A and C.

6.4.5. LAB MEDIA: Figure 5 B and D.

# Conclusion

**7. Conclusion Interview Statements**

7.1. **Qianye Chen:** The quality of the paraffin sections is the base of the histological analysis. Sagittal paraffin sections in which the cartilage layer was symmetrical and showed a clear M-shaped line was used here. NOTE: This one is uploaded to AWS.

7.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 4.5.1. Videographer: Please skip this, Qianye Chen will record her own video statement.*

7.2. **Yiling Yang:** Further studies will include more characteristics of the skeletal system, such as mechanical properties.

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