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Adult mouse digit amputation and regeneration; A simple model to investigate mammalian blastema formation and intramembranous ossification

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Cover Letter

Lindsay Dawson, PhD
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TEXAS A&M UNIVERSITY

February 28, 2019

Dr. Phillip Steindel
Editor, **Journal of Visualized Experiments**

Dear Dr. Steindel,

We are submitting the revised original manuscript entitled “Adult mouse digit amputation and regeneration; A simple model to investigate mammalian blastema formation and intramembranous ossification” by Lindsay A. Dawson, Regina Brunauer, Katherine N. Zimmel, Osama Qureshi, Alyssa R. Falck, Patrick Kim, Connor P. Dolan, Ling Yu, Yu-Lieh Lin, Benjamin Daniel, Mingquan Yan, and Ken Muneoka to the **Journal of Visualized Experiments**. We are submitting a protocol of adult mouse distal terminal phalanx amputation, which is a procedurally simple and reproducible model of epimorphic regeneration characterized by blastema formation and bone regeneration via intramembranous ossification. Our protocol demonstrates the standardized terminal phalanx amputation plane, the processing of amputated digits for immunofluorescent analysis, and a detailed description of fluorescent immunohistochemistry to investigate blastema formation, revascularization in the context of regeneration, and bone regeneration via intramembranous ossification. Moreover, we demonstrate the use of in-vivo microcomputed tomography to create high resolution images and for the quantification of bone changes in the same digit over the course of regeneration. We believe this protocol will add to the somewhat limited database for mammalian epimorphic regeneration and the manuscript is of interest to the **Journal of Visualized Experiments** readership.

In this resubmission, we have edited the document to address all of your and the reviewers comments, as well added an additional figure and a second panel to Figure 3 to further address the reviewer comments.

We have no conflicts of interest and this work has not been previously published nor is it under review at another journal. All authors meet the qualifications for authorship and have had opportunity to read and comment upon the manuscript.

All experimental animal studies were performed in accordance with approval from the Institutional Animal Care and Use Committee at the College of Veterinary Medicine and Biomedical Sciences at Texas A&M University.

Sincerely,

Lindsay Dawson

TITLE:

Adult Mouse Digit Amputation and Regeneration, a Simple Model to Investigate Mammalian Blastema Formation and Intramembranous Ossification

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KEYWORDS:

digit, amputation, epimorphic regeneration, intramembranous ossification, osteoprogenitors, blastema, decalcification, fluorescence immunohistochemistry, microcomputed tomography

SUMMARY:

Here, we present a protocol of adult mouse terminal phalanx amputation to investigate mammalian blastema formation and intramembranous ossification, analyzed by fluorescent immunohistochemistry and sequential in-vivo microcomputed tomography.

ABSTRACT:

Here, we present a protocol of adult mouse distal terminal phalanx (P3) amputation, a procedurally simple and reproducible mammalian model of epimorphic regeneration, which

involves blastema formation and intramembranous ossification analyzed by fluorescence immunohistochemistry and sequential in-vivo microcomputed tomography (μCT). Mammalian regeneration is restricted to amputations transecting the distal region of the terminal phalanx (P3); digits amputated at more proximal levels fail to regenerate and undergo fibrotic healing and scar formation. The regeneration response is mediated by the formation of a proliferative blastema, followed by bone regeneration via intramembranous ossification to restore the amputated skeletal length. P3 amputation is a preclinical model to investigate epimorphic regeneration in mammals, and is a powerful tool for the design of therapeutic strategies to replace fibrotic healing with a successful regenerative response. Our protocol uses fluorescence immunohistochemistry to 1) identify early-and-late blastema cell populations, 2) study revascularization in the context of regeneration, and 3) investigate intramembranous ossification without the need for complex bone stabilization devices. We also demonstrate the use of sequential in vivo μCT to create high resolution images to examine morphological changes after amputation, as well as quantify volume and length changes in the same digit over the course of regeneration. We believe this protocol offers tremendous utility to investigate both epimorphic and tissue regenerative responses in mammals.

INTRODUCTION:

Mammals, including humans and mice, have the capacity to regenerate the tips of their digits after distal amputation of the terminal phalanx (P3)[1-3]. In mice, the regeneration response is amputation-level-dependent; increasingly proximal digit amputations display a progressively attenuated regenerative response until complete regenerative failure at amputations transecting and proximal to the P3 nail matrix[4-8]. P3 regeneration is mediated by the formation of a blastema, defined as a population of proliferating cells that undergo morphogenesis to regenerate the amputated structures[9]. The formation of a blastema to regenerate the structures lost by amputation, a process termed epimorphic regeneration, distinguishes the multi-tissue-level P3 regeneration response from traditional tissue repair after injury[6,10]. P3 regeneration is a reproducible and procedurally simple model to investigate complex regenerative processes including wound healing[11,12], bone histolysis[11,12], revascularization[13], peripheral nerve regeneration[14], and blastemal conversion to bone via intramembranous ossification[15].

Previous studies using immunohistochemistry have demonstrated that the blastema is heterogeneous, avascular, hypoxic, and highly proliferative[11,13,15,16]. Following distal P3 amputation, the early blastema is initially associated with the P3 periosteum and endosteum and is characterized by robust proliferation and nascent osteogenesis adjacent to the bone surface[15]. Subsequent to bone degradation and wound closure, the heterogeneous blastema is formed by the merging of periosteal and endosteal-associated cells, followed by the differentiation of blastemal components including bone via intramembranous ossification[15].

Bone repair in response to injury typically occurs by endochondral ossification, i.e. via an initial cartilaginous callus that forms a template for subsequent bone formation[17,18]. Long bone intramembranous ossification, i.e., bone formation without a cartilaginous intermediate, is commonly induced using complex distraction devices or surgical fixation[19,20]. The digit regeneration response is a pre-clinical model that offers advantages over conventional

intramembranous ossification models: 1) it does not require external or internal fixation post injury to stimulate intramembranous ossification, 2) it is performed using 4 digits from each animal, thus maximizing samples while minimizing animal use, and 3) sequential in vivo microcomputed tomography (μCT) analysis can be performed with ease and speed.

In the present study, we show the standardized P3 amputation plane to achieve a reproducible and robust regeneration response. Additionally, we demonstrate an optimized fluorescence immunohistochemistry protocol using paraffin sections to visualize blastema formation, revascularization in the context of regeneration, and blastemal conversion to bone via intramembranous ossification. We also demonstrate the use of sequential in-vivo μCT to identify changes in bone morphology, volume, and length in the same digit over the course of regeneration. The goal of this protocol is to investigate mammalian blastema formation after amputation and to demonstrate 2 techniques, fluorescence immunohistochemistry and sequential in-vivo μCT, for the study of intramembranous bone regeneration.

PROTOCOL:

All animal use and techniques were in compliance with the standard operating procedures of the Institutional Animal Care and Use Committee of Texas A&M University.

1. Adult Mouse Hind Limb Distal P3 Amputation

1.1. Anesthetize an 8-to-12-week-old CD-1 mouse (**Table of Materials**) using isoflurane gas in oxygen; initially anesthetize at 3% in a chamber, followed by 2% isoflurane supplied by a nosecone over the duration of the surgery. Apply ophthalmic ointment on eyes to prevent dryness while under anesthesia.

NOTE: The adult P3 amputation studies standardized in our lab are performed on 8-to-12-week-old mice, and the regeneration response is conserved in all tested strains.

1.2. Under a 10x dissection microscope, sterilize the digits of the hind limb with surgical Povidone-iodine and 70% ethanol. Trim hair away from the surgical site using micro-scissors. Apply 1 μL of topical Bupivacaine locally to anesthetize the surgical site.

1.3. Amputate the distal tip of the terminal phalanx (P3) on digits 2 and 4 of each hind limb using a sterile #10 scalpel (**Figure 1A**). Amputation must be performed under aseptic conditions, including sterilization of surgical instruments. Under the dissection microscope, gently splay the hind paw to expose the medial digit surface. Hold the scalpel at an angle parallel to the fatpad to perform the amputation shown in **Figure 1B**.

NOTE: Amputation transects the nail organ, dermis, the P3 bone, vasculature, and nerves, but does not transect the marrow cavity or the digit fat pad. If bleeding is observed, apply direct pressure using a sterile cotton tip applicator.

1.4. The standard P3 distal amputation plane removes approximately 15%–20% of the P3 bone volume[21]. MicroCT scanning (see below) can be performed to confirm the correct amputation length.

1.5. Return the mouse to a clean cage and allow the wound to heal without wound dressing. Monitor the mouse for 3 days to ensure the mouse returns to normal activity.

2. Digit Collection and Tissue Preparation

2.1. Euthanize the mouse using carbon dioxide (CO_2) in a closed chamber, followed by cervical dislocation.

2.2. Use a scalpel to sever the digit mid-way through the adjacent bone segment, the middle phalanx (P2) bone, at approximately the second ventral fat pad indent. Transfer the digit(s) to a 20 mL scintillation vial containing 10 mL fresh buffered zinc formalin fixative, an 18% formaldehyde solution (see **Table of Materials**). Fixative volume should be at least 20x the volume of the tissue present.

NOTE: Digits are collected at various timepoints after amputation to investigate the full regeneration response. Generally, for early blastema formation, bone histolysis, and wound closure the collection timepoints are 4–8 days post amputation (DPA). To investigate the early osteogenic blastema the collection timepoints are 9 and 10 DPA, and to visualize continued bone regeneration and mineralization, the collection timepoints are 14, 21, and 28 DPA. Unamputated digits are collected as well.

2.3. Fix the digit for 24–48 h at room temperature with gentle mixing on a shaker.

2.4. Remove the fixative and wash the digit 2x for 5 min in 5 mL of 1x phosphate buffered saline (PBS) at room temperature.

2.5. Place 10 mL of fresh Decalcifier I (see **Table of Materials**), a 10% formic acid solution, in the scintillation vial and gently mix on a shaker for 2 h at room temperature. After 2 h, replace Decalcifier I with fresh solution and decalcify digit overnight at room temperature with gentle mixing on shaker.

2.6. Remove the Decalcifier I and wash the digit 2x for 5 min in 5 mL of 1x PBS at room temperature.

2.7. Process the digit through a graded ethanol series. This process can be performed manually in a 20 mL scintillation vial, using 10 mL of each liquid if less than 40 total digits.

2.7.1. Begin with 2 immersions in fresh 70% ethanol at room temperature with gentle mixing on a shaker, 1 h each, followed by 2 immersions in fresh 95% ethanol at room temperature with gentle mixing on a shaker, 1 h each.

2.7.2. Complete the digit dehydration with 2 washes of fresh 100% ethanol at room temperature with gentle mixing on a shaker, 1 h each. The final 100% ethanol wash can be left overnight.

2.8. In the same scintillation vial, replace the 100% ethanol with 10 mL of fresh xylenes, and place the vial in a chemical fume hood for 1.5 h at room temperature. Repeat with a fresh wash of xylenes in a chemical fume hood for 1.5 h at room temperature for a total of 2 washes.

2.9. Replace the xylenes with liquid paraffin wax melted to 68 °C, and place the vial in an incubator at 68 °C for 2 h, 2 times. After 4 total h of immersion in paraffin wax, embed the digit for paraffin sectioning.

NOTE: Digit processing for histology can be performed in an automated processor, following these same guidelines.

2.10. Section digits at 4–5 µm thickness using a microtome. Place sectioned samples on adhesive slides, using a 38–41 °C water bath supplemented with a histological adhesive solution to ensure samples adhere to the slide. Place slides on a 37 °C slide warmer to dry.

3. Immunohistochemical staining of adult mouse digits to investigate blastema formation and intramembranous ossification

3.1. Heat sectioned slides at 65 °C for 45 min, followed by heating at 37 °C for no less than 15 min to ensure samples adhere to the slide.

3.2. Deparaffinize and rehydrate slides 2x with 5 min washes of xylenes, a graded ethanol series, and submersion in water. Place slides in a 50–100 mL capacity staining jar and keep immersed in 1x Tris Buffered Saline with Tween 20 (TBST).

NOTE: Do not allow samples to dry throughout the staining process. At any TBST step, slides can be incubated up to 2 h.

3.3. Prepare a humidifying chamber. A 1-inch deep covered plastic slide box with moistened tissue paper at the base is sufficient.

3.4. Prepare the heat retrieval solution for Runx2, Osterix (OSX), and Proliferating Cell Nuclear Antigen (PCNA). For the antigen retrieval of the early osteoprogenitor marker, Runx2, prepare a 1x solution of Tris-EDTA, pH 8. For the osteoblast marker, OSX, prepare a 1x solution of citrate buffer, pH 6. PCNA immunostaining can be performed in either solution.

3.5. Prepare Proteinase K antigen retrieval solution for the blastema marker CXCR4[22] and the endothelial cell marker von Willebrand Factor 8 (vWF) by placing 100 µL of Proteinase K solution per slide in a microcentrifuge tube and heating to 37 °C (approximately 5 min).

NOTE: Antigen retrieval for Runx2, OSX, and PCNA is performed using heat retrieval, and CXCR4 and vWF antigen retrieval is performed via proteinase K treatment.

3.6. For antibody retrieval requiring heat, immerse slides in a staining jar in the appropriate antigen retrieval solution. Heat slides to 95 °C for 25 min, ensuring boiling does not occur. Remove staining jar from heat source and allow to cool at room temperature for 35 min.

3.7. For Proteinase K antigen retrieval, lay slides flat in the humidifying chamber, and place 100 µL of pre-warmed Proteinase K on the slide. Cover the slides with a small strip of parafilm to ensure the slides do not become dry. Incubate slides for 12 min at 37 °C.

3.8. Wash slides 3 times for 5 min in 1X TBST in staining jar after antigen retrieval.

3.9. Remove slides from the staining jar and place in humidifying chamber. Place 6–7 drops of blocking solution (**Table of Materials**) on the slide, and cover the slide with fresh paraffin film to ensure the slide does not become dry. Incubate slides for 1 h at room temperature.

3.10. Prepare the primary antibodies by diluting the antibodies into antibody diluent (**Table of Materials**). Vortex each primary antibody solution for 3 s. Primary antibodies derived from different host species may be combined.

NOTE: Immunohistochemical staining for Runx2 (rabbit anti-Runx2 antibody, 1:250 dilution, at a final concentration of 4 µg/mL) combined with PCNA (monoclonal mouse anti-PCNA antibody, 1:2000 dilution, at a final concentration of 0.5 µg/mL), and OSX (rabbit anti-OSX antibody, 1:400 dilution, at a final concentration of 0.125 µg/mL) combined with PCNA is shown in **Figure 2**. The mouse anti-PCNA antibody used in this study is highly specific and requires no additional blocking steps beyond those outlined in this protocol. Immunohistochemical staining using CXCR4 (rat anti-CXCR4 antibody, 1:500 dilution at a final concentration of 2 µg/mL) and vWF (rabbit anti-human vWF VIII antibody, 1:800 dilution, at a final concentration of 41.25 µg/mL) is shown in **Figure 2**. Primary antibodies were optimized and tested by our lab under the conditions in this protocol and are listed in the **Table of Materials**.

3.11. Carefully remove the paraffin film, and gently drain the slide onto tissue paper to remove excess blocking solution. Place 100–200 µL of primary antibody solution on the slide, replace parafilm cover, and return to the humidifying chamber. Incubate the slides in the closed humidifying chamber overnight at 4 °C.

NOTE: Do not let the slides become dry while draining the blocking solution. This step is performed quickly.

3.12. Carefully remove the parafilm, and gently drain the primary antibody solution from the slide. Place the slide in a staining jar containing fresh 1X TBST. Wash with fresh 1X TBST for 5 min 3 times.

3.13. Prepare the secondary antibodies by combining with antibody diluent (**Table of Materials**). Vortex the secondary antibody solution for 3 s. Secondary antibodies conjugated to different fluorophores derived from different species may be combined.

NOTE: Fluorescent secondary antibodies are light sensitive. Alexa Fluor 488-conjugated goat anti-rabbit IgG (H+L) for Runx2, OSX, and vWF, Alexa Fluor 568-conjugated goat anti-rat IgG (H+L) for CXCR4, and Alexa Fluor 647-conjugated goat anti-mouse IgG (H+L) for PCNA, diluted at 1:500 with a final concentration of 4 µg/mL, were used in **Figure 2**.

3.14. Place 200 µL of secondary antibody solution on the slide, cover slide with fresh paraffin film, and return to humidifying chamber. Incubate the slides in the closed humidifying chamber for 45 min at room temperature. Ensure the humidifying chamber is closed to avoid light.

3.15. Carefully remove the paraffin film and gently drain the secondary antibody solution from the slide. Place the slide in a staining jar containing fresh 1x TBST. Wash with fresh 1x TBST for 5 min 3x. Avoid light.

3.16. Prepare nuclear stain. Add 20 µL of DAPI (stock DAPI solution is 5 mg/mL in H_2O) to 200 mL of 1x PBS and shake vigorously to ensure homogeneity. Immerse slides for 5 min in DAPI-PBS solution. Avoid light.

3.17. Wash slides in dH_2O for 3 min. Decant the water and allow to slides to air-dry or gently aspirate the water from the slide, ensuring that the samples are not disturbed while vacuuming. Avoid light.

3.18. Carefully coverslip dry slides using 100 µL of anti-fade mounting medium (**Table of Materials**); avoid the formation of air bubbles on the slide during the mounting step. Store the slides flat and allow the mounting medium to dry overnight at room temperature in a light-proof container. After the mounting medium is dry, store slides flat in a light-proof container in 4 °C until ready to view.

NOTE: If unacceptable levels of air bubbles are present after mounting, the mounted slide can be gently placed in 1x PBS, the coverslip can be removed, and once the slide is rinsed again in water and re-dried, can be re-mounted.

4. Microscopy and Image Analysis

NOTE: Imaging and analysis using a fluorescence deconvolution microscope and associated software, equipped with 3 fluorescent filters (to visualize Alexa Fluor 488, 568, and 647 nm signals), plus DAPI (419 nm) is used in this experiment.

4.1. Image the slide at 10x magnification to capture the entire blastema region.

NOTE: Proliferating osteoblast primary antibody detection utilizes secondary antibodies (Alexa

Fluor 488 and 647) with non-overlapping emission spectra to minimize incorrect identification of co-labeled cells. Background subtraction of autofluorescent signal can be performed using the 568 nm filter to ensure integrity of the 488 or 647 signals. Blastema primary antibody detection utilizes secondary antibodies (Alexa Fluor 488 or 568). Background subtraction of autofluorescent signal can be performed with the 568 filter if using the 488-conjugated antibody, and 488 filter is using the 568-conjugated antibody. Autofluorescent signal is typically associated with the nail plate and erythrocytes throughout the digit, and is observed in the 488, 568, and 647 filters.

5. Sequential in-vivo microcomputed tomography (µCT)

5.1. Regenerating digits of the same animal scanned at 1 day prior to amputation (unamputated), 1 DPA, and at various time points until complete regeneration at 28 DPA using the vivaCT 40 (**Table of Materials**) is performed in this experiment and shown in **Figure 3A**.

5.2. Outfit the µCT with tubing to enable oxygen flow into and out of the µCT animal chamber, ensuring that the out-flowing oxygen is equipped with an activated charcoal filter to trap the isoflurane.

NOTE: Ensure that the µCT animal chamber is tightly enclosed; in this way, an animal nose-cone is not required for supplying isoflurane to the mouse during the scan.

5.3. Prepare the µCT scanning parameters. Digits are scanned at a voxel size of 10.5 µm, at 45kVp, 145 uA with 1000 projections per 180° using continuous rotation, and with an integration time of 200 msec, resulting in a maximum of 213 slices per scan. Scans performed in this experiment are approximately 9 min long. A decreased electric current can be compensated with proportionally increased integration time but will result in longer scan times.

5.4. Anesthetize the adult mouse using isoflurane; initially anesthetize at 3% in a chamber, followed by 1.5% isoflurane supplied to the µCT animal chamber over the duration of the scan. Gently place the mouse in the µCT animal chamber with the hindlimb digits arranged in close association, flat, ventral side-up with left paw on left side and right paw on right side on the scanning platform, followed by gently securing the digits in place using surgical tape. Apply ophthalmic ointment on eyes to prevent dryness while under anesthesia.

5.5. Return the mouse to a clean cage and monitor the mouse until awake. Continue scanning the same mouse at bi-weekly to weekly time points to visualize the entire bone regeneration response.

5.6. After scanning, allow up to several hours for µCT image reconstruction to occur. Using the software provided with the µCT, convert the files to a series of dicom files; each dicom file will correspond to one slice of the scan, therefore, if 213 slices have been scanned, 213 dicom files will be generated and uploaded to the company's server.

5.7. Download the dicom series in a single folder using a batch file downloader in a web browser.

5.8. Download the BoneJ plugin[23] and drag the file into the ImageJ plugin folder. In ImageJ, open the dicom file series as a stack by dragging and dropping the folder to the ImageJ bar. A 16-bit image stack displaying all the gray values will be opened. To display bone only, perform image thresholding (**Image > Adjust > Threshold**).

NOTE: When viewing ImageJ files, the ImageJ output will correspond to an inverted image of the digits; i.e., digits arranged in the scanning platform ventral side-up, with left paw on left side of platform and right paw on right side of platform, will appear as dorsal side-up, right paw on left side and left paw on right side of the ImageJ image stack.

5.9. Once the threshold dialog box is opened, slide the bottom bar, corresponding to the upper threshold, to the far right, and adjust the top bar, corresponding to the lower threshold, until only the bone is highlighted red. The lower threshold value will be approximately 10,000–13,000 at a maximum signal intensity of 32,767. Click **apply**, and in the new dialog box click **black background**. Click **OK**.

5.10. An 8-bit image displaying black and white values will be generated, with white corresponding to bone. Select the P3 bone by drawing a rectangle around it, and duplicate the stack. Generate a 3D image (**Plugins** > **3D Viewer**). To crop any unnecessary bone from the image, click the freehand selection tool, and circle the bone to be deleted. Right click, and select **Fill Selection** to delete bone.

NOTE: The steps described above apply to ImageJ 1.52h, Java 8 and may differ slightly when using a different version of ImageJ. For thresholding, we recommend the determination of an optimal value and using that value consistently.

5.11. Quantify bone volume from the 3D rendering by using the BoneJ Volume Fraction Plugin (**Plugins** > **BoneJ** > **Volume Fraction**). A result window will appear, and bone volume (BV) will be displayed in mm^3.

5.12. Quantify bone length from the 3D rendering by using the ImageJ multipoint tool.

NOTE: Bone length is dynamic over the course of P3 regeneration; the length decreases between 7-10 DPA associated with osteoclast-mediated bone degradation[11], and is followed by a period of distal bone regrowth between 14-28 DPA (**Figure 3B**). Length is measured from the central base of the P2/P3 joint to the farthest point of mineralization at the distal bone apex, but does not include degraded bone that has been completely detached (**Figure 3B**). BoneJ allows for the complete 3D assessment of the bone architecture; thus, the angle in which the bone is scanned will not alter the ability to measure length.

5.13. Capture an image of the 3D rendering by clicking **View**, and take snapshot. Save image as a tif or jpeg file.

REPRESENTATIVE RESULTS:

Adult mouse regenerating P3 digits at 6/7 DPA (**Figure 2A–D**), 9 DPA (**Figure 2E–H**), and 10 DPA (**Figure 2I–L**) were immunostained with antibodies to Runx2, OSX, and PCNA to visualize intramembranous bone regeneration, and immunostained with antibodies to CXCR4 and vWF to visualize blastema formation. Representative µCT renderings of digits scanned prior to amputation and at various timepoints over the course of regeneration (**Figure 3A,B**) and identification of the landmarks used to identify length measurements (**Figure 3B**) are also shown.

FIGURE AND TABLE LEGENDS:

Figure 1: Digit number and identification of adult mouse distal P3 amputation plane. The adult mouse right hind paw is shown with digits numbered 1-5; amputations performed in this study are carried out on digits 2 and 4 (**A**). The distal P3 amputation plane is shown (dashed line) on digits 2 and 4 (**B**).

Figure 2: Early intramembranous ossification and blastema formation in the regenerating adult mouse P3 digit. Runx2 (green) and PCNA (magenta) double immunofluorescence show that proliferating osteoprogenitors are initially localized to the periosteal and endosteal bone surfaces at 6/7 DPA, and expand to the distal blastema at 9 and 10 DPA (**A, E, I**). OSX (green) and PCNA (magenta) double immunofluorescence show few OSX-positive osteoblasts and broad proliferation at 6/7 (**B**), and enhanced OSX immunostaining distally bounded by proliferating cells at 9 and 10 DPA (**F, J**). CXCR4 (red) immunofluorescence identifies early blastema formation at 6/7 DPA (**C**), followed by robust CXCR4 immunostaining in the 9 and 10 DPA regenerating digits (**G, K**). vWF (green) immunostaining identifies intact vasculature in the marrow of amputated digits, and few positive cells associated with the avascular blastema (**D, H, L**). Samples counterstained with DAPI. Dorsal is to the top, distal is to the right. Bl = blastema, m = marrow. Scale bars: 100 µm.

Figure 3: P3 bone regeneration visualized by sequential in-vivo µCT scanning. Representative µCT renderings of one digit scanned prior to amputation (unamp), and at 1, 7, 10, 14, 21, and 28 DPA. µCT scanning demonstrates P3 regeneration is characterized by an initial bone histolysis response, followed by bone island formation at 14 DPA and robust bone regeneration at 21 and 28 DPA (**A**). Representative µCT renderings of one digit scanned prior to amputation and at 1, 7, 10, 14, 21 and 28 DPA illustrating the regions in which digit length is measured at each time point. (**B**) Green dots indicate the total length measured, whereas the red X denotes the region of expelled bone excluded from the length measurement. Individual digit images created by ImageJ can be cropped to standardize the digit size to enable creation of images seen in this figure. Distal is to the right, dorsal is to the top.

DISCUSSION:

This protocol describes a standardized procedure of adult mouse distal P3 amputation, fluorescent immunohistochemical staining to visualize and investigate blastema formation and intramembranous ossification, and sequential in-vivo µCT scanning to identify bone morphological, volume, and length changes post amputation. P3 amputation is a unique, procedurally simple, and reproducible model to analyze a pro-regenerative wound environment

that triggers blastema formation. Furthermore, the P3 digit model offers numerous advantages over traditional bone injury models to investigate intramembranous ossification.

To ensure success of this protocol, complete digit decalcification must be performed. In the event the digit is not completely decalcified, it will crumble and shred during the sectioning process. Additionally, heat retrieval immunohistochemistry can be problematic in that the tissues may detach slightly or become entirely dislodged from the slide. To alleviate this issue, samples should be mounted on adhesive slides using a water bath supplemented with a histological adhesive agent, as well as baking the dry slides to 65 °C prior to use. Lastly, the mouse digits are relatively small; therefore, in order to accurately visualize morphology and quantify changes in bone volume and length, the µCT must be of sufficient resolution to function at the appropriate scanning parameters.

One limitation of this method is that not all primary antibodies are compatible with tissue fixation and paraffin processing. In this event, standard frozen tissue cryosectioning can be performed to ensure the integrity of the primary antibody antigen[11]. Cryosectioning also eliminates the need for the decalcification step, however we have found that cryosectioning of digits is technically more challenging than paraffin sectioning.

The entire blastema can be visualized at 10x magnification by deconvolution microscopy, and, using the appropriate image quantification software, the immunostaining results can be easily quantified. Fluorescent immunohistochemistry probing for osteogenic markers of the regenerating digit provides a unique view of blastemal differentiation via intramembranous ossification. Immunohistochemical staining reveals the P3 bone regeneration response is polarized and results in organized proximal-to-distal bone formation (**Figure 2**). At later regeneration stages, non-proliferative osteoblasts derived from the blastema are localized adjacent to the bone stump and are distally bounded by proliferating osteoblasts. The proliferating osteoblasts, in turn, are distally bounded by proliferating undifferentiated blastemal cells. Fluorescent immunohistochemistry probing for the blastema marker CXCR4 identifies early blastema cells associated with the injured bone surface followed by enhanced immunostaining at later regeneration stages (**Figure 2**). The blastema is avascular[13] and immunofluorescence for the endothelial cell marker vWF identifies few positive cells within the blastema region (**Figure 2**).

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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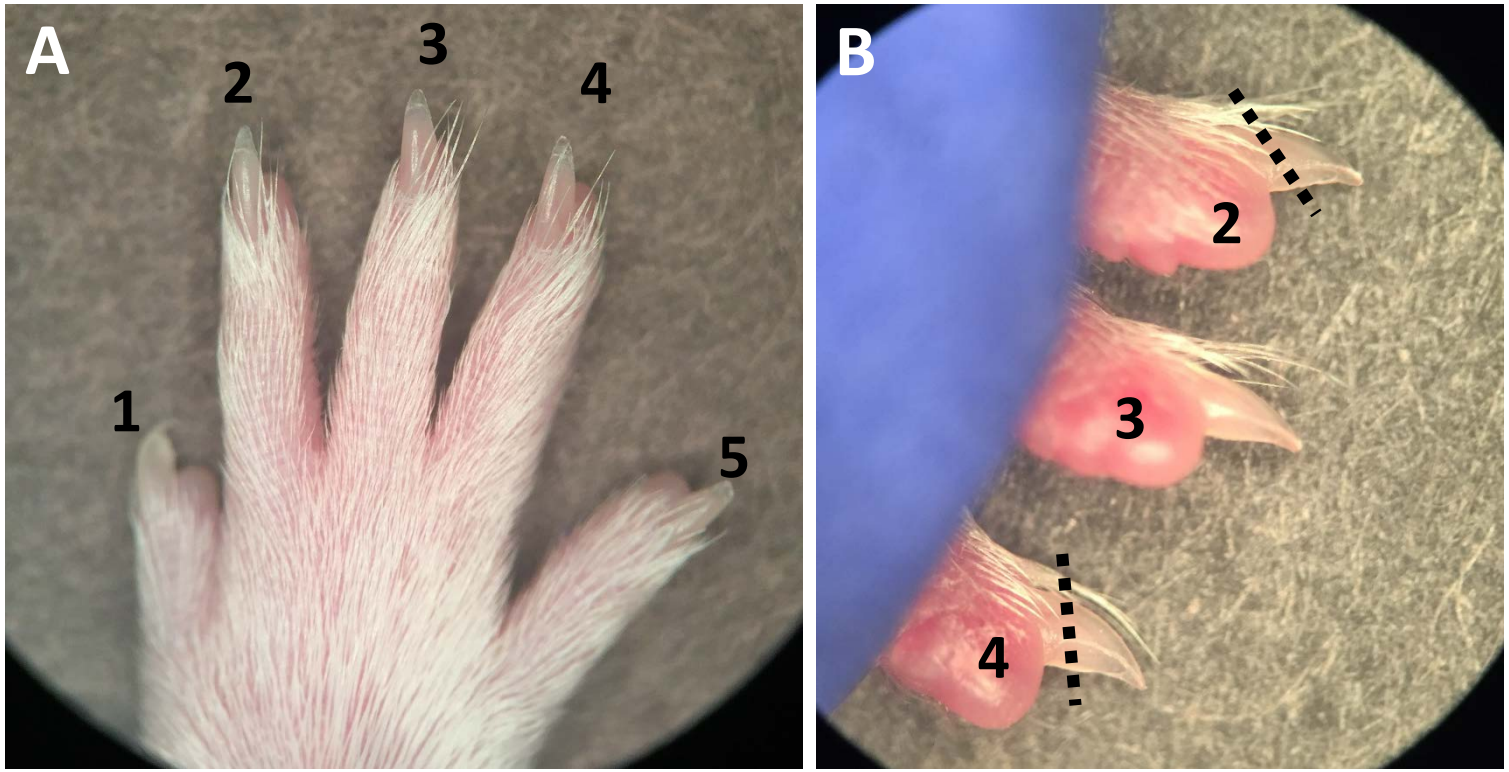


Figure 2

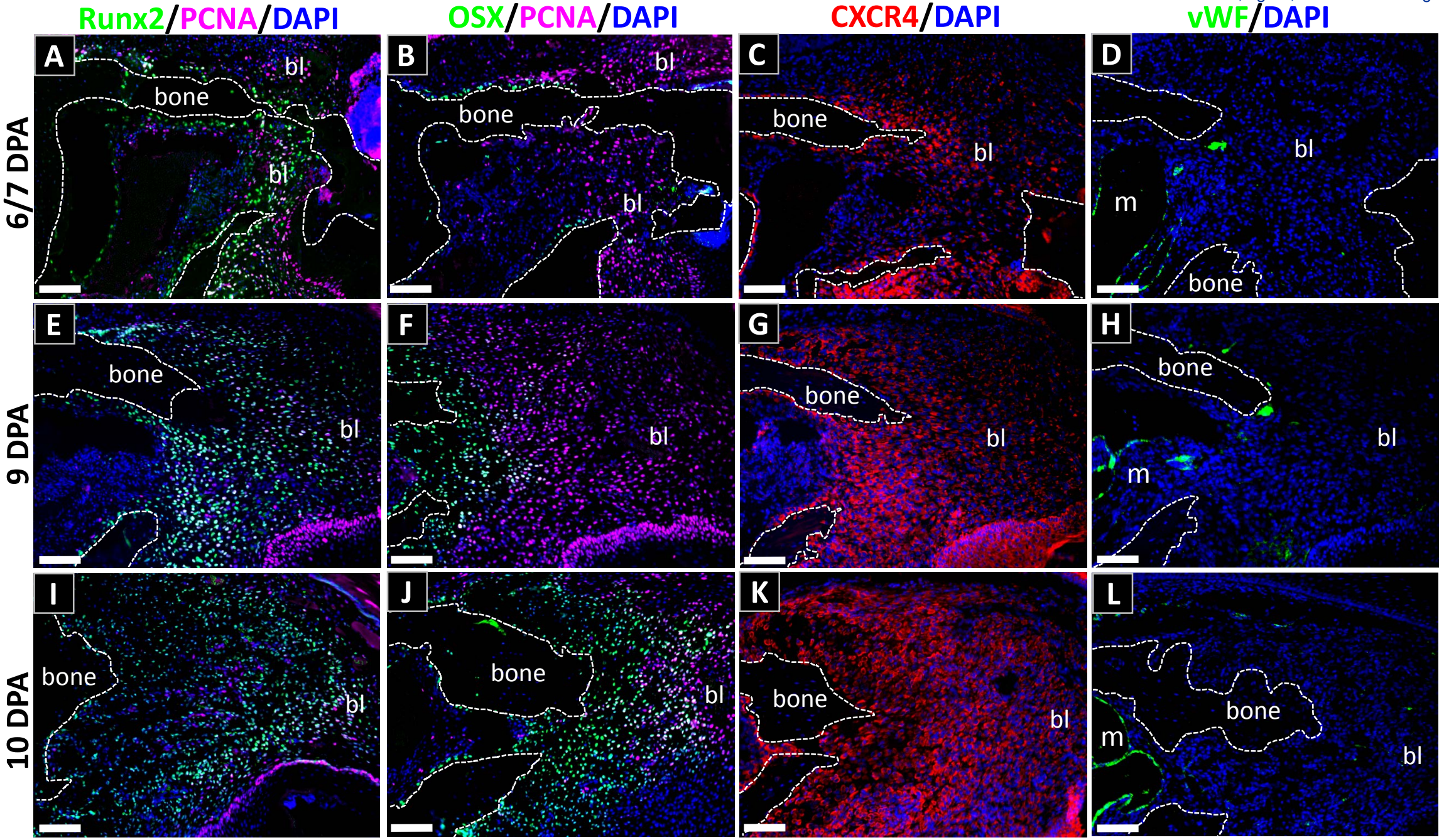
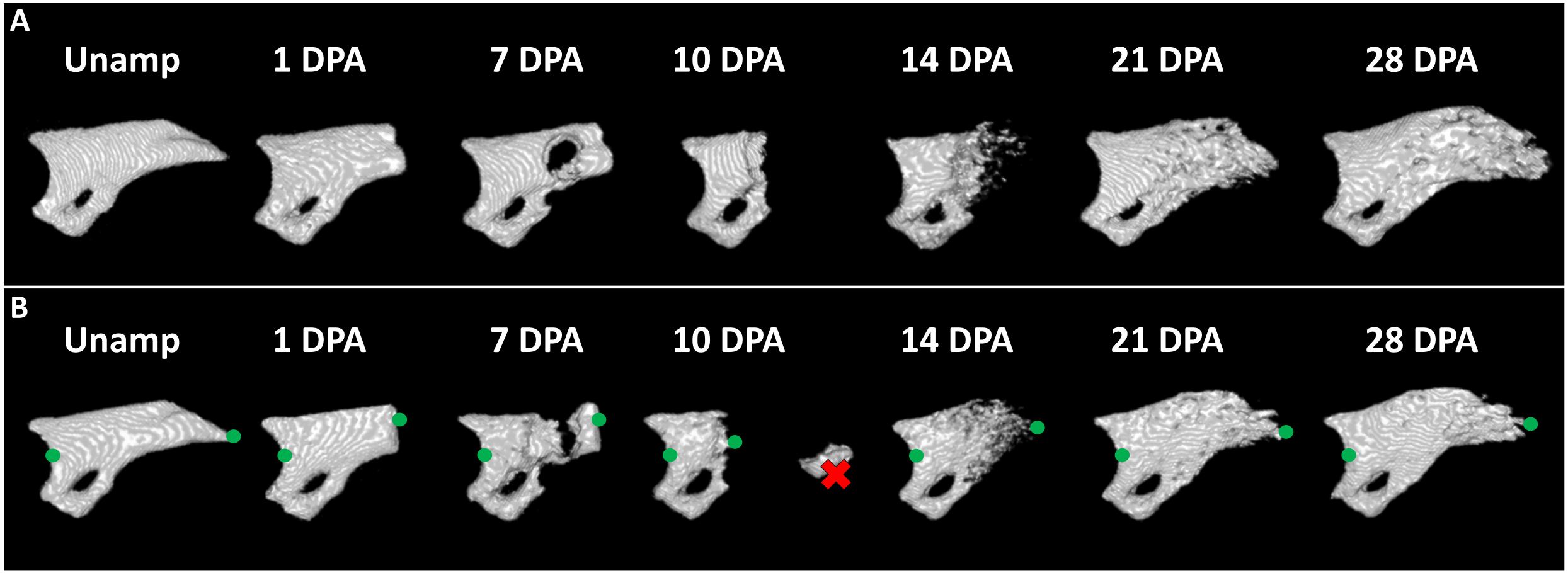


Figure 3



Name of Material/ Equipment	Company	Catalog Number
Protein Block Serum Free	DAKO	X0909
Mouse anti-PCNA antibody	Abcam	ab29
Rat anti-CXCR4 antibody	R&D Systems	MAB21651
Rabbit anti-human vWF XIII antibody	DAKO	A0082
Rabbit anti-osterix, SP7 antibody	Abcam	ab22552
Rabbit anti-Runx2 antibody	Sigma-Aldrich Co.	HPA022040
Alexa Fluor 647-conjugated goat anti-mouse IgG (H+L)	Invitrogen	A21235
Alexa Fluor 488-conjugated goat anti-rabbit IgG (H+L)	Invitrogen	A11008
Alexa Fluor 568-conjugated goat anti-rat IgG (H+L)	Invitrogen	A11077
Prolong Gold antifade reagent	Invitrogen	P36930
Surgipath Decalicifier 1	Leica Biosystems	3800400
Z-Fix, Aqueous buffered zinc formalin fixative	Anatech LTD	174
CD-1 Female Mouse	Envigo	ICR(CD-1)
vivaCT 40	SCANCO Medical	

Comments/Description
Ready to use
1:2000 dilution
1:500 dilution
1:800 dilution
1:400 dilution
1:250 dilution
1:500 dilution
1:500 dilution
1:500 dilution
Ready to use
Ready to use
Ready to use
8-12-weeks-old

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Author(s): Lindsay A. Dawson, Regina Brunauer, Katherine N. Zimmel, Osama Qureshi, Alyssa R. Falck, Patrick Kim, Conor P. Dolan, Ling Yu, Yu-Lieh Lin, Benjamin Daniel, Mingquan Yan + Ken Muneoka.

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CORRESPONDING AUTHOR

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Institution:	Texas A+M University
Title:	Research Assitant Professor
Signature:	 Date: 1.29.2019

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Dear Editor and Reviewers,

Please see our comments below. We have revised the document and added an additional figure to address all of your concerns. In our comments, we direct you to specific number lines in the revised document; these lines correspond to the document when viewed in track changes, with "all markup" selected, however we apologize if the number lines do not match up perfectly due to various versions of Microsoft Word. We thank you for your time and appreciate your insight.

Sincerely,
Lindsay Dawson

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

We thoroughly proofread the article.

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

All reference numbers have been changed to superscript. The references were created using the "JoVE EndNote style file" provided to authors on the instructions for Authors template.

Protocol:
1. Being a video based journal, JoVE authors must be very specific when it comes to the humane treatment of animals. Regarding animal treatment in the protocol, please add the following information to the text:
a) Please specify the use of vet ointment on eyes to prevent dryness while under anesthesia.

The text was edited to make this clear, please see lines 123-124, and 355-356.

b) Discuss maintenance of sterile conditions during survival surgery.

The text was edited to include sterile conditions during surgery, please see lines 134-135, 144.

2. Please split up Protocol steps so that individual steps contain only 2–3 actions and a maximum of 4 sentences.

The text was edited to follow these guidelines.

3. For each step, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

The text was edited to follow these guidelines.

Figures:
1. Please remove 'Dawson et al.' from the Figures.

Dawson et al. was removed from all figures.

2. Figure 2: As there is only one panel, labelling with 'A' doesn't seem necessary.

Figure 2 (which is now figure 3 due to revisions), has been edited and now includes another panel.

References:
1. Please do not abbreviate journal titles.

We have edited the references to include full journal titles. The references were created using the "JoVE EndNote style file" provided to authors on the instructions for Authors template.

Table of Materials:

1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

We have updated the Table of Materials to include all relevant information.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript nicely describes the process of amputating the mouse digit, performing microCT and staining. I would have liked to have this manuscript available 6 years ago! Below are some concerns

Major Concerns:

Under Adult Mouse Hind Limb Distal P3 Amputation:

1. How do authors confirm the correct percentage of bone removal? Authors should include sentence in this paragraph that amputation length is confirmed via microCT..

We have added a step to clarify that the standard distal amputation plane removes approximately 15-20% of the P3 volume, and that this can be confirmed via microCT scanning. Please see lines 146-148.

Also, are there any landmarks (aside from the first 1/3) to help the surgeon determine where the amputation should be made? At what angle should the scapel blade be relative to the amputation. Please add more detail to this section. Do the authors splay the paw before cutting?

We have added a figure to identify the amputation plane. Additionally, we have edited the text to clarify landmarks, the angle of the scalpel, and laying the paw flat, please see Figure 1B and lines 135-137.

Under the table of materials:

1. The use of mouse PCNA is used on mouse tissue, but no mention of any extra blocking steps were noted to prevent mouse on mouse background staining

We have edited the document to clarify that no additional blocking steps are required, since the antibody is very specific and we have never encountered any non-specific binding issues. Please see lines 256-258.

Under Sequential in-vivo microcomputed tomography (μCT)

In #9, it is recommended that author describes in greater detail how length will be measured, especially during healing. Will the blastema be considered in the length (assuming no if its bone length). How many measurements will be obtained per digit. Will the measurement be collected at the longest portion? Where is the starting and end point of lengths. This becomes more complicated with amputated limbs. How will you determine the correct angle of the digit is the same each time in the CT scan when measuring?

We have provided an additional panel in Figure 3 (was Figure 2 prior to editing). This figure includes an additional set of MicroCT images with green dots identifying the regions in which we measure length, as well as the bone region (marked by a red X) that we do not measure (the bone with a red X corresponds to the expelled bone segment after bone degradation.) Also, BoneJ allows for the complete 3D assessment of the bone architecture, therefore the angle in which the bone is scanned will not alter the ability to measure length. We've clarified all of this in the manuscript, please see lines 402-409. Please also see Figure 3B, as well as the figure legend, lines 449-452.

In discussion, author mentions that cryosectioning can be performed on the digits without decalcification. Has the author ever performed this task? It's quite difficult to get nice sections even with that small amount of bone present.

Indeed, cryosectioning is difficult! But yes, we have performed this task, and also provided a citation in which the majority of immunostaining was performed on cryosectioned digits. We have edited the manuscript to include a sentence explaining that cryosectioning is challenging compared to paraffin sectioning, please see lines 488-489.

Minor Concerns:

Under Adult Mouse Hind Limb Distal P3 Amputation:

1. Author should state why there is an age range for the animals. Is regeneration limited to that age.

We simply wanted to state that the standardized adult model for our lab is usually performed on 8-12 week old mice, except in aging studies. The P3 regeneration aging study is ongoing and the publication is in preparation. We have edited the document to clarify that we gave this age-range since it has been tested and optimized by us, please see lines 126-127.

2. Is the strain of mouse important?

To our knowledge, the strain of mouse is not important as the regeneration response is conserved in all strains tested in our lab. We have edited the document to clarify this, please see line 127.

Under Digit Collection and Tissue Preparation

1. Is the zinc formalin (vs neutral buffered formalin, etc) important for achieving optimal staining.

In our lab, we only perform immunostaining using Z-Fix, and we have not tested the antibodies using any other fixative. Since we have not used any other fixative, we included the Z-Fix information in the Table of Materials as well as added a sentence to the methods section to clarify that our optimization of antibodies was performed under the exact conditions of this protocol. Please see lines 261-262.

2. Is the decalcifier specifically mentioned for optimal decalcification (or can EDTA, etc be used instead)

The decalcifier mentioned was used to optimize these specific antibodies, and we have not tested these antibodies using any other decalcifier. While we have used EDTA decalcification for other antibodies and it has worked fine, we believe we should leave the manuscript text as-is since this protocol has been optimized.

Under Sequential in-vivo microcomputed tomography (μCT)

1. Authors may want to mention if hindlimbs are taped down to ensure no movement (animals still twitch under anesthesia process)

We have edited the manuscript to include the use of tape to secure the digits in place. Please see line 355.

2. Authors may also want to mention to place tail on specific side of the animal with each CT scan as a landmark to distinguish between left and right side. This will aid in identifying side of animal when viewing CT scans later.

The amount of slices in our digit scans is too small to include a region for the tail (this would be very helpful for limb scans though), so the text has been edited to clarify that the digits must always be placed ventral-side up, left paw on left side, right paw on right side, and the Image J output will correspond to an inverted image of the digits. Please see lines 352-355, and 377-380.

3. In the Image J description, does the "set scale" section also need calibrated and adjusted

No, the "set scale" section does not need to be calibrated and adjusted.

4. Also want to include somewhere that all images need to be the same size when making measurements (to accurate comparisons).

We edited the document to include this, please see lines 452-453.

Thank you for your time and insight, it is greatly appreciated!

Reviewer #2:

Manuscript Summary:

This is a straightforward description of a reliable protocol in use for several years which allows for study of a complex and important process which is rare in mammals. It has formed the foundation of several significant publications and promoted great scientific progress.

To be completely forthcoming it is important to note that I have not myself used this protocol.

Major Concerns:

none

Minor Concerns:
my only scientific argument is with the statement made in the introduction that regenerative failure occurs just as one encounters the middle phalanx. This overstates the regenerative capacity of the mouse digit tip. Regenerative failure commences in the distal phalanx proximal to the nail field and persists proximal to this point.

We have edited the text to clarify this point, please see line 71.

Thank you for your time and insight, it is greatly appreciated!

Reviewer #3:
Summary:
Dawson,….,Muneoka present a detailed protocol for performing mouse digit tip amputations as well as analyzing the regenerating tissue by immunohistochemistry and microCT. Many groups are beginning to study digit tip regeneration, thus this protocol will be a valuable resource coming from the lab that has arguably done the most work to technically develop and scientifically understand the model. The manuscript is largely publishable as-is, though addressing the minor comments listed below would help clarify some points throughout.

Minor comments:
1. If there is flexibility re: number of figures, a schematics of the paw/digit detailing digit counting, phalanx numbering and landmarks for amputation would be helpful.

We have added a figure to identify the digit numbers and amputation plane. Additionally, we have edited the text to clarify landmarks, the angle of the scalpel, and laying the paw flat, please see Figure 1 and lines 135-137.

2. Lines 82-84: it is unclear what "blastema proper" is, aren't more cell types than periosteal and endosteal cells represented in the blastema?

Since the initial blastemal populations are compartmentalized to the periosteal and endosteal bone surfaces prior to bone degradation and wound closure, we call the merging of these two populations the blastema proper. We understand how this is unclear, and have edited the text to clarify that the blastema is heterogeneous, and that the regenerated bone is derived from the heterogeneous blastema, please see lines 85-87.

3. Protocol 1.1: vendor and catalog number should be provided for CD-1 mouse

We edited the table of materials to include this information.

4. Protocol 1.2: dissection microscopes vary so optimal magnification should be noted

We edited the document to include this information, please see line 129.

5. Protocol 1.3: as noted in comment #1, a schematic with landmarks or measurements would help understand what "distal 1/3" is. Scalpel # would also be useful.

We have added a figure to identify the amputation plane, please see figure 1B. Additionally, we have edited the text to clarify landmarks, the number of scalpel used, and laying the paw flat, please see lines 135-137. We have also added a step to clarify that the standard distal amputation plane removes approximately 15-20% of the P3 volume, and that this can be confirmed via microCT scanning. Please see lines 146-148.

6: Protocol 2.2: concentration of formalin should be included

We have edited the text to include this information, please see lines 160-161.

7: Concentration of PBS and TBST should be included throughout

We have edited the text to include this information.

8: Protocol 4.2: the authors bring up background subtraction—it may be useful to describe or show what background in the different channels could be expected.

We have edited the text to clarify the locations of the usual autofluorescent signal, including the nail plate and the red blood cells. Please see lines 328-329.

9: Protocol 5.1: vivaCT 40 manufacturer should be noted

We have edited the table of materials to include this information.

Thank you for your time and insight, it is greatly appreciated!