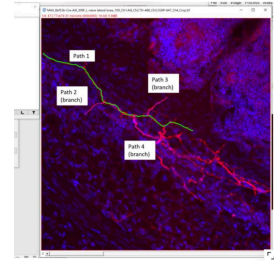


Jan 03, 2025

Protocol for quantification of bone indices, calcein labels, and nerve axon density in multi-channel confocal images

DOI

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Protocol status: Working

We use this protocol and it's working

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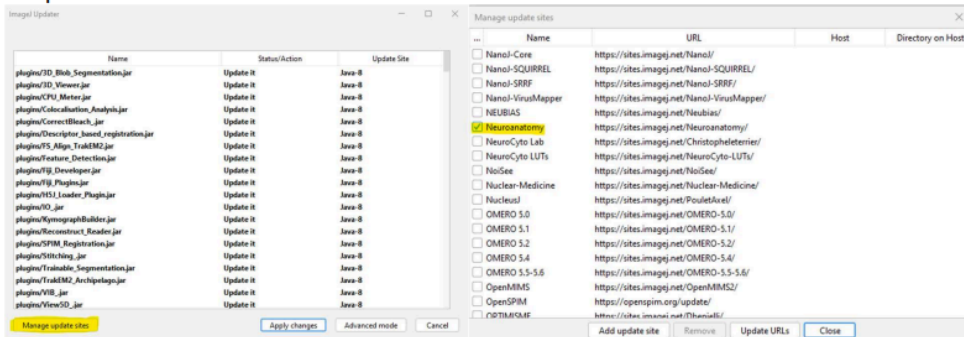
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Abstract

This protocol details the quantification of basic bone parameters including bone area, bone area fraction, periosteal perimeter, etc., dynamic indices of bone formation using calcein double labels, and the length, density, and branching of nerve endings present in long bone cross-sections imaged by confocal microscopy.

Guidelines

- In case you didn't find the neuroanatomy Plugin in the FIJI Plugins menu
 1. Go to: Help menu → Update
 2. Go to: Help menu → Update ow → Manage update sites
 3. Select Neuroanatomy tool → Add update site
 4. The software will run the updating process → restart the software when it finishes the update.



The protocol consists of 4 main steps:

The quantification protocol includes 4 main stages:

1. Creation of bone masks
2. Quantification of bone measurements
3. Quantification of calcein labeling
4. Quantification of nerve length and density (and branching, optional)

Note

- The examples in this protocol used a multi-channel tiff image derived from a Nikon Spinning Disk microscope. Specifically, a non-decalcified mouse tibia with prior calcein injection from a Baf53b-Cre; Ai9 reporter mouse was cut transversely and immunostained for CGRP with Dapi added to highlight nuclei. The resulting image has nuclei in blue (Dapi), new bone formation in green (calcein double labels), all nerves in red (Baf-Ai9, pan-neuronal genetic reporter), and sensory nerves in pink (CGRP). You can apply these protocols on any images with various stains, locations, and ROIs in which masking and/or nerve quantification is needed.
- You can also skip the first 2 steps if your quantification process doesn't require the development of bone masks, or the first 3 if you are only interested in tracing nerves.



Materials

- **Operating System:**

Windows or MacOS

- **Software:**

Fiji or ImageJ (with Neuroanatomy Plugin)

Image Preparation

1 Importing Image into Fiji Or ImageJ:

- For raw images (with stacks):
 1. Import the raw image file into Fiji.
 2. Go to: Image → stacks → Z project.
 3. Select the slices you would like to flatten (and choose the maximum intensity from the box).
 4. Save the new max projection image as TIFF (File → save as → TIFF).

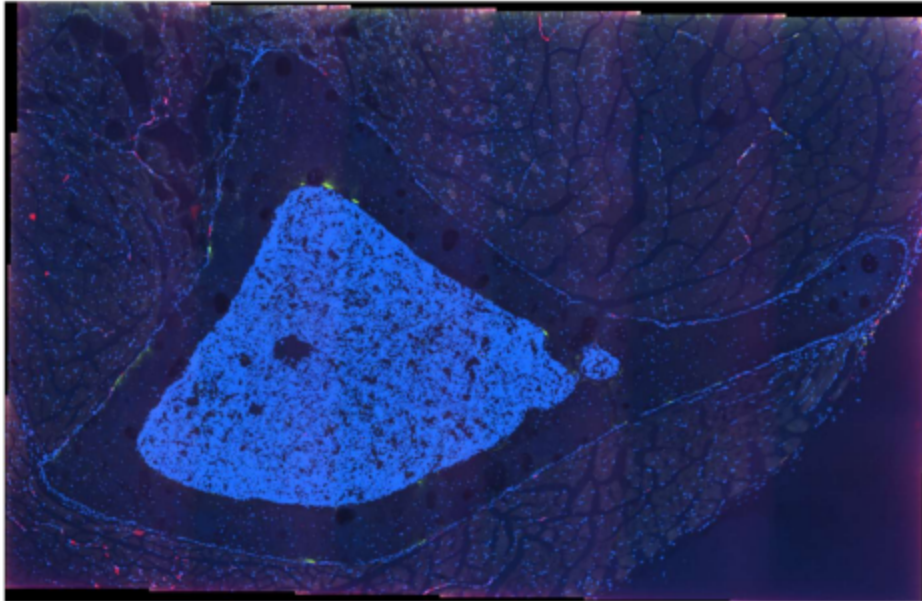
Note

If you already have the maximum projection TIFF file, go directly to the next step.

Creation of bone masks

2 For Maximum projection TIFF files:

- Import the TIFF image file into Fiji



3 Bone mask creation

3.1 Select the DAPI channel window

Open the channels tool (Image → Color → Channels Tool). This allows you to click a button to view or hide channels, and to figure out which channel has a specific fluorophore. In our test image, Dapi is in Channel #1.

Note

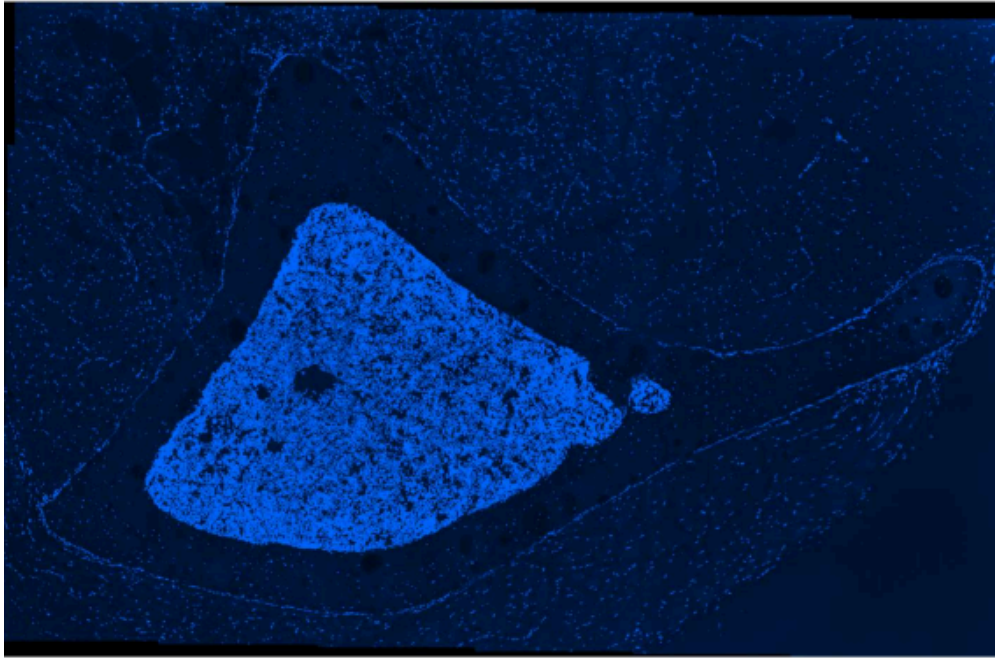
This channel is helpful for the visualization of the periosteum and the creation of the periosteal mask later.

3.2 Duplicate DAPI channel

Select the DAPI channel, then go to the menu (Image → Duplicate). Add the number of the DAPI channel (or channel of interest) to the "Channels (c)" box.

Note

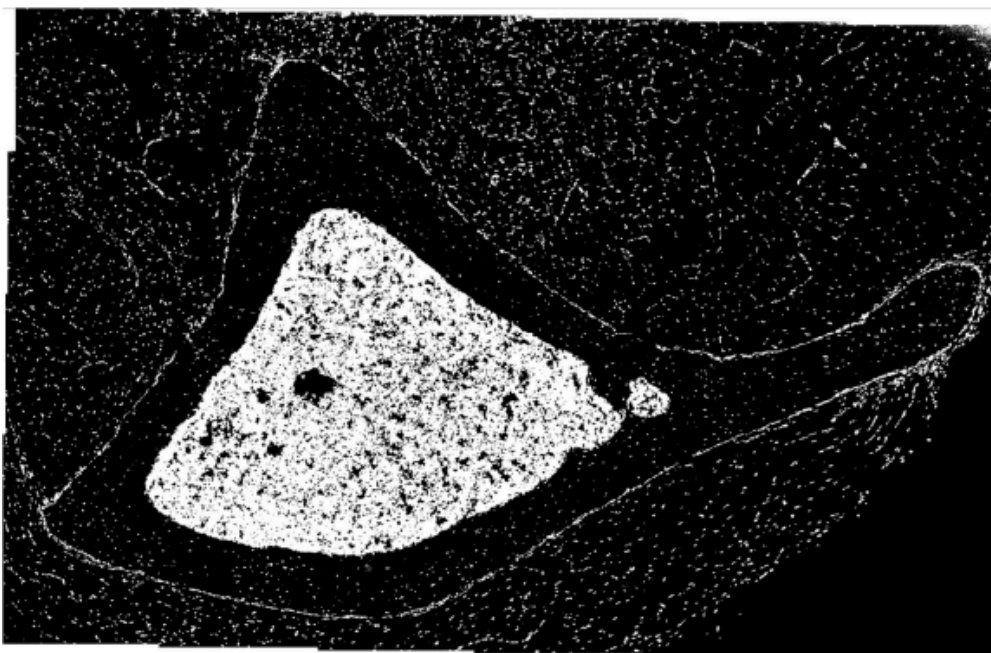
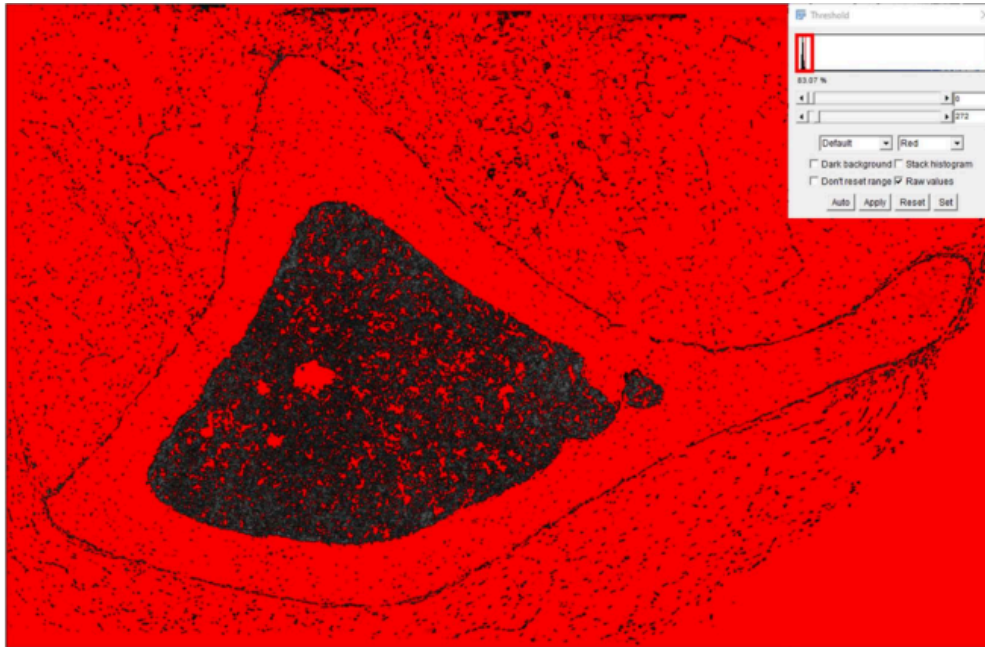
De-select duplicate hyperstack (if you do not, it will duplicate all four channels).



- 3.3 To create a threshold for the bone mask, go to the menu (Image → Adjust → Threshold), adjust the threshold, then press (Apply).

Note

Adjust the upper and lower thresholds until you start de-selecting bone. Everything in red will be kept after the threshold, everything else will be cleared by the threshold and become a white background.



3.4 To clear the periosteum and the outside of the bone.

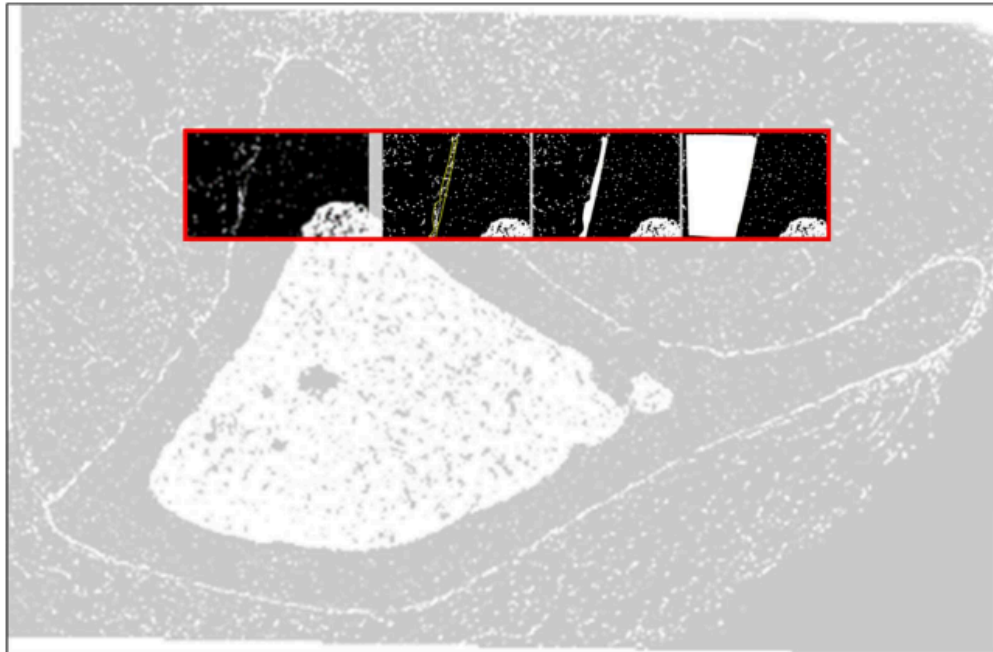
1. You may need to zoom in and clear the periosteum and the outside of bone 'region by region' or in one step
2. To clear the periosteum and the outside region by region: use the polygon (



) or freehand (



) selection tools, select the region that you would like to clear, then Ctrl+F.



- 3.5 After you clear all the periosteum and outside the bone. Smooth out the bone mask and fill in the holes.
- Select (Process → Filters → Median → Radius = 10 pixels).
 - Then to remove holes within the cortical bone, select (Process → Binary → Fill holes).

Note

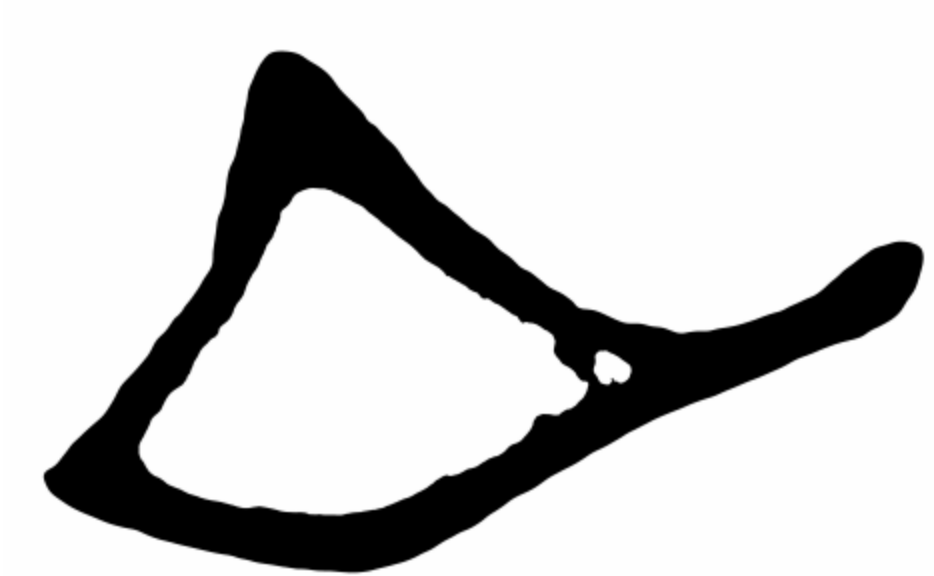
You need to create a white path through the cortical bone to the marrow cavity to avoid filling the marrow space (e.g. use the paint brush tool to draw a white line through the cortical bone).

- Finally, select (Process → Filters → Median → Radius = 30 pixels) to smooth out the bone mask.

Note

Remove any residual bone marrow artifacts if needed using the selection tools and Ctrl+F as above.

- Save the mask as TIFF file. This mask will be used to quantify cortical bone surface area.



3.6 To fill the bone marrow in the bone:

- Select (Process → Binary → Fill Holes)

- Save the mask as TIFF file. This mask will be used to quantify total bone surface area and perimeter.



- 3.7 To check the accuracy of the bone mask(s), move back to the original 4-channel TIFF file, DAPI channel, then:
- On the bone mask image: select (Edit → Selection → Create Selection).
 - Then move back to the original 4-channel TIFF file, DAPI channel, then select (Edit → Selection → Restore Selection).
 - Once you are satisfied with your final selection, save the bone masks as TIFF file (File → Save as → TIFF).

4 Periosteal mask creation:

- 4.1 Select the DAPI channel window.

- 4.2 Duplicate DAPI channel again

Select the DAPI channel, then go to the menu (Image → Duplicate)

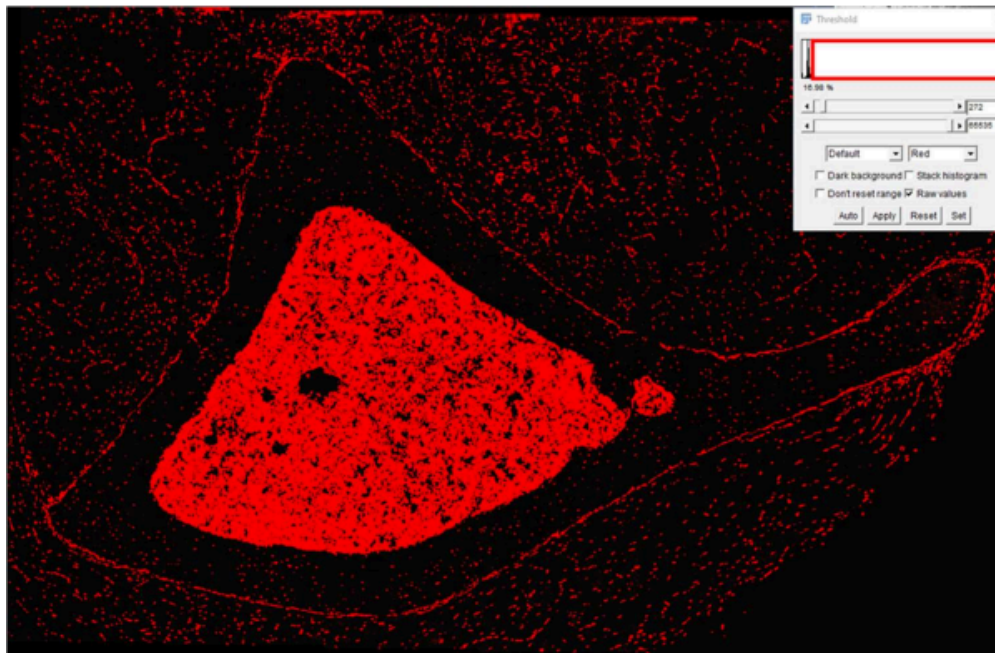
Note

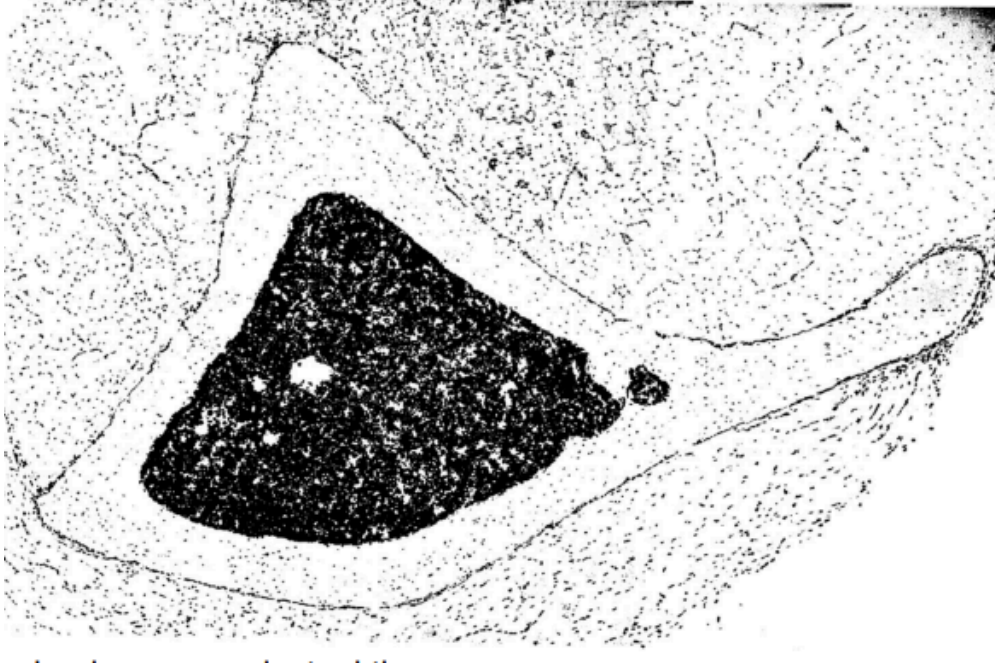
De-select duplicate hyperstack (if you do not, it will duplicate the four channels)

- 4.3 Move to the duplicated DAPI window.
- 4.4 To threshold the periosteal mask: select (Image → Adjust → Threshold), adjust the threshold, then press (Apply).

Note

Adjust the upper and lower thresholds individually just before you start deselecting the periosteum. You want to make sure that you are deselecting non-periosteal tissue as much as possible to make manual corrections minimal.



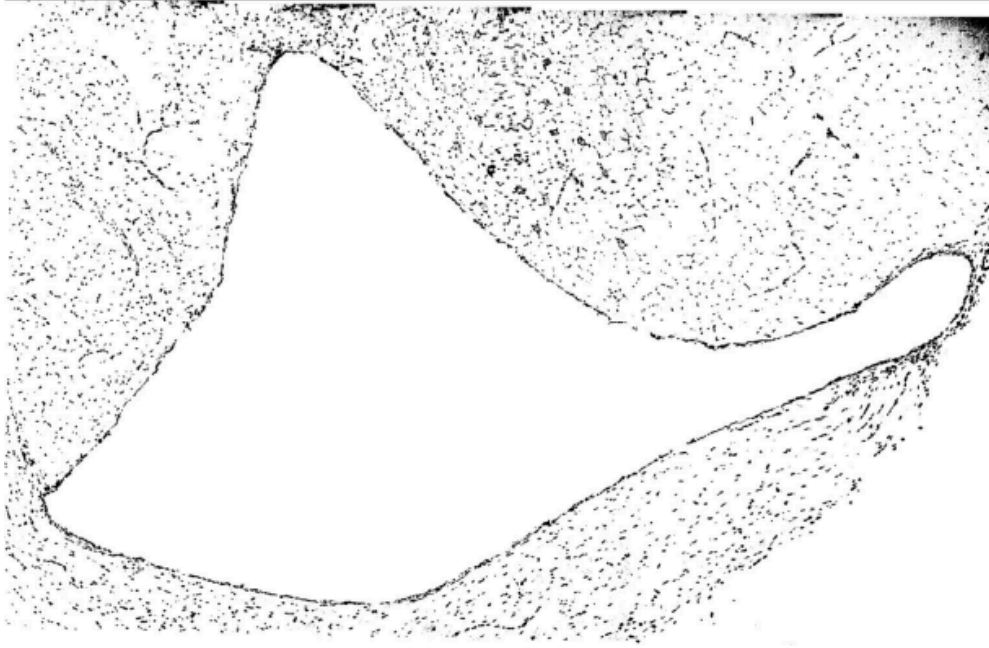


4.5 Start by clearing non-periosteal tissues:

- Go to the previously saved total bone mask TIFF,
- Create selection of the bone mask (Edit → Selection → Create Selection)
- Then move back to the duplicated channel, press (Edit → Selection → Restore Selection), then press Ctrl+F.

Note

This step will help to remove the bone in one step and will facilitate highlighting the periosteum.



4.6 To de-select the remaining non-periosteum tissues:

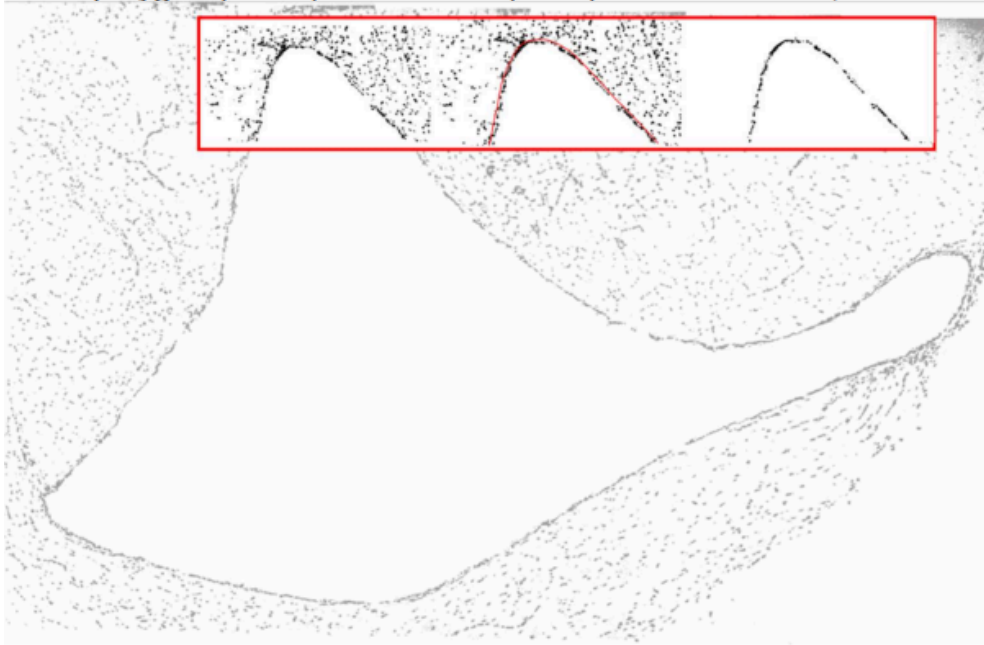
- Zoom in and clear around the periosteum region by region.
- Use the polygon (



) or freehand (



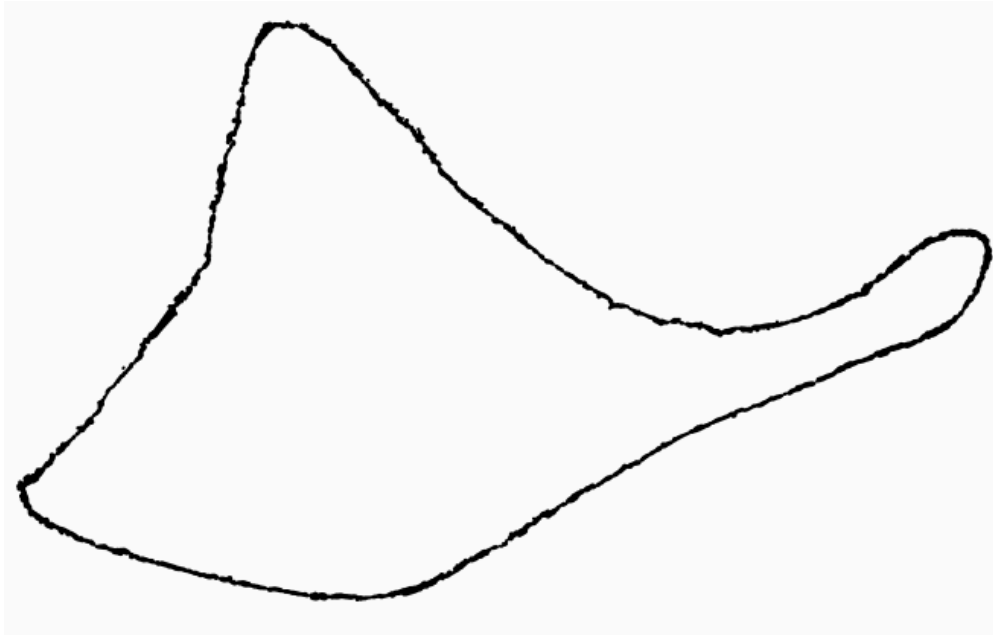
) selections tools, then Ctrl+F.



- 4.7 After clearing all around the periosteum, smooth out the periosteal mask and fill in the holes, select (Process → Filters → Gaussian blur → 5 blur nuclei).
- 4.8 Then re-threshold to create the periosteal mask.

Note

This step aims to connect the nuclei of the periosteum to make one continuous layer as much as you can. However, please note that it is ok if the periosteum is discontinuous as long as this accurately reflects the contents of your image. Be careful while adjusting the threshold not to expand the thickness of the periosteum.



4.9 To check the accuracy of the periosteal mask, move back to the original 4-channel TIFF file, DAPI channel, then:

- On the periosteal mask image: select (Edit → Selection → Create Selection).
- Then move back to the original 4-channel TIFF file, DAPI channel, then select (Edit → Selection → Restore Selection)
- Once you are satisfied with your final selection, save the bone mask as TIFF file (File → Save as → TIFF)
- Once you have the bone and periosteal masks, you can move into the quantification process.

Note

By the end of this section, we created 3 available masks that are going to be used in the following quantification steps (Cortical Bone Mask, Total Bone Mask, Periosteal Mask).



Quantification of bone measurements

5 To measure cortical bone surface area:

5.1 Open the saved cortical bone mask TIFF file.

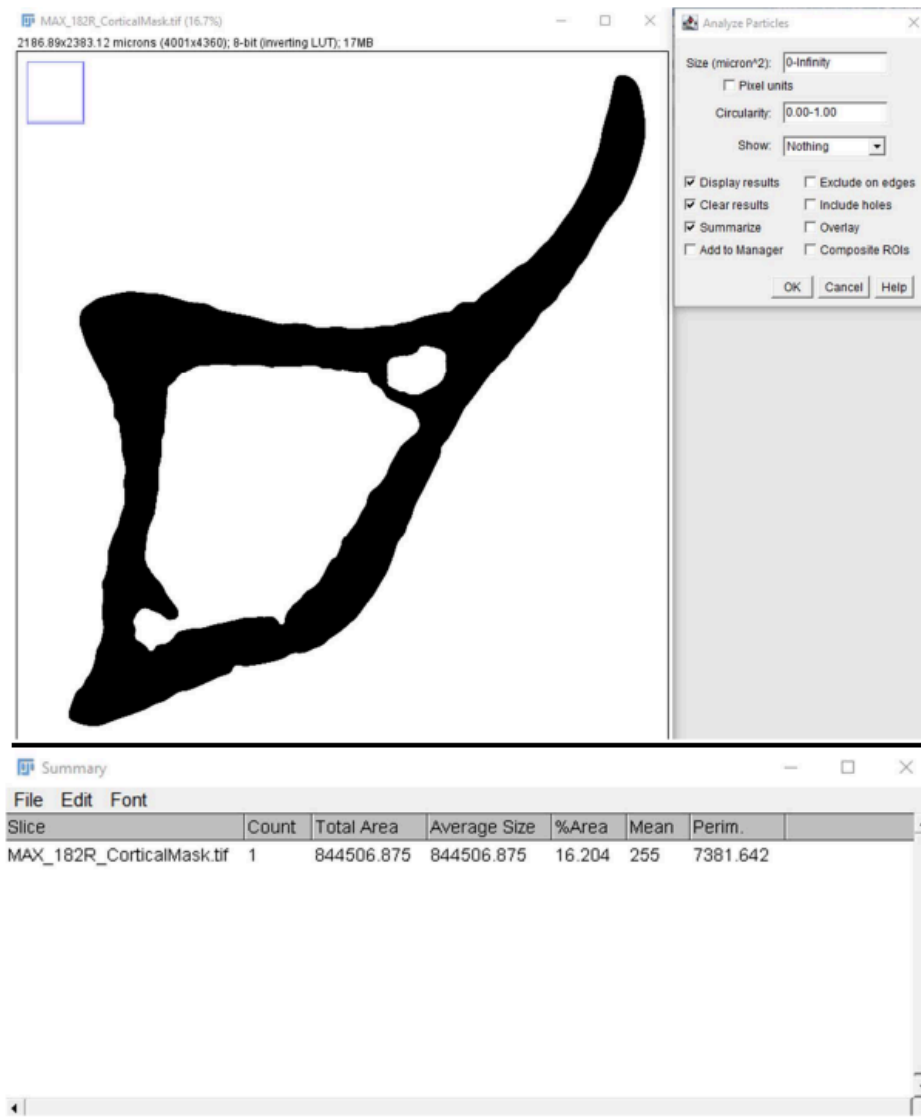
5.2 Select (Analyze → Analyze Particles), mark (Display results, Clear results, Summarize), then press Ok.

5.3 The total area represents the area of the masked bone.

Note

- Make sure that the scale of your image is appropriately calibrated. Ideally, this should be pulled from the metadata associated with the image file.
- The scale information is displayed at the top of the image. If the scale in microns is not automatically set, you can use a scale bar or a known pixel to micron conversion ratio to set the scale using the (Analyze → Set Scale) function.

5.4 Save the value in your excel sheet.



- 6 To measure Total Area and Cortical Perimeter Length:
 - 6.1 Open the saved total bone mask TIFF file.
 - 6.2 Select (Analyze → Analyze Particles), mark (Display results, Clear results, Summarize), then press Ok
 - 6.3 The total area represents the area of the bone + bone marrow.

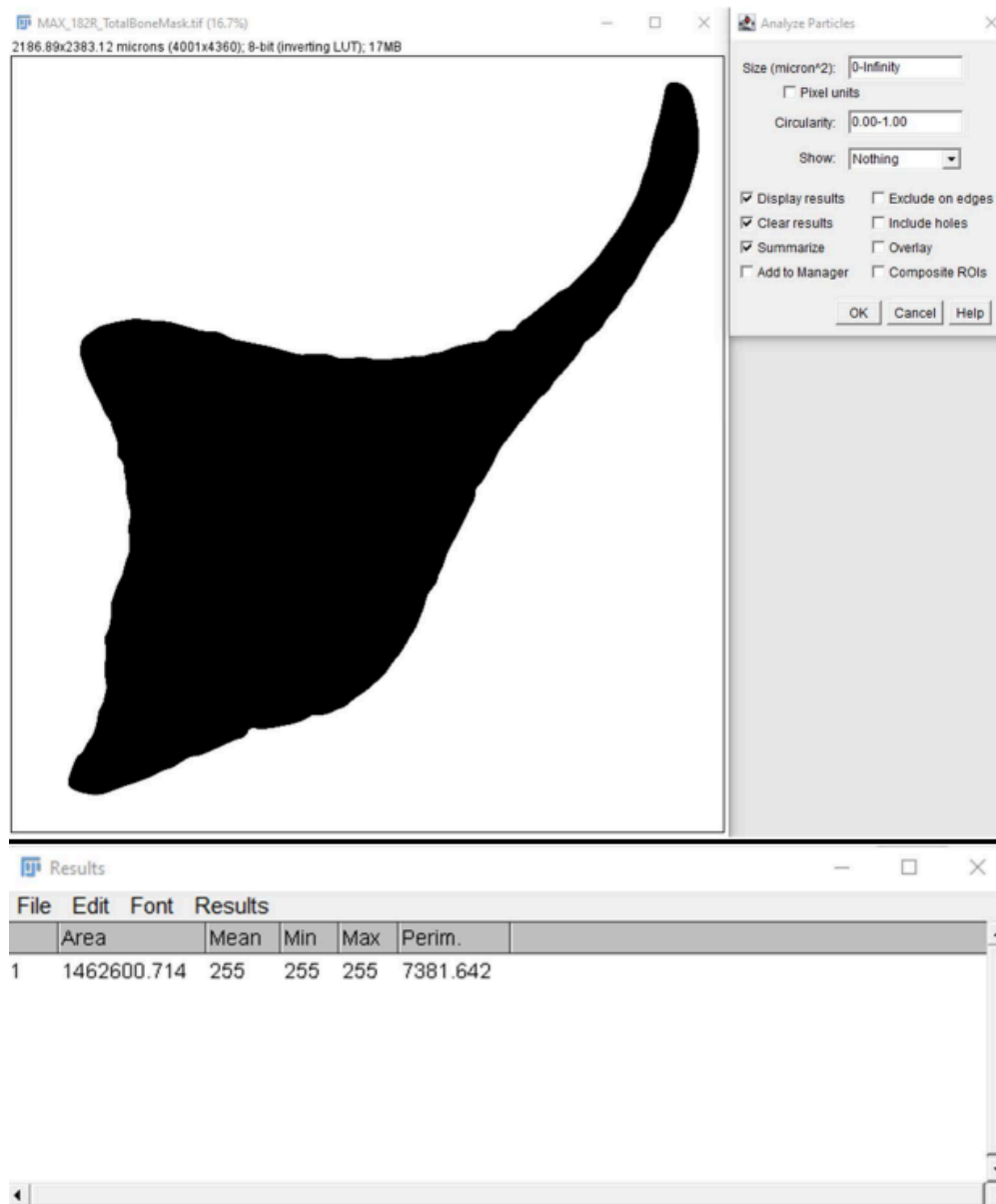


6.4 The perim represents the length of the cortical bone perimeter.

6.5 Save the values in your excel sheet.

6.6 In your excel sheet, you can calculate the:

Marrow Area as follow: $\text{Marrow Area} = \text{Total Area} - \text{Bone Area}$.



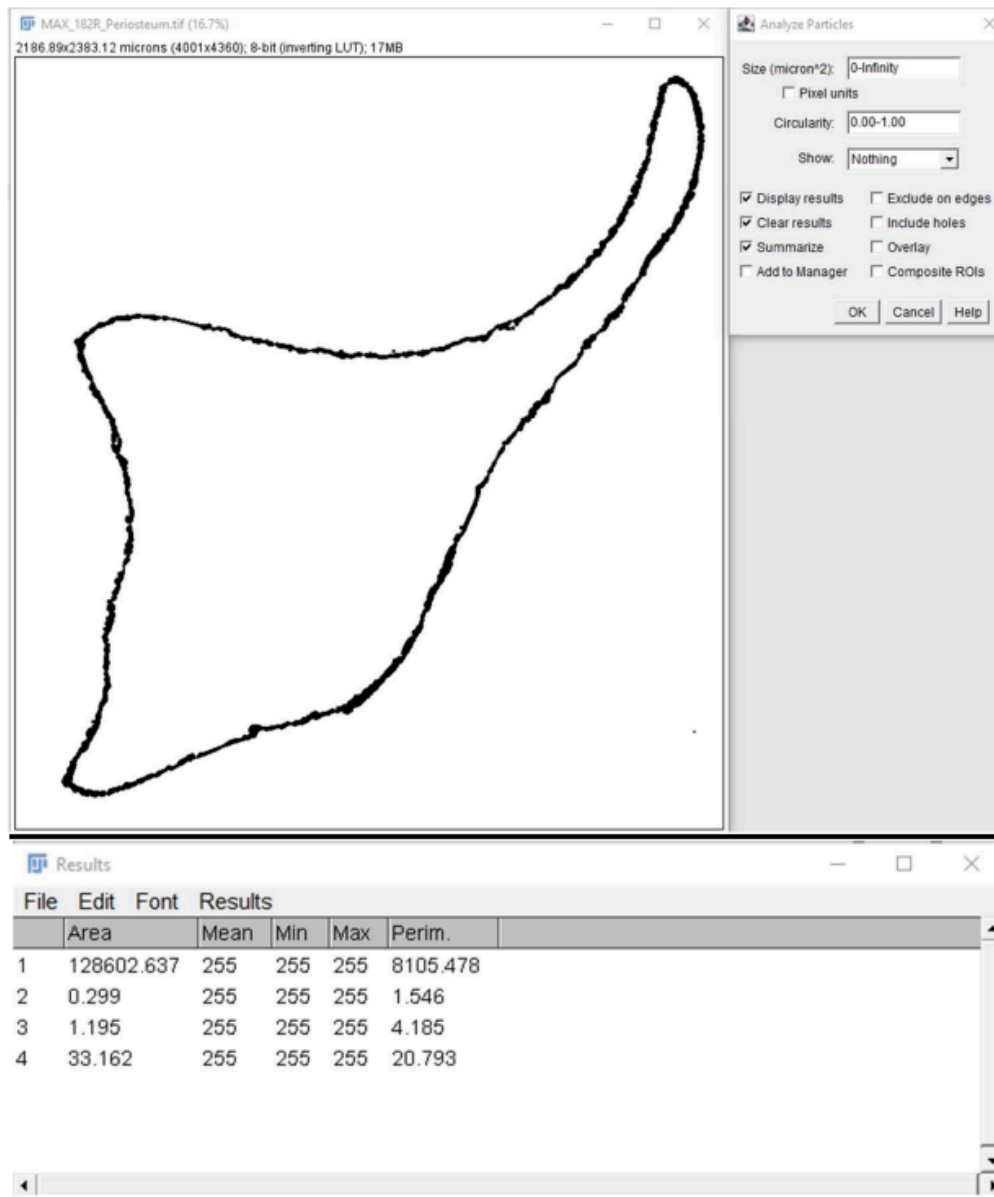
- 7 To measure Periosteum Area:
 - 7.1 Open the saved periosteum mask TIFF file.
 - 7.2 Select (Analyze → Analyze Particles), mark (Display results, Clear results, Summarize), then press Ok.

7.3 The area represents the area of the periosteum.

Note

Sometimes small areas of periosteum will be separated due to sectioning or bone morphology. Sum all values to get the total.

7.4 Save the value in your excel sheet.



Note

By the end of this section, we quantified the total area, bone area, cortical bone perimeter, and periosteal area using the 3 available bone masks. Next, we are going to quantify bone formation indices using calcein labels and nerves using the Baf53b-Cre;Ai9 reporter.

Quantification of calcein labeling

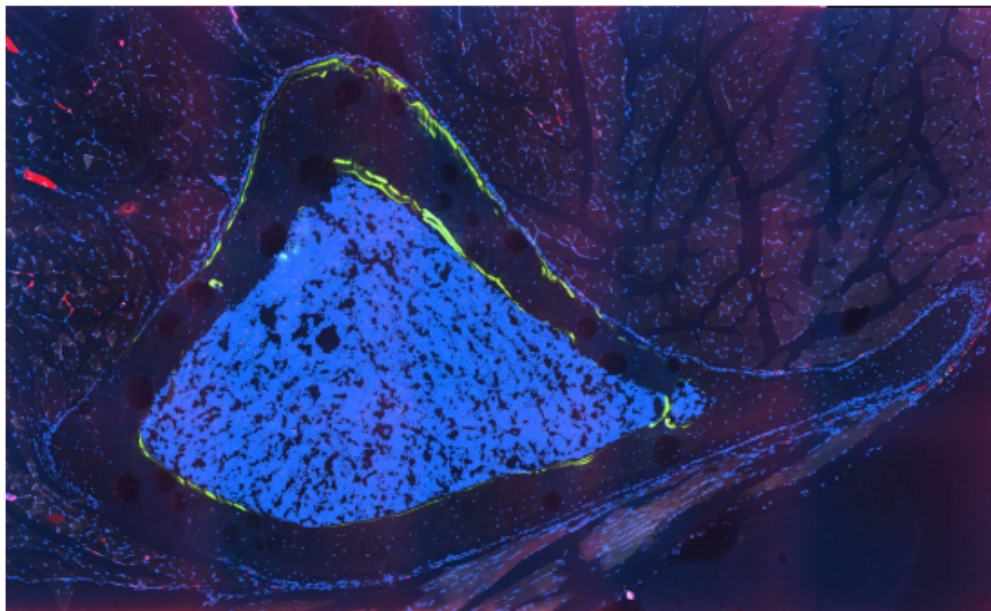
8

Note

Calcein labeling can be single or double labeling. In the following analysis, we are going to illustrate how you can quantify both.

9 Single Labeled Surface (Ps.sLS)

9.1 Open your TIFF files in FIJI



- 9.2 Select the FITC channel (green channel) and start adjusting the brightness and contrast (Image → Adjust → Brightness/Contrast) to distinguish the calcein label on the bone surface of interest. In this case, we will be quantifying single labeled surface with calcein on the periosteal surface.

Note

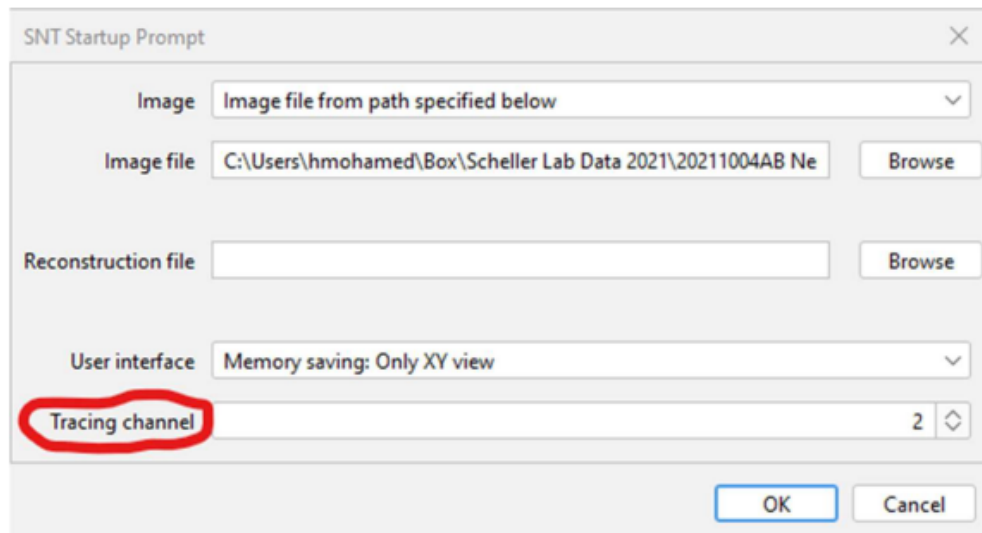
To adjust the brightness/contrast of your channel of interest, make sure that the “c” slider bar at the bottom of the image is in the correct position. You can also use the channels tool (Image → Color → Channels Tool) to view or hide channels.

- 9.3 Go to Plugins, and select (Neuroanatomy → SNT).

- 9.4 A pop-up window will appear, select the proper channel that you are going to trace, specifically, if you are tracing more than one channel or label (ex. Calcein and nerves).

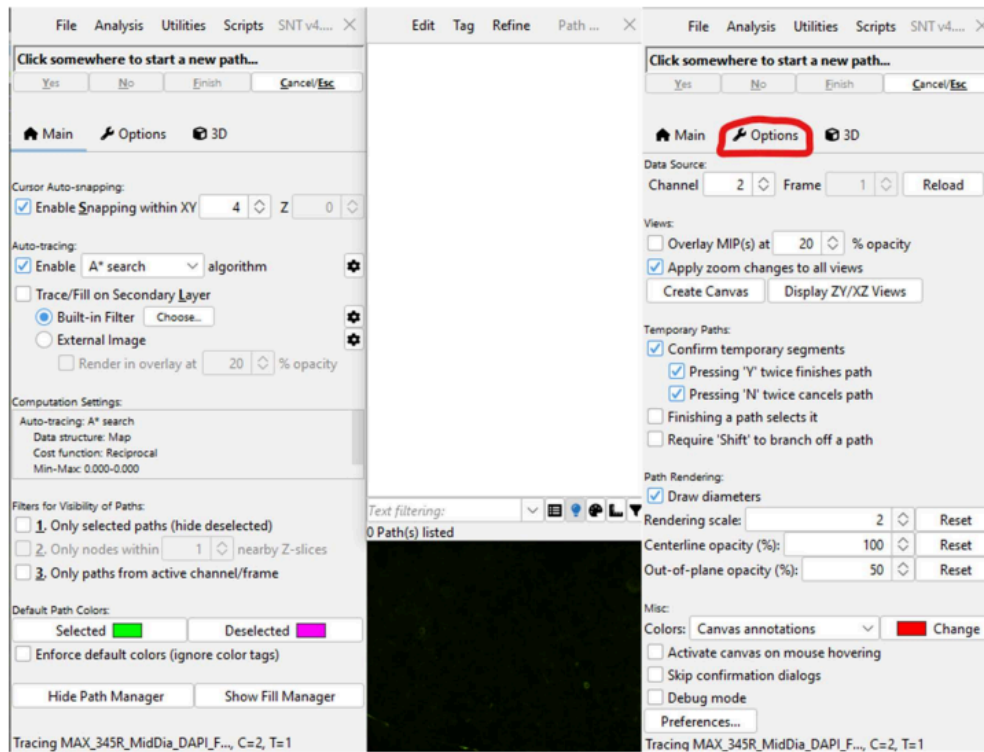
Note

This is an important point for not labeling the wrong channel.



- 9.5 The main plugin window will pop-up.

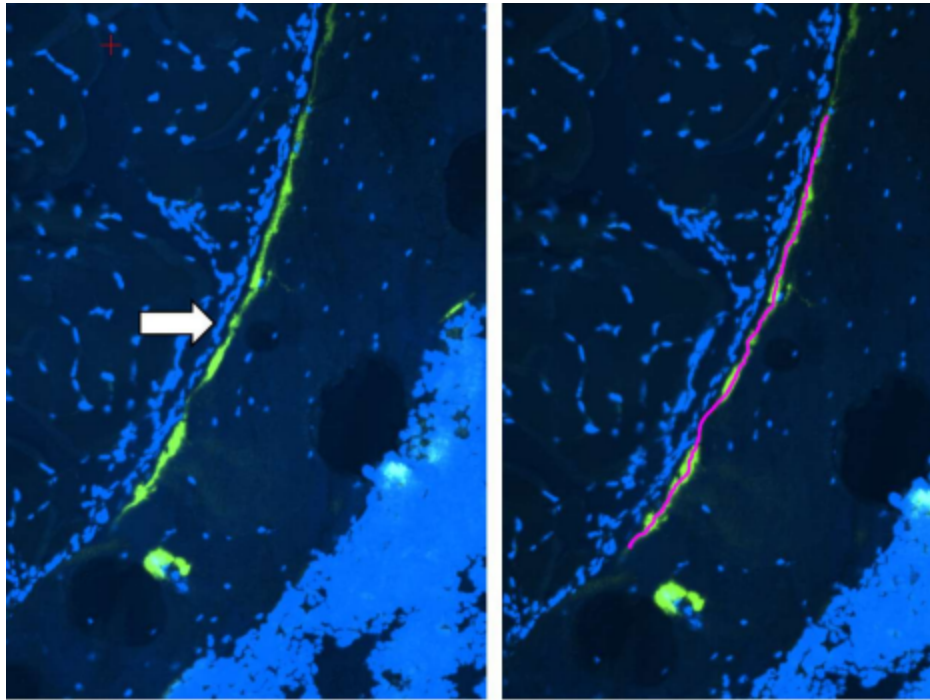
- 9.6 In the options section, you can confirm and change the channel you are tracing. Then you can move back to the main section.



- 9.7 Trace the calcein label in areas where there is only a single label (single labeled surface).
- To do this click the pointer to start the segment, then move a distance away down the line and click again. The program will automatically trace the line.
 - Press "Y" to keep the segment or "N" to delete it and try again. If you would like to extend the segment further, click again farther down the line and repeat.
 - Once you are done with the segment click "F" to finish it. Repeat for all single labeled surface calcein labels.

Note

You may need to adjust the brightness and contrast on the original image to check for the presence of single vs double labels.



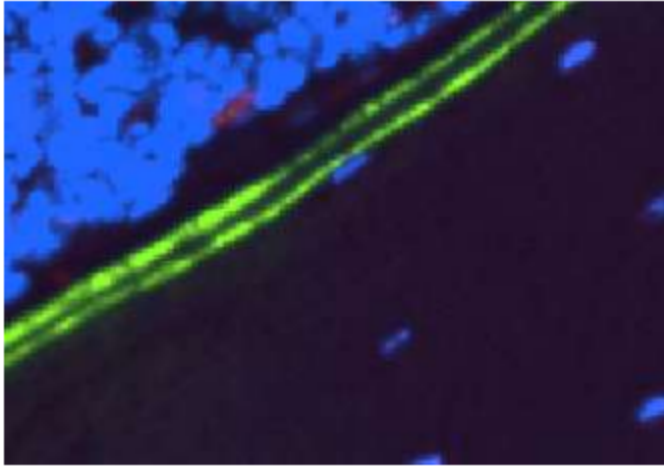
- 9.8 After finishing all tracing, export "Ps.sLS" traces file. [SNT Main Window: File → Export as... > TRACES] – this has the tracing data if you need it again in the future.
- 9.9 Export the rendered path image, [Path Manager Window: Analyze → Skeletonize...]. This will look like a white line on a black background wherever you did the tracing. You can use this file to create overlays of the traced areas with your original image.
- 9.10 Save the skeletonized imaged as "Ps.sLS" TIFF. (This step allows you to transform the tracing file into a TIFF file, so you can process it with normal FIJI functions).
- 9.11 To measure the length of the traced segments, [SNT Main Window: Analysis → Measure... → Check "Cable length" → OK].

SNT Measurements		
	SWC Type(s)	Cable length (μm)
Cell 1 rooted in Path (1)	Undefined	362.3692587386345

9.12 Save the values in your excel sheet.

10 Double Labeled Surface (Ps.dLS)

10.1 Open the TIFF file and the plugin, as described in the previous section.



10.2 Start tracing ONLY the outer double labeled surface first, after finishing every segment, press Y then F. This will allow you measure the length of the dLS before quantifying the interlabel width/area.

Note

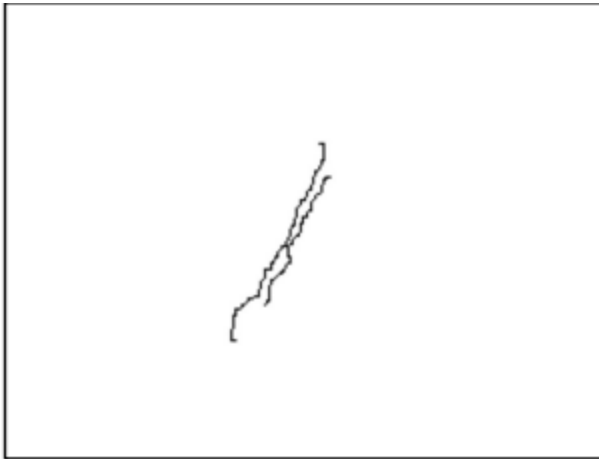
You have to be sure to press Y and F to keep the traced segment. This step is essential to keep tracing all segments.

10.3 After finishing tracing the outer label of the dLS, export the "Ps.dLS" traces file (with only the outer label traced). [SNT Main Window: File → Export as... > TRACES].

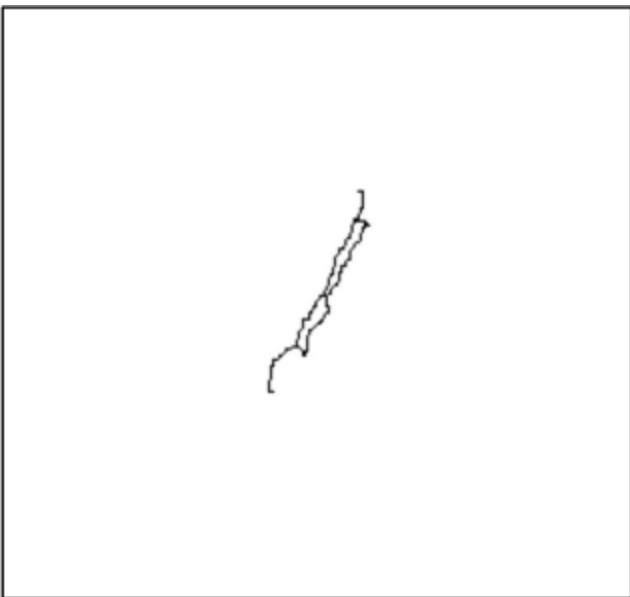
10.4 To export the rendered path, [Path Manager Window: Analyze → Skeletonize...].

10.5 Save the skeletonized imaged as "Ps.dLS" TIFF.

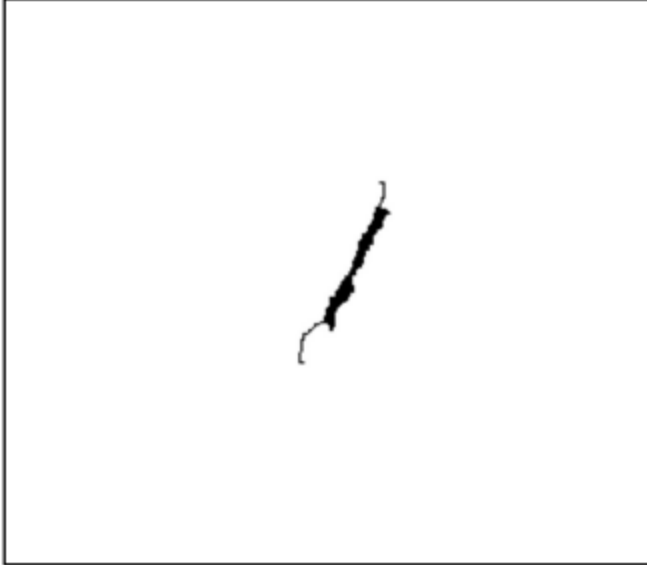
- 10.6 To measure the length of dLS, [SNT Main Window: Analysis → Measure... → Check "Cable length" → OK].
- 10.7 Save the values in your excel spreadsheet.
- 11 Double Labelled Surface: Interlabel Area (Ps.Ir.L.Ar)
 - 11.1 Import the tracing file you used to quantify the Ps.dLS into simple neurite tracer.
 - 11.2 Now trace the inner double labelled surface.
 - 11.3 After finishing all tracing, export traces file. [SNT Main Window: File → Export as... > TRACES].
 - 11.4 To export the rendered path, [Path Manager Window: Analyze → Skeletonize...].
 - 11.5 Save the skeletonized image as "Ps.Ir.L.Ar" TIFF.
 - 11.6 Activate the Ps.Ir.L.Ar TIFF file in FIJI.
 - 11.7 Go to: Edit → Invert (this step will allow you to visualize all double labelled spots and prepare them for interlabel area quantification).



- 11.8 Zoom in close and use the line drawing tool to connect the ends of the double labels to enclose the area between the two labels.



- 11.9 After enclosing all the double labels, select fill holes to fill in the interlabel area.



11.10 Repeat the previous steps with all double labelled spots.

11.11 Analyze Particles to calculate total Interlabel Area.

11.12 Save the value in your excel spreadsheet.

11.13 Save as a .TIFF file.

Quantification of nerve length and density (and branching, optional)

12

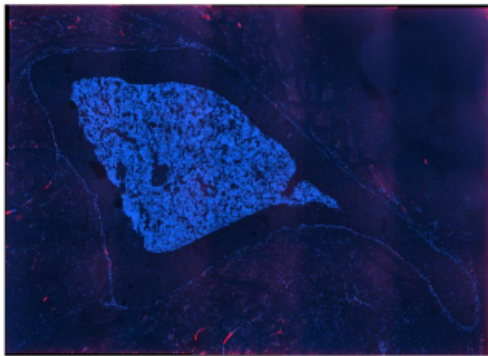
Note

Quantification of nerve endings is like the quantification of single calcein labels. In the following section, we are going to summarize the steps to calculate nerve length, nerve density, and nerve branching (optional).

Note

- To start, we recommend that you familiarize yourself with nerve axon morphology. Nerve axons are defined as long, slender projections of a neuron.
- The most common mistake made by people new to tracing is to quantify non-specific autofluorescence or staining as “nerve”.
- For example, the extracellular matrix can have linear auto-fluorescent fibers that resemble nerves. However, these can easily be ruled out by comparing to a negative control tissue that does not have your reporter or primary antibody of interest (required).
- Similarly, cellular signals can be avoided using strict morphologic criteria and a nuclear counterstain such as dapi.
- Nerves in bone do not have nuclei since the nerve cell bodies are located in the ganglia near the spinal cord. Nerve axons are also not rounded like immune cells or fibroblastic appearing.
- Example images and atlases of nerves in and around the mouse skeleton can be found here in our Neuroskeltal Atlas manuscript (Lorenz et al. 2021. JBMR. 36(5):1012-1025. PMID: PMC8252627).

- 13 Open your TIFF files in FIJI.
- 14 Select the channel(s) that contains your neural marker(s). In this case we are using the TRITC channel (red channel) to map the Baf53b-Cre;Ai9+ nerves. Adjust the brightness and contrast to distinguish the nerve label.
- 15 Open the mask of interest (in this case we will use the Periosteum Mask from Step 4.1 of mask creation).



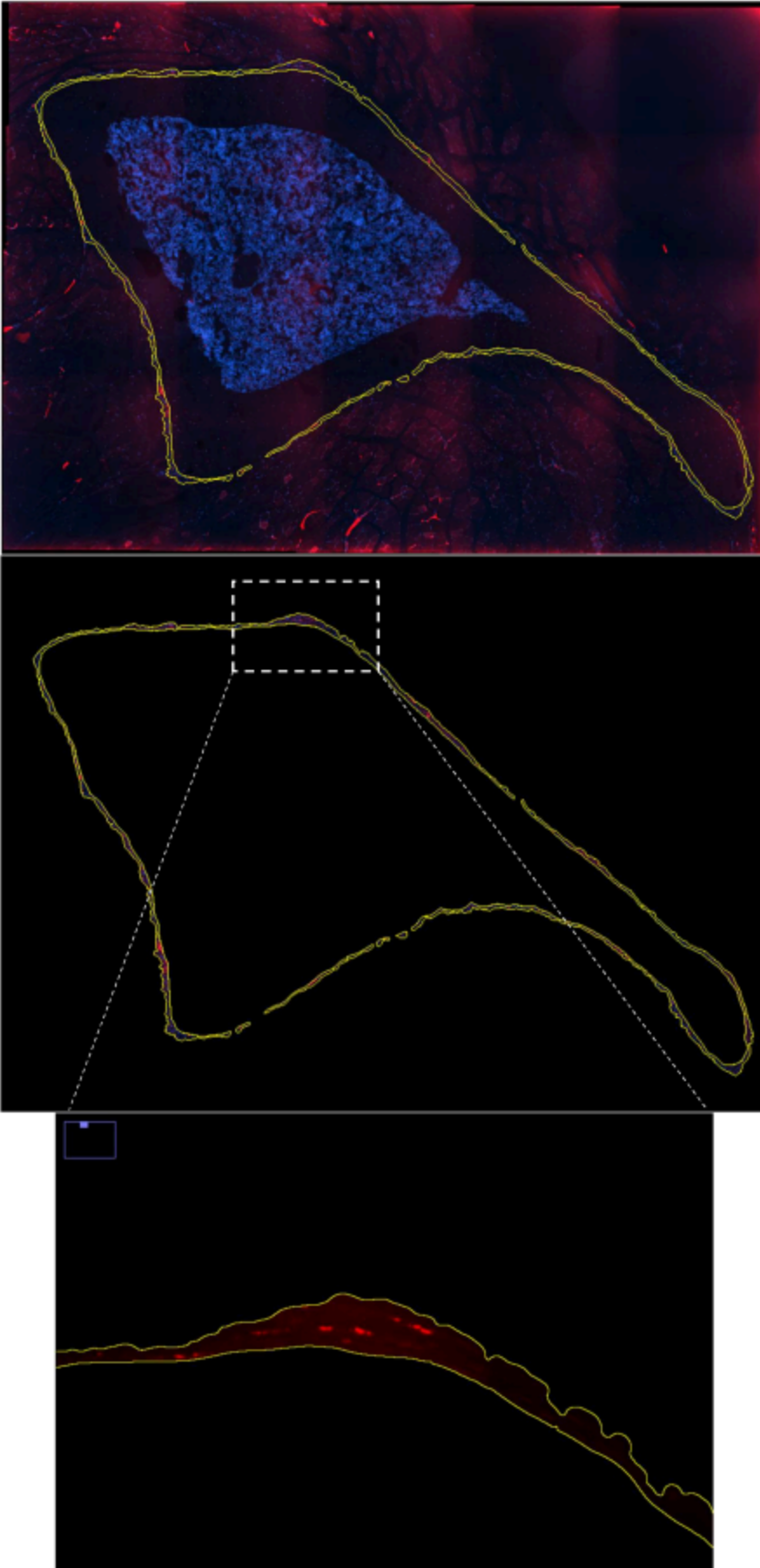
- 16 In the Periosteum mask window, Select (Edit → Selection → Create Selection).



- 17 Go to the main TIFF file, select (Edit → Selection → Restore Selection), then clear outside.

Note

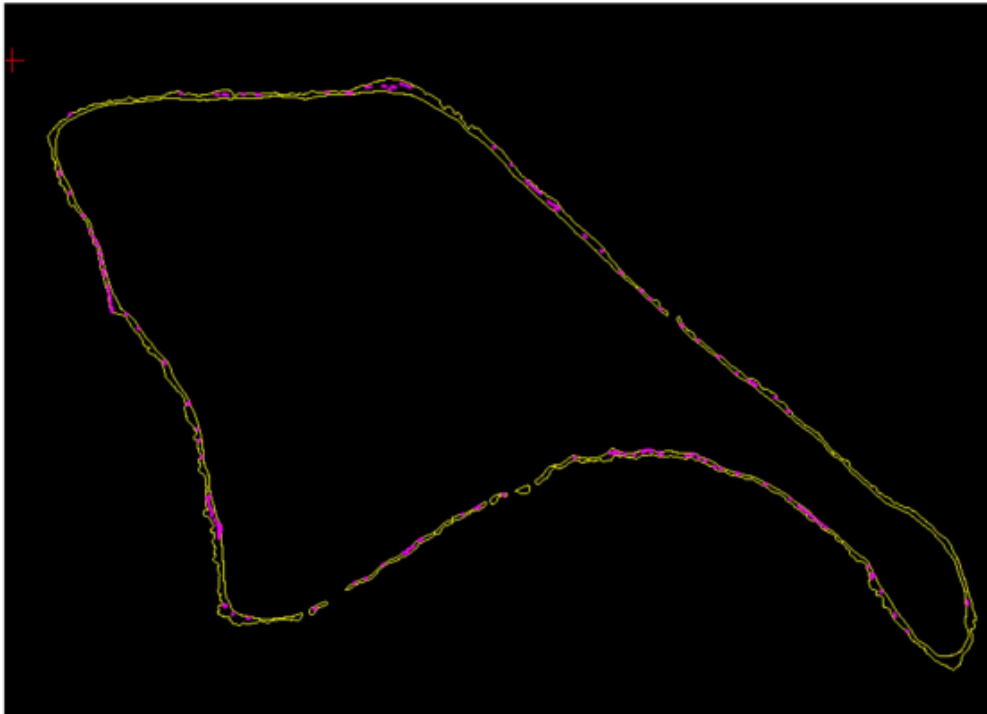
This step will delete everything outside the periosteum region. Pre-selection of the periosteum facilitates unbiased quantification of the nerves within this region of interest. We can also express the length of the nerves relative to the area of the tissue to calculate periosteal nerve density.



- 18 Go to Plugins, and select (Neuroanatomy → SNT).
- 19 Start tracing the nerve endings the same way we described earlier in the measurement of Calcein Labels.

Note

Check that you are choosing the channel corresponding to the nerve stain of interest (in case you are tracing multiple channels).



- 20 After finishing all tracing, export the traces file. [SNT Main Window: File → Export as... > TRACES].
- 21 To export the rendered path image, [Path Manager Window: Analyze → Skeletonize...].



- 22 Save the skeletonized imaged as TIFF.
- 23 To measure the length of the traced segments, [SNT Main Window: Analysis → Measure... → Check "Cable length" → OK].
- 24 Save the value in your excel speardsheet.
- 25 To calculate Periosteal nerve density:

Nerve Density = Nerve Length / Periosteal Area

Note

This can be repeated with multiple channels. A reference pan-neuronal channel (e.g. Baf53b-Cre;Ai9) can also be used to identify and trace axons that co-express nerve identifying markers such as CGRP and TH. Please contact us for further information if needed (scheller@wustl.edu).

Calculate nerve branching (optional)

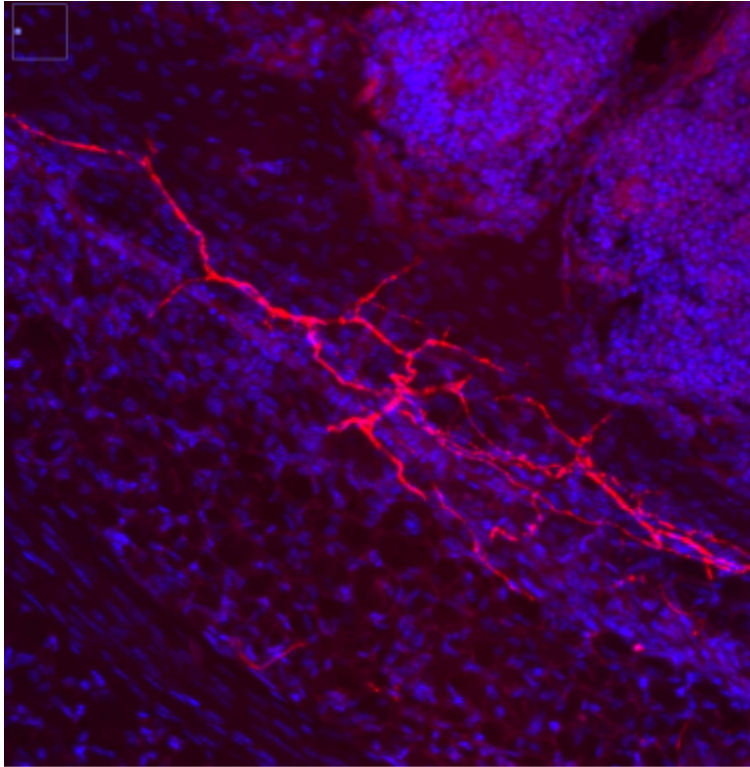
26



Note

- In this protocol, our analyses have focused on nerve length and density as the primary tracing outcome.
- Though the simple neurite tracer program can provide additional information, we do not recommend calculating axon diameter or thickness since this varies depending on the brightness/contrast settings and may not reflect actual differences in nerve size.
- In addition, the majority of the nerves in bone fall into the small axon class (0.1 to 5 μm). Given this, quantification of size, even if accurate, may not provide much additional information.
- Images with matched contrasting may be suitable if axon diameter is a metric that is needed for your study.
- Beyond nerve length and caliber, axon branching is a commonly used metric indicating the abundance of sprouts or local network formation. This may not be reliable in thin sections ($<10\ \mu\text{m}$), as the axons move in and out of plane.
- However, with thicker sections (30-50 μm), these patterns can be more easily appreciated and may represent an important outcome for your study.
- To quantify axon branching, you will need to create nodes during the tracing step in addition to tracing the length of the individual fibers.

- 27 Open your TIFF files in FIJI.



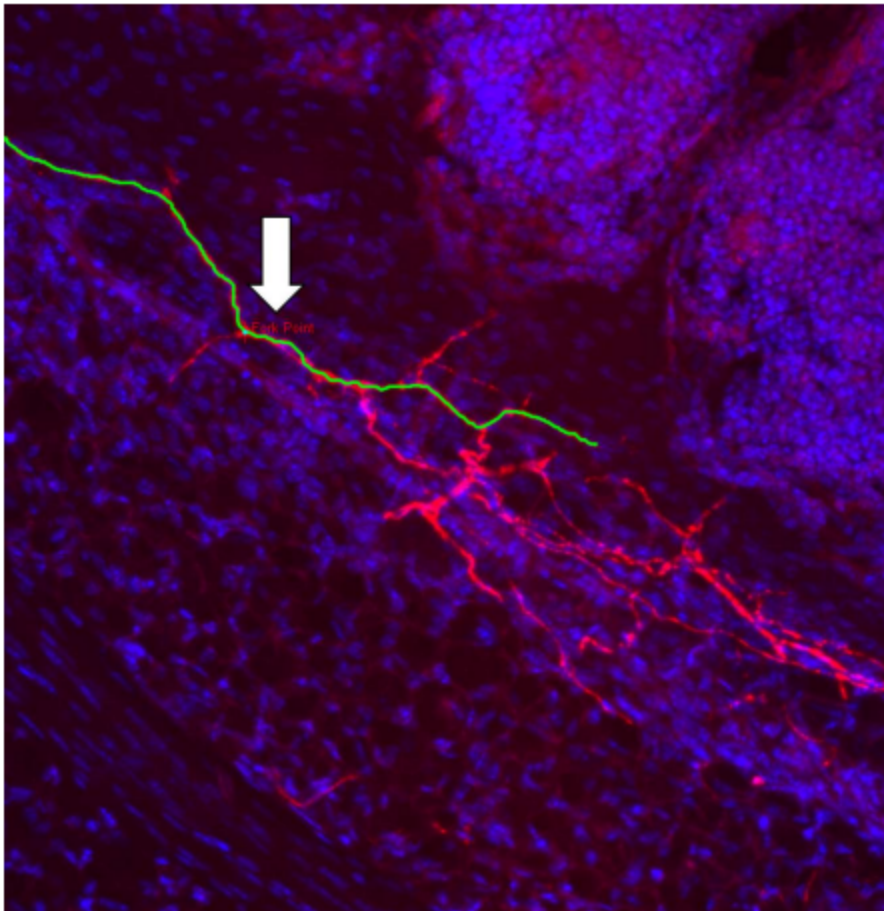
- 28 Select the channel(s) that contains your neural marker(s). In this case we are using the TRITC channel (red channel). Adjust the brightness and contrast to distinguish the nerve label on the periosteal surface.
- 29 Go to Plugins, and select (Neuroanatomy → SNT).
- 30 Start tracing the nerve endings like we described earlier in the quantification of nerve endings.

Note

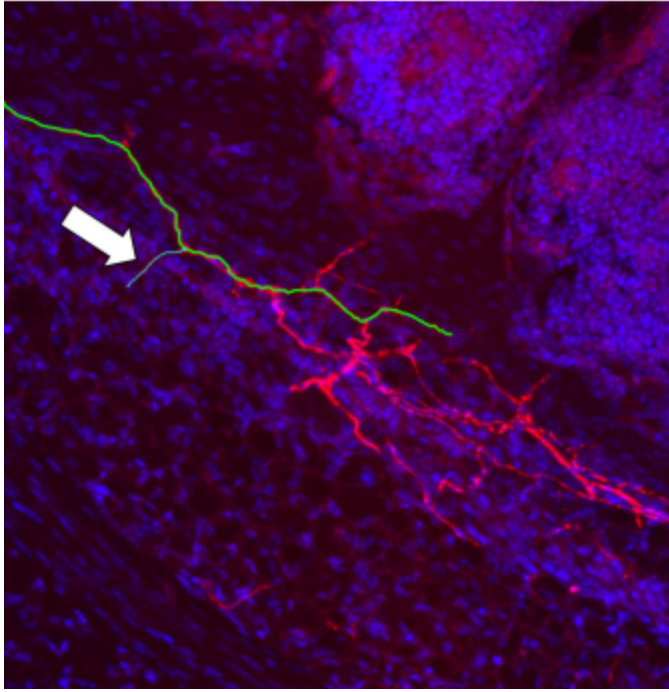
Check that you are choosing the channel corresponding to the nerve stain of interest (in case you are tracing multiple channels).

- 31 Trace the longest path of the spit nerve.

- 32 Select the drawn nerve using the Path Manager, or by press G on keyboard with pointer near the nerve. Once selected, the nerve should be green.
- 33 Holding down Atl + Shift (or just alt), a red marker with a "Fork Point" label should appear, and the selector should only be able to move up and down the traced nerve path.
- 34 Bring the Fork Point marker to the location of the branch point, then click at that location, an indicator should be placed at that point.



- 35 Once the Fork point is placed, lift the Alt + Shift keys and click to the end of the branch.



- 36 Press Y, then F to confirm the trace of the branch.
- 37 The trace should show up in the Path Manager as a tiered nerve with the branch, Path(2), as a part of the longer nerve, Path(1). (See Path manager Window in Image below).
- 38 After finishing all tracing, export traces file. [SNT Main Window: File → Export as... > TRACES].
- 39 To export the rendered path, [Path Manager Window: Analyze → Skeletonize...].
- 40 Save the skeletonized image as TIFF.
- 41 To measure the traced segments' length and branching, [SNT Main Window: Analysis ◇ Measure...(select target outputs)].
- 42 Save output data into your excel spreadsheet.

