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# Axon tracing and node segmentation of myelinated bladder afferents in the pelvic nerve

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**Protocol status:** Working
**We use this protocol and it's working**

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## Abstract

This protocol describes method for tracing myelinated axons labelled with the adeno-associated virus, AAV-PHP.S and neurofascin immunohistochemistry (to identify paranodes) in pelvic nerve of rat, using Neurolucida 360. This tracing method can be applied to various types of peripheral nerves. In rats, AAV-PHP.S has a high tropism for myelinated but not unmyelinated afferents. Cholera toxin subunit B (CTB) microinjected into the bladder was used to identify bladder afferents in the pelvic nerve. This protocol does not include details of the immunohistochemistry or procedures involving animals (viral labelling and CTB tracing). Other antibodies relevant to myelinated axons can be used instead of neurofascin.

## Materials

| Software | |
|---|---|
| **Neurolucida 360** | NAME |
| MBF Bioscience | DEVELOPER |

| Software | |
|---|---|
| **Neurolucida Explorer** | NAME |
| MicroBrightField Bioscience | DEVELOPER |

## Image acquisition

1 On a confocal microscope, scan the nerve fascicle with sufficient magnification and resolution to achieve a voxel size of at least 0.099 x 0.099 x 1 µm (XYZ). This will require multiple tiles acquired with 10% overlap.

## Image pre-processing

2 Stitch together the tiled image stack dataset into a new single image.

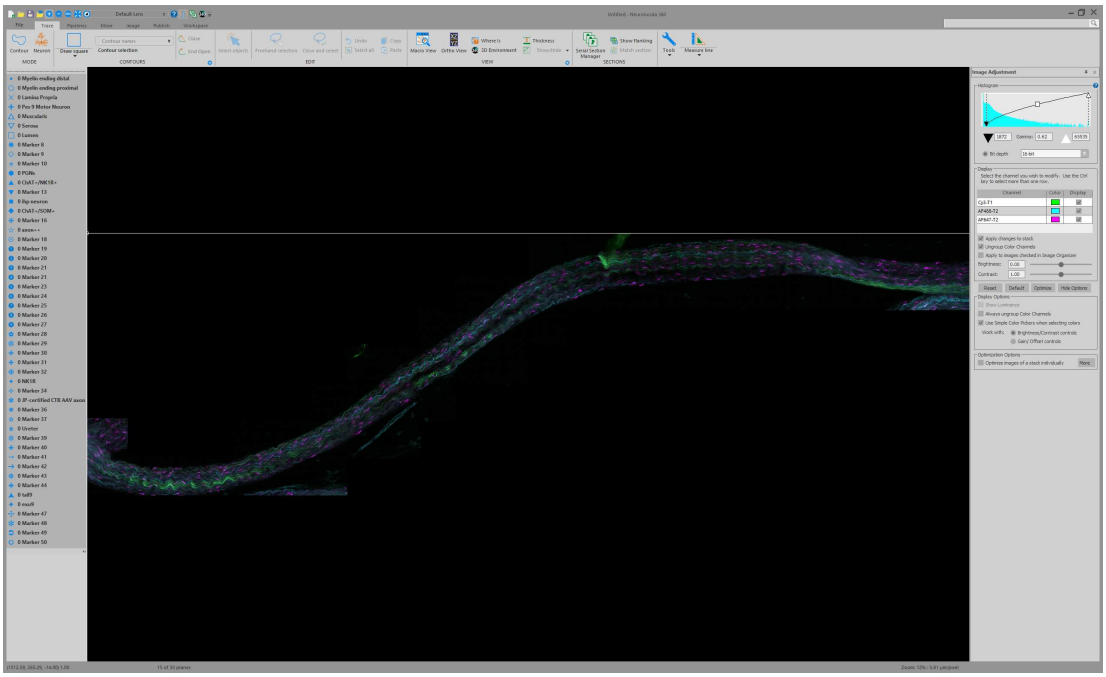
3 Convert the stitched image to JPEG2000 (JPX) using **MicroFile+.**

| Software | |
|---|---|
| **MicroFile+ (RRID: SCR_018724)** | NAME |
| MBF Bioscience | DEVELOPER |

## Opening file in Neurolucida

4 Open the converted nerve JPEG2000 dataset in **Neurolucida 360**.

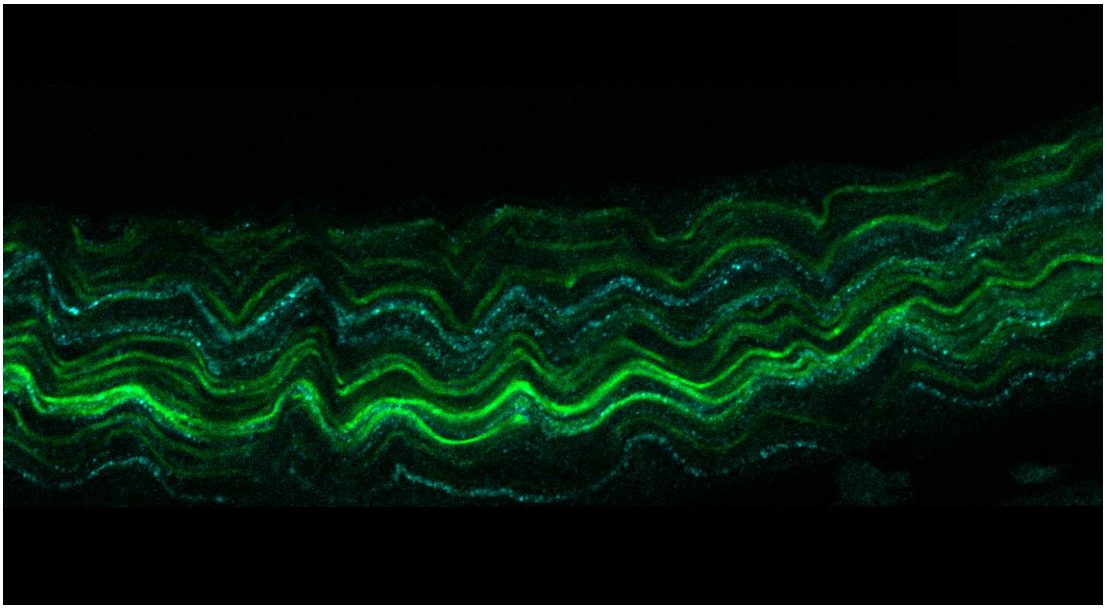
| Software | |
|---|---|
| **Neurolucida 360** | NAME |
| MBF Bioscience | DEVELOPER |



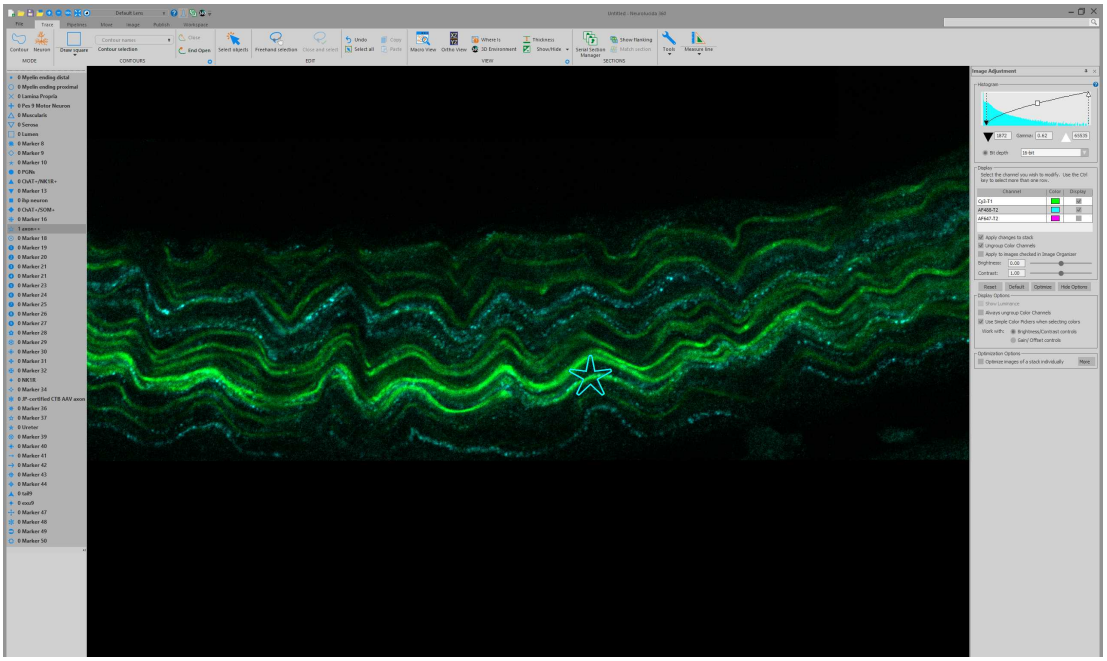
5 Thoughout all the subsequent Steps, save progress. This will prompt a saving of the JPEG2000 (if display settings were altered) and the saving of a data file. Choose .XML as the file type for saving the data file. Keep the .XML in the same file location as the associated JPEG2000.

## Identification of specific axon classes

6 In the 2D view, zoom in to the nerve dataset until individual axons can be discerned.

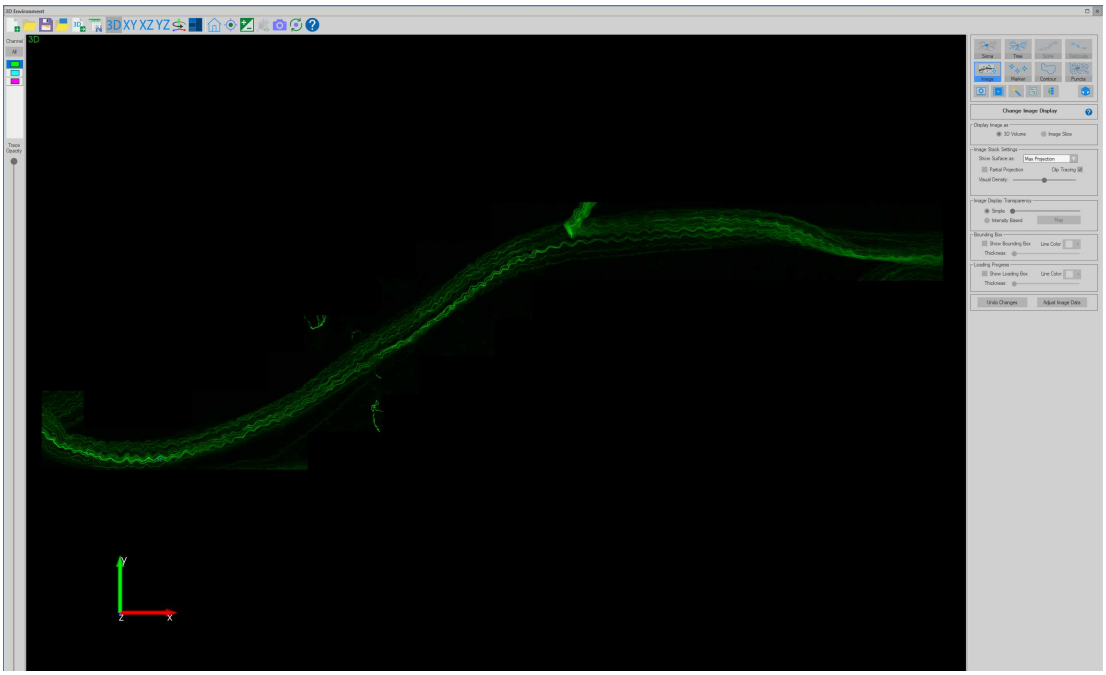


7 Search the nerve fascicle by moving around in XYZ for myelinated (AAV-PHP.S+) bladder (CTB+) axons and mark them with a marker from the panel on the left.

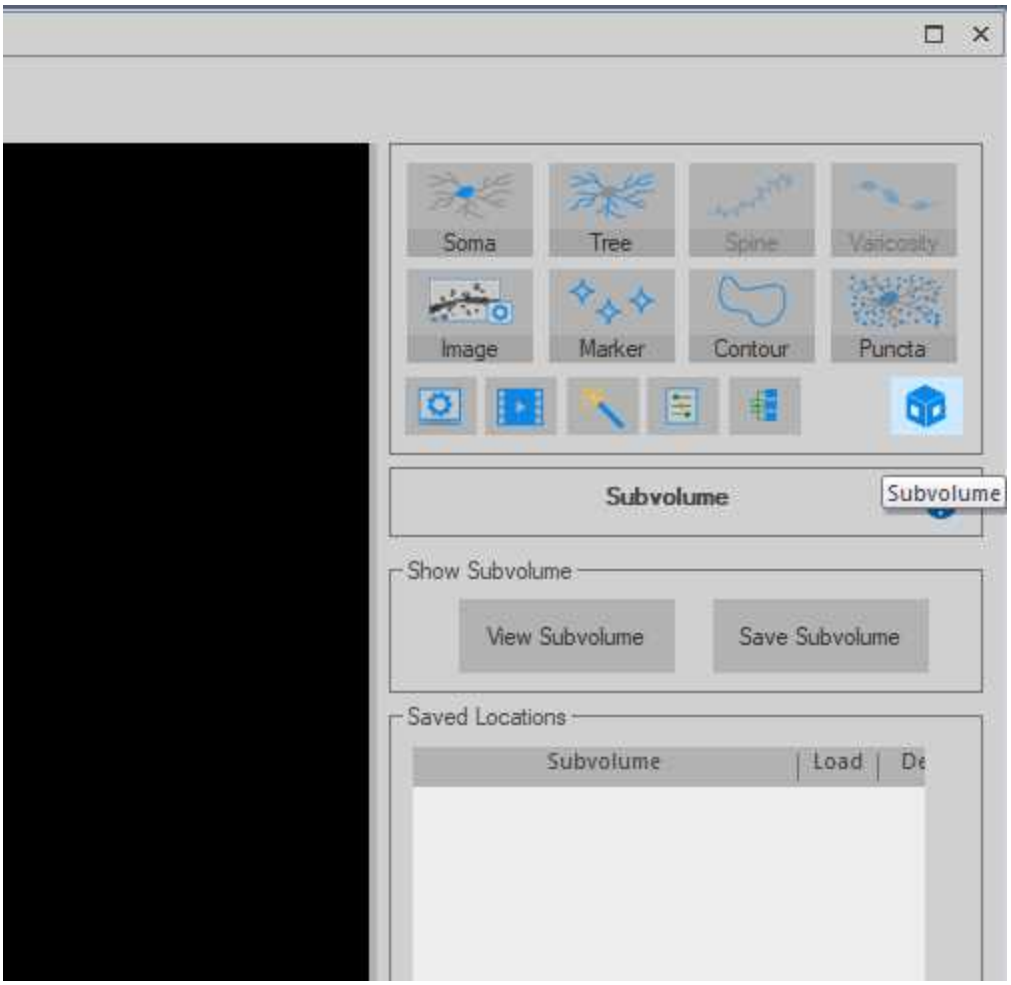


## Subvolume 3D viewing of axons

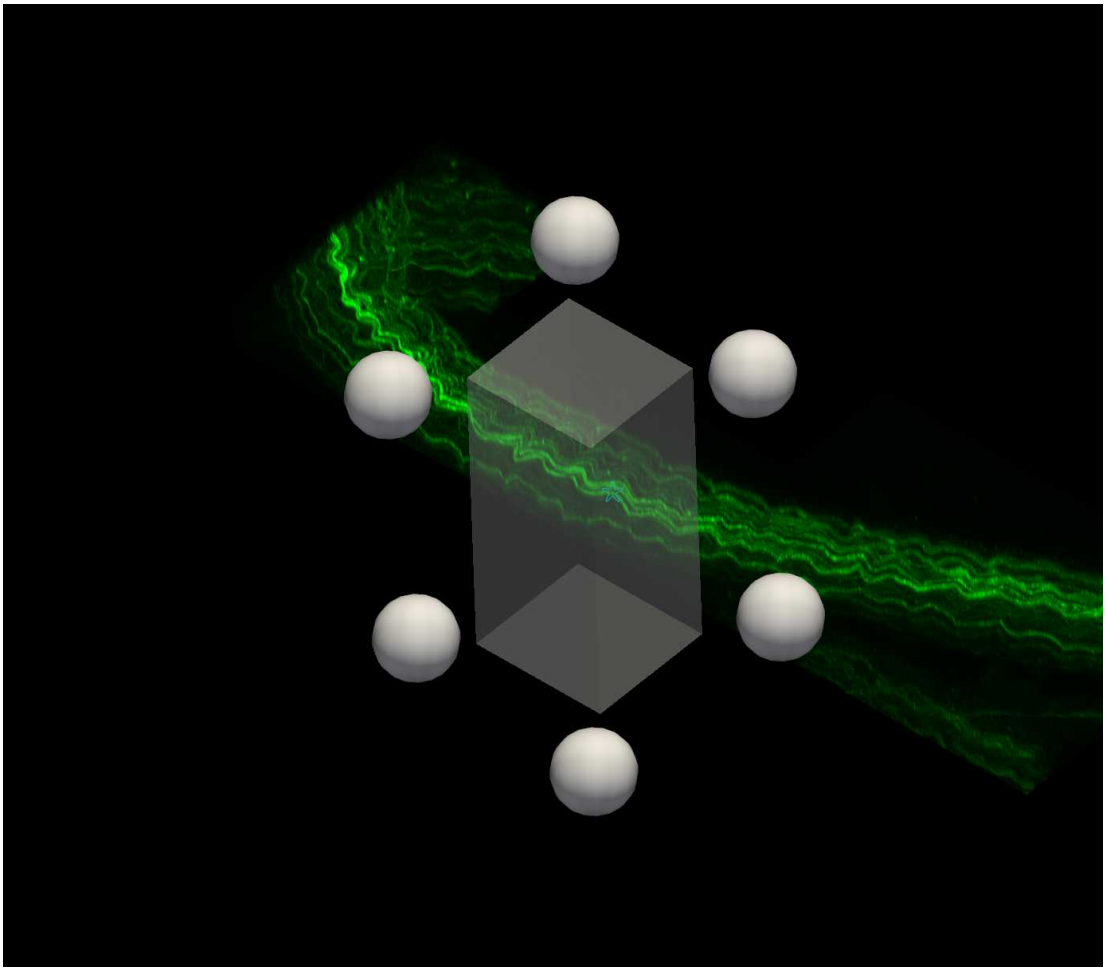
8 Open the **3D Environment** to view the dataset in 3D.



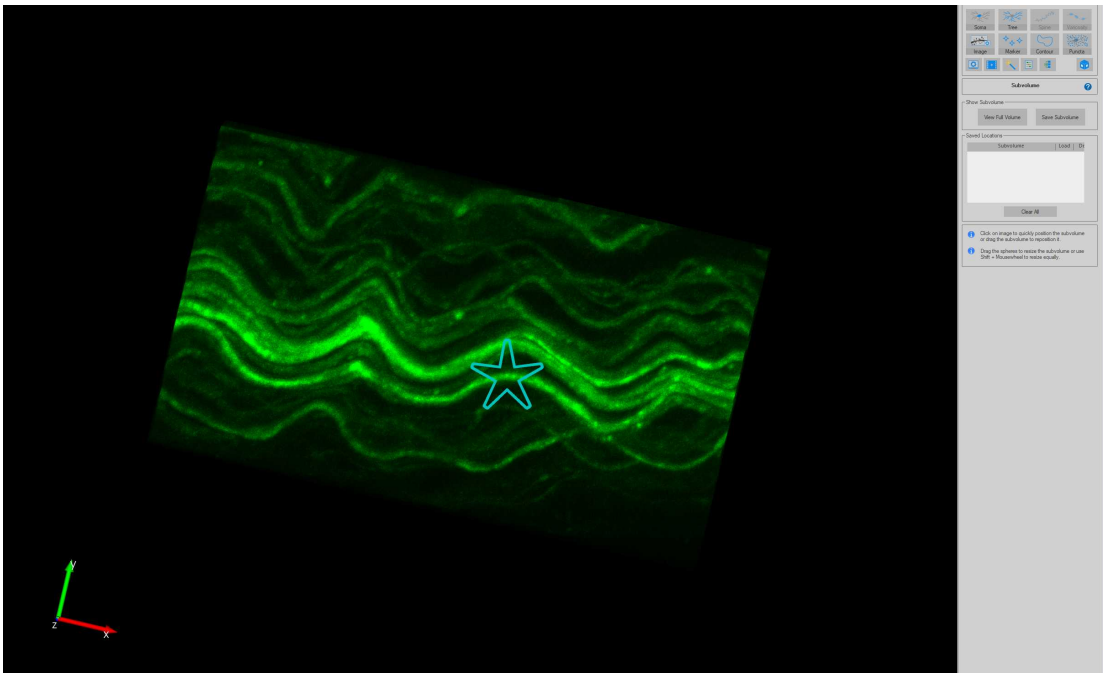
9 In the top right-hand menu, click on **Subvolume**.




10 Move the **Subvolume** region to cover one of the markers established in Step 6.

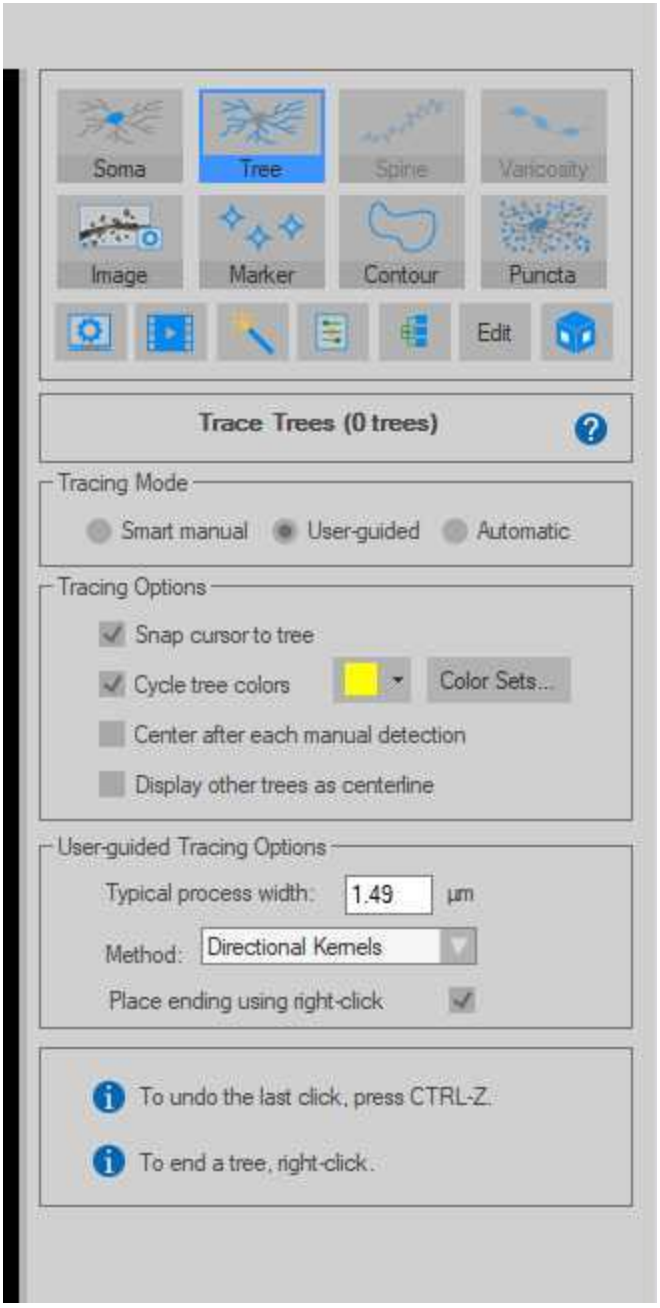


11 Click **View Subvolume** to view only the region selected.

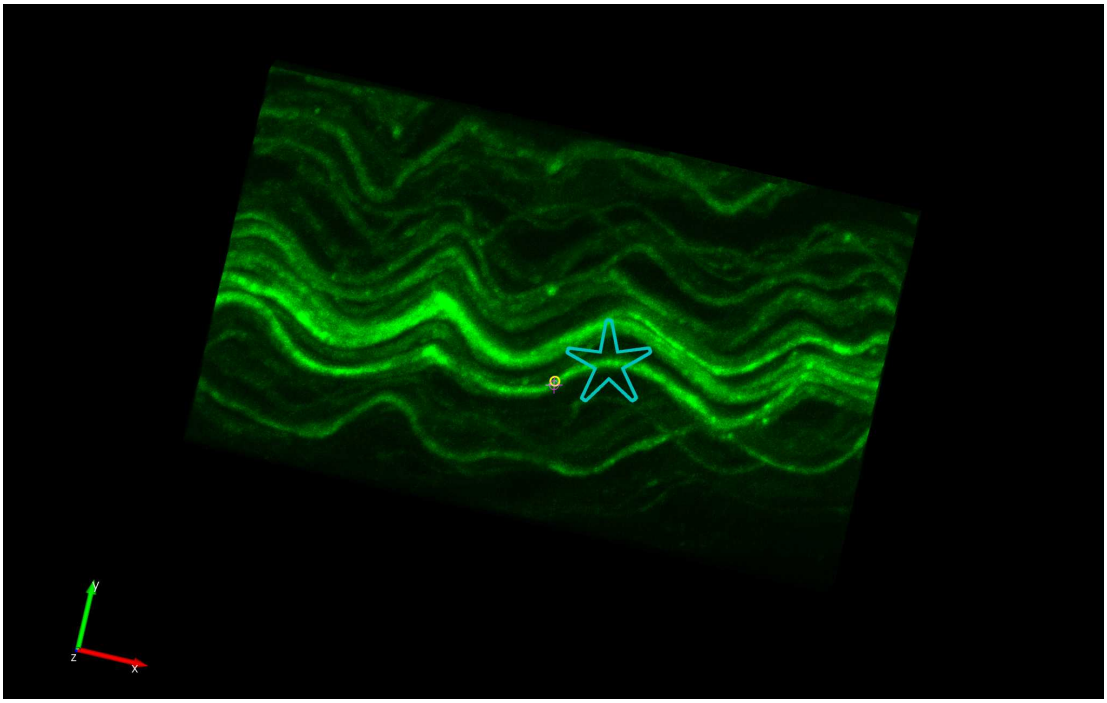


## Tracing axons using AAV-PHP.S fluorescence

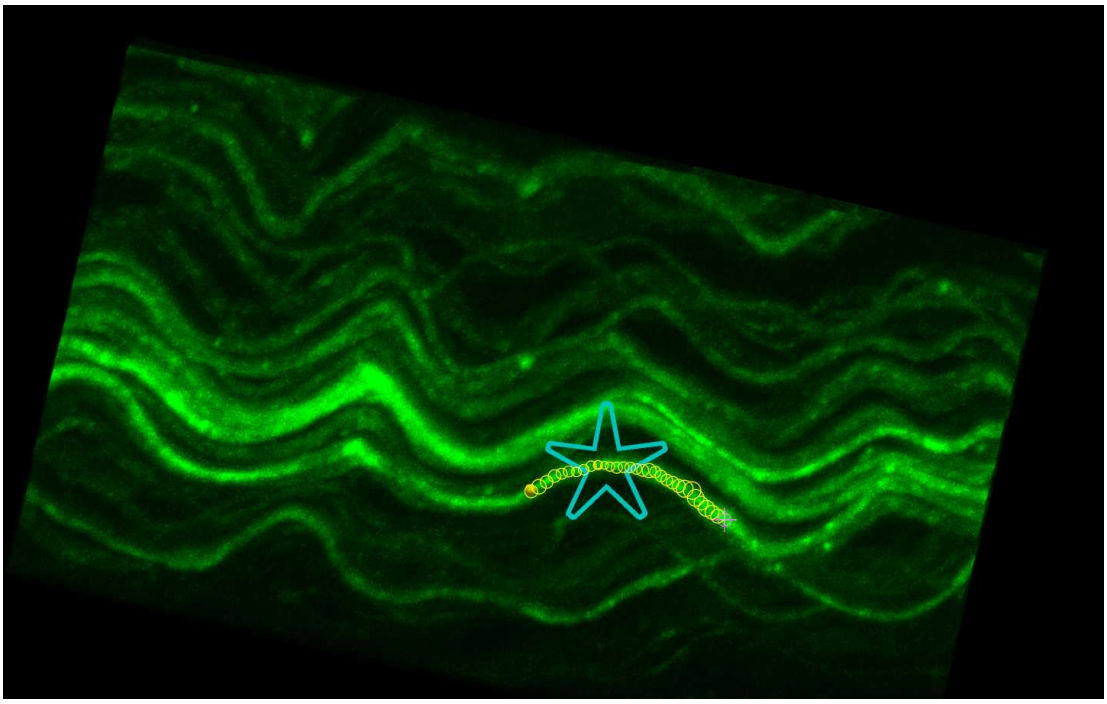
12 Using the channel in which the AAV-PHP.S fluorescence was acquired, start the tracing by clicking on **Tree** in the right-hand side panel. Select **User-guided** and under the **User-guided Tracing Options** select **Directional Kernels** as the **Method**.




13 Move the cursor onto the desired axon to be traced in the **Subvolume** image stack, a small circle should appear on the axon of approximately the same diameter as the axon. This is the starting point of the tracing. **Left click** to start tracing.



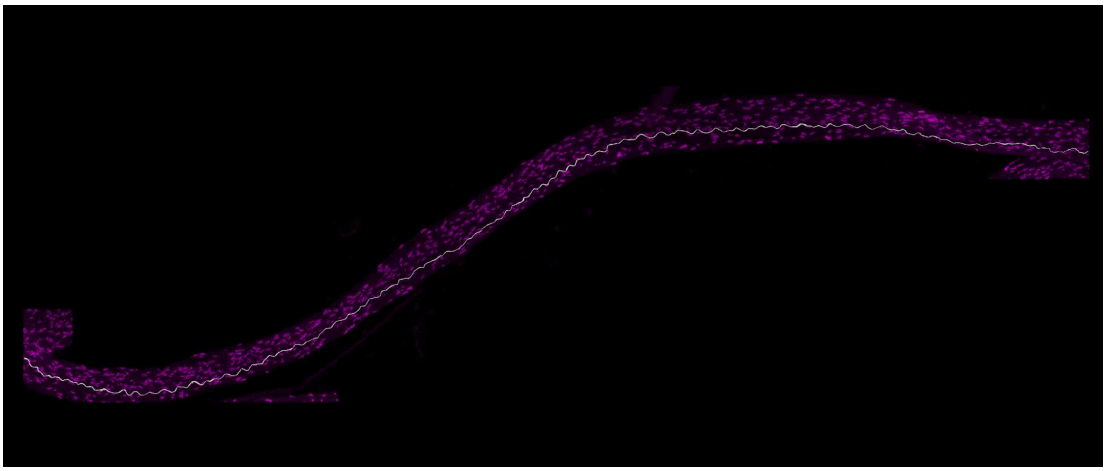

14 Move the cursor along the axon. A string of circles of the axon diameter should follow the cursor, these are individual points of the axon tracing. Go as far as possible without the points deviating from the axon. **Left click** to confirm points, current position will become the new start position.



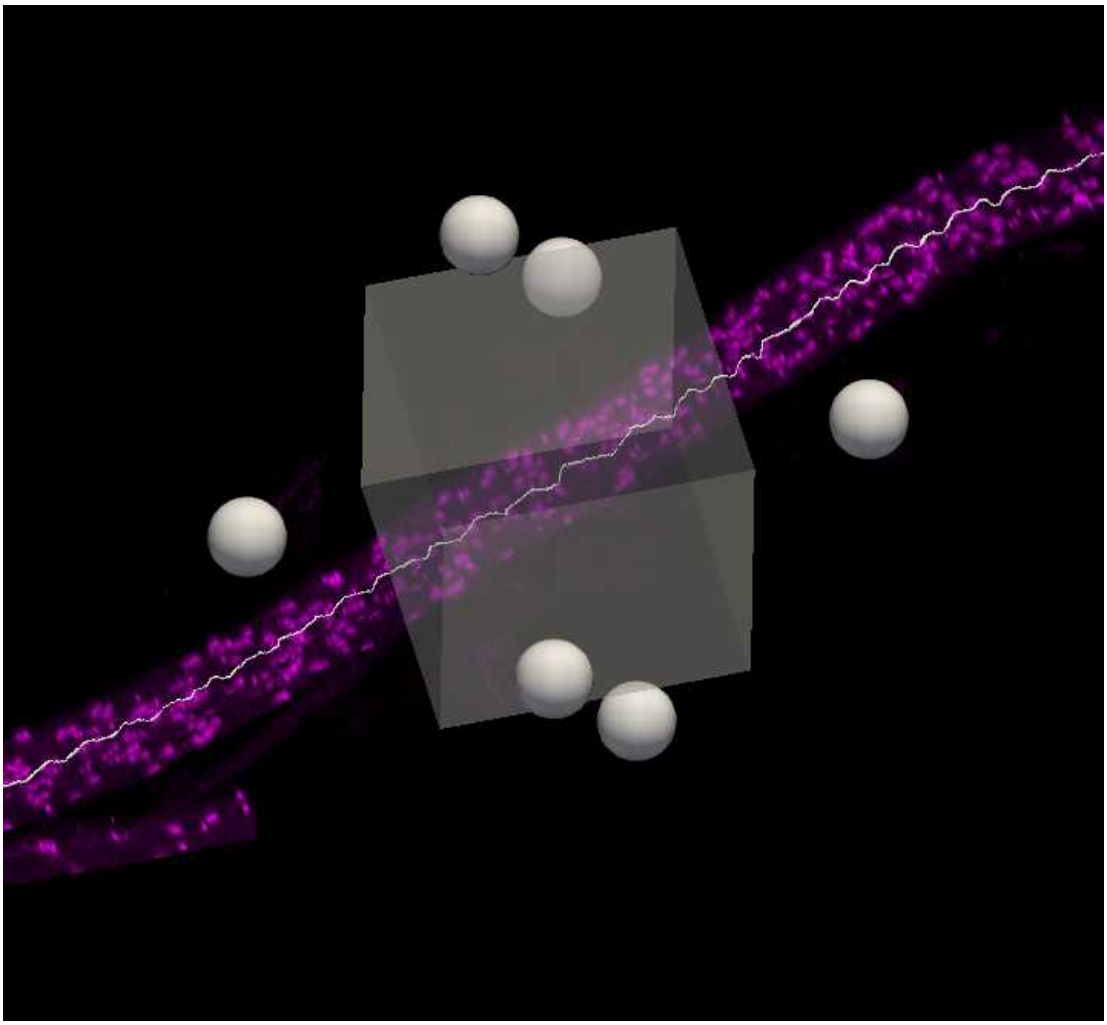
15 When the tracing approaches the edge of the **Subvolume** region, the **Subvolume** region will automatically shift in the direction of the tracing to allow for seamless tracing of axons without the need to view the entire image stack. Continue tracing until the end of the axon (or when the axon cannot confidently be traced).

## Paranode segmentation on traced axons

16 Swap the displayed channel to that in which the Neurofascin immunofluorescence was acquired.

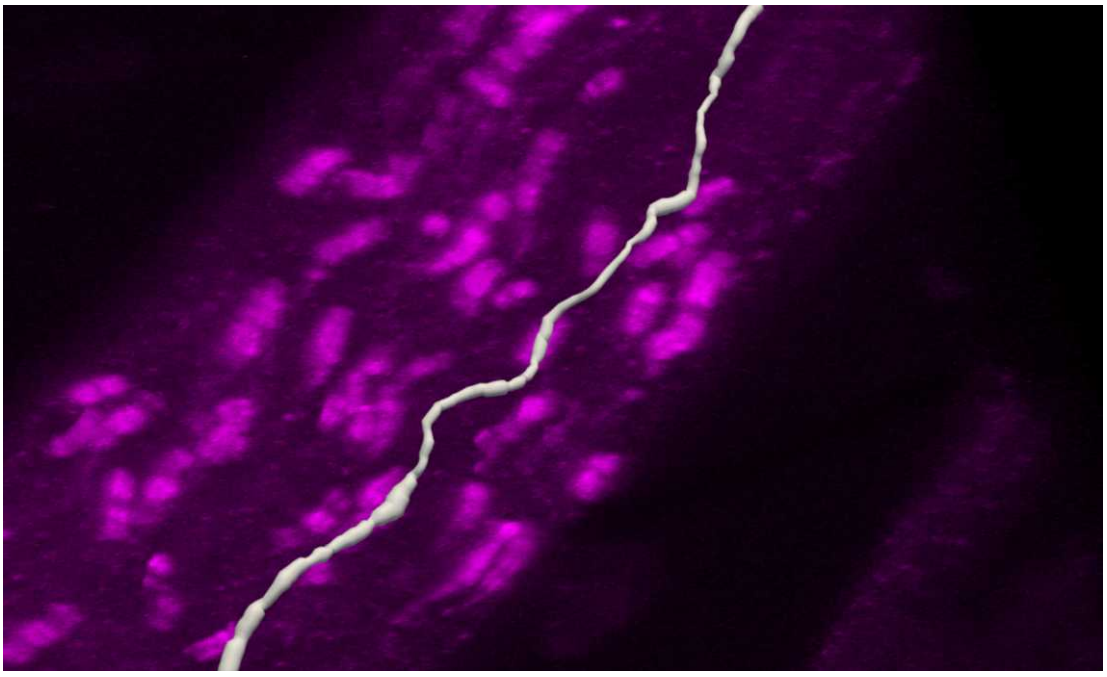


17 As in Steps 8 and 9, select a region of the image to view via **Subvolume**.



18 In the **Subvolume** region, search along the traced axon for Neurofascin labelling that completely overlaps with the axon. Move the image to see the paranodes from multiple angles to ensure correct identification of nodes on the axon. Move the **Subvolume** region along the axon progressively until a paranode is found.

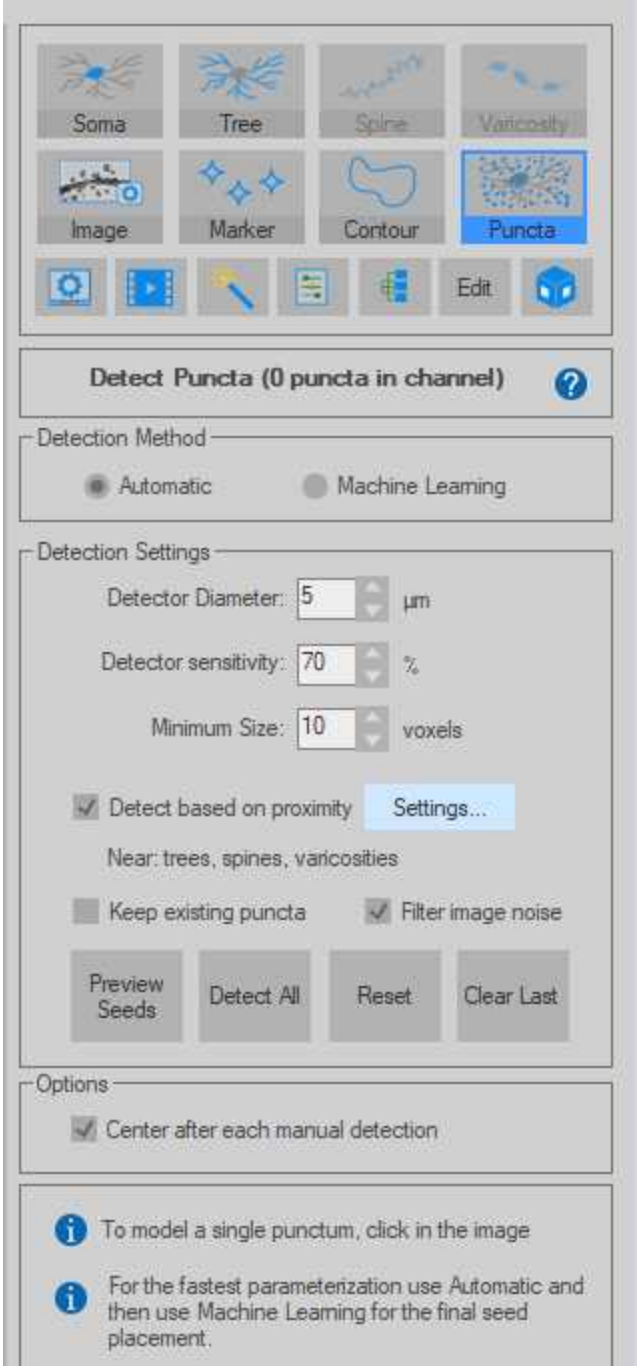
Paranode on axon (middle of image).



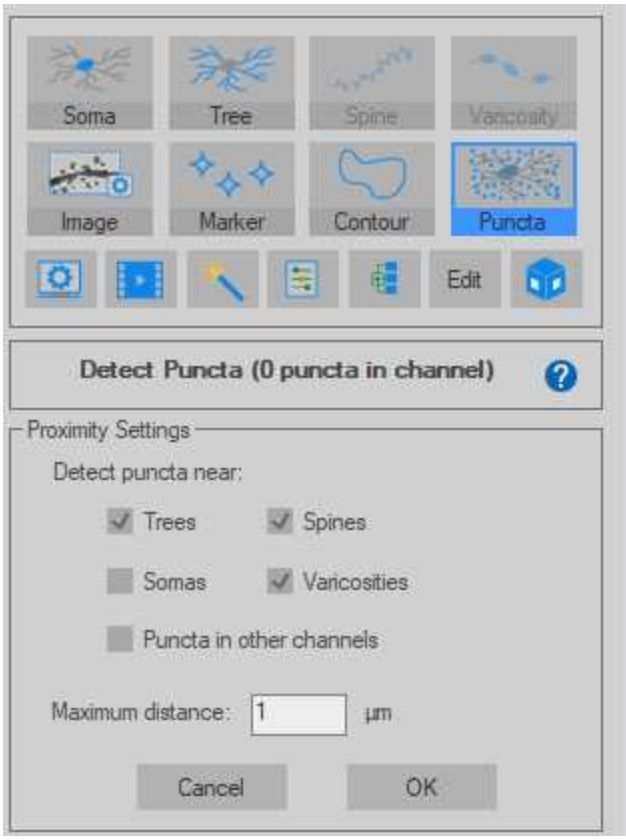
Turning to the side, the paranode is clearly still on the axon.

19 With a paranode identified, in the right-hand panel click on **Puncta**. Keep the **Detection Method** set to **Automatic**. In **Detection Settings**, select Detect based on proximity and click on

**Settings...**



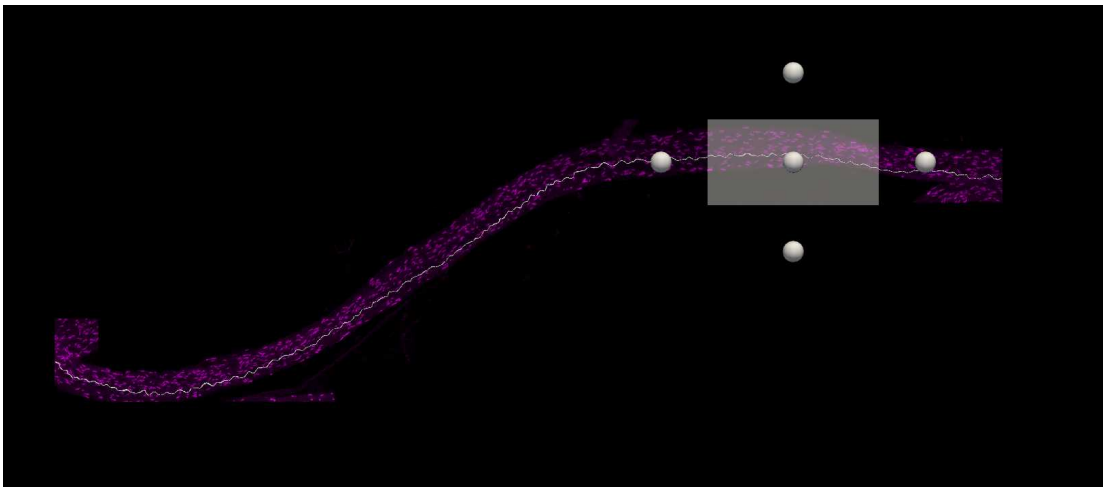

20 Under **Proximity Settings** make sure **Trees** are ticked and set the **Maximum distance** to 1 µm. Click OK.




21 Left click on the paranode in the image to segment that specific paranode. This will create a **Punctum** that represents the paranode.

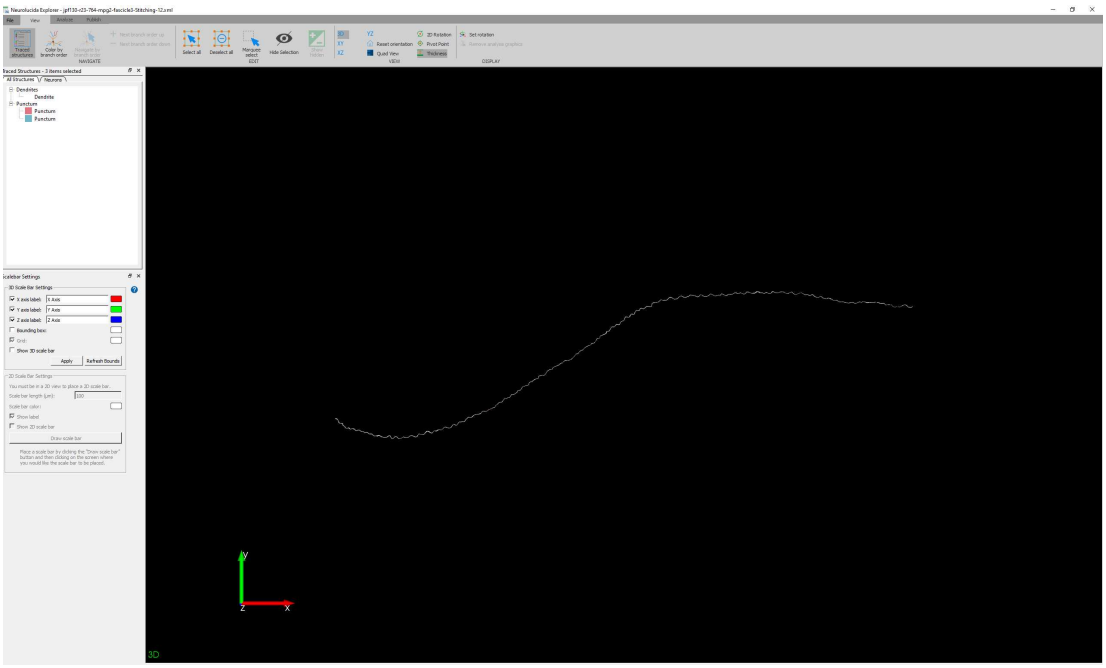
If the detection fails, this is unlikely to be a paranode along the axon (too far away). Sometimes **Detector Sensitivity** can be adjusted for signal that is not being detected but is clearly along the axon.

22 Continue inspecting the dataset for paranodes along the axon, moving the **Subvolume**. For each paranode identified, re-select **Puncta** and repeat Step 20 to segment the paranode.

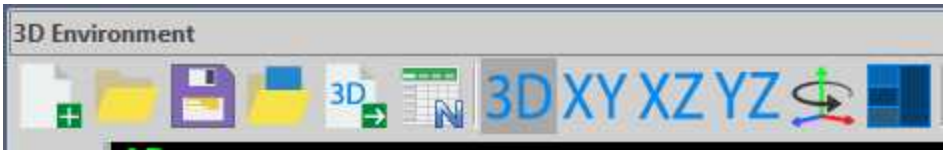


## Axon and paranode data extraction

23 To extract data, open the .XML file created by **Neurolucida 360** in **Neurolucida Explorer**.

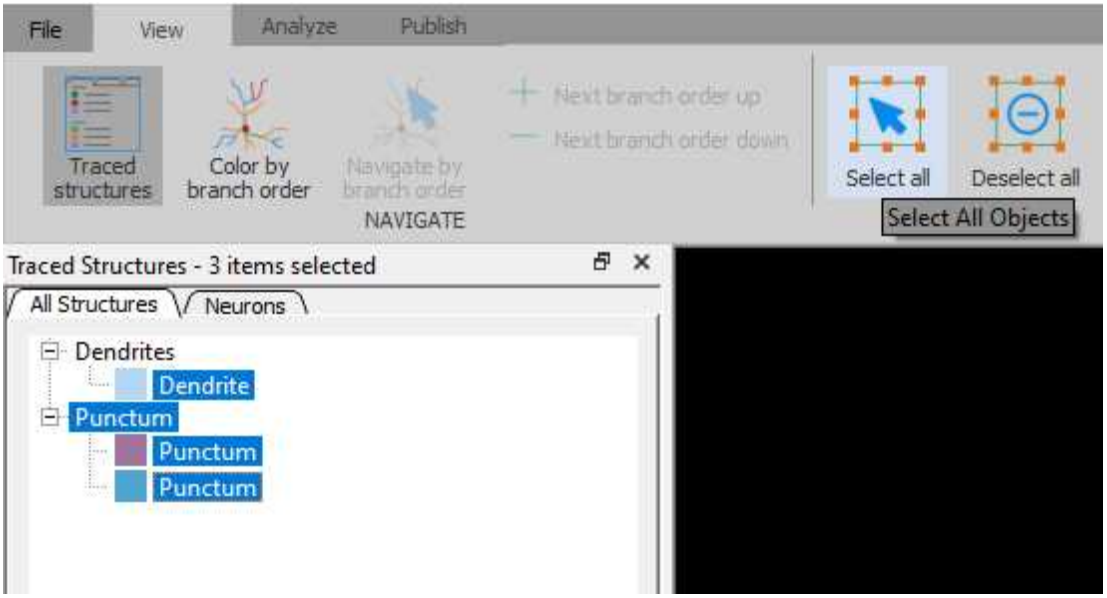


This can be done directly from the **3D Environment** of **Neurolucida 360** by clicking on the icon that looks like spreadsheet with a superimposed 'N'. This will open the current .XML in **Neurolucida Explorer**.

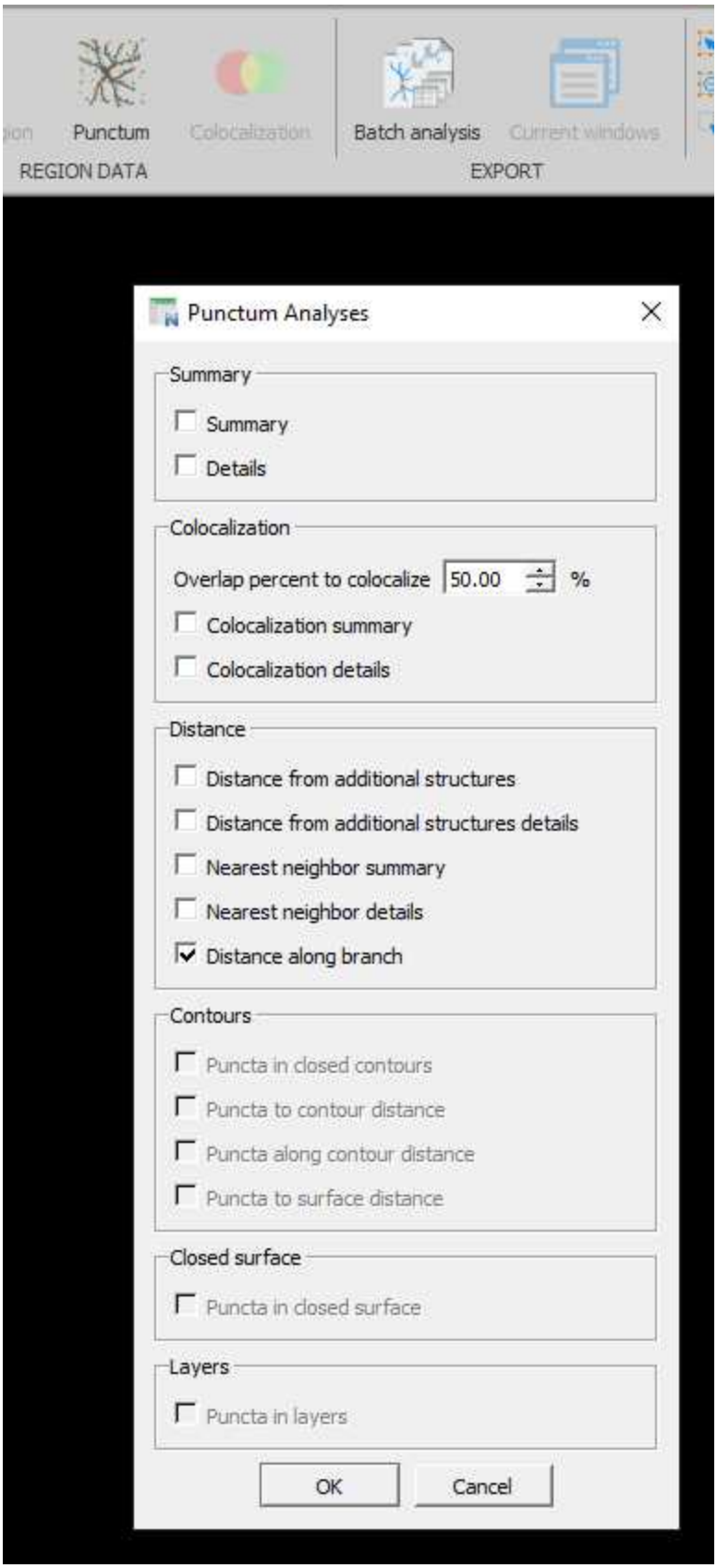



| Software | |
|---|---|
| **Neurolucida Explorer** | NAME |
| MicroBrightField Bioscience | DEVELOPER |

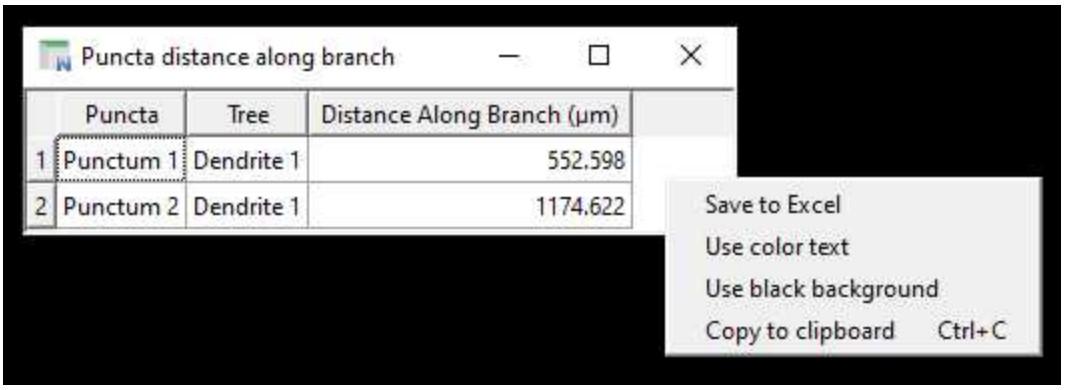
24 Select all the axons and punctum, either manually or by clicking **Select all**.




25 Click on **Punctum** to bring up the **Punctum Analyses** panel and select **Distance along branch**. Click OK.


Punctum
Colocalization
Batch analysis
Current windows
REGION DATA
EXPORT
Punctum Analyses
Summary
Summary
Details
Colocalization
Overlap percent to colocalize
50.00
%
Colocalization summary
Colocalization details
Distance
Distance from additional structures
Distance from additional structures details
Nearest neighbor summary
Nearest neighbor details
Distance along branch
Contours
Puncta in closed contours
Puncta to contour distance
Puncta along contour distance
Puncta to surface distance
Closed surface
Puncta in closed surface
Layers
Puncta in layers
OK
Cancel

26 A window titled **Puncta distance along branch** will appear with an interactable spreadsheet listing each **Puncta** (paranode) and its distance along the **Tree** (axon). To export this table, right click and select **Save to Excel** to save a .CSV.




## Protocol references

Wiedmann, N.M., Fuller-Jackson, J.-P., Osborne, P.B., Keast, J.R., 2024. An adeno-associated viral labeling approach to visualize the meso- and microanatomy of mechanosensory afferents and autonomic innervation of the rat urinary bladder. The FASEB Journal 38, e23380. https://doi.org/10.1096/fj.202301113R