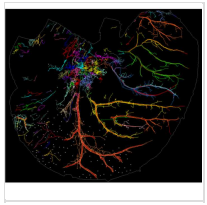


Oct 06, 2021

Mapping CGRP-IR innervation of male mice stomach with Neurolucida 360

Duyen Nguyen[1], Jichao Ma[1]

[1]University of Central Florida

dx.doi.org/10.17504/protocols.io.bygmptu6

This protocol describes the process of using Neurolucida 360 software to map the topographical organization of Calcitonin gene related peptide – immunoreactive axons and terminals in the muscular layer of mice stomach. Stomachs were removed, layers were separated and gone under immunohistochemistry as whole mounts, then scanned using confocal microscopy.

DOI

dx.doi.org/10.17504/protocols.io.bygmptu6

Duyen Nguyen, Jichao Ma 2021. Mapping CGRP-IR innervation of male mice stomach with Neurolucida 360. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bygmptu6

protocol ,

Sep 22, 2021

Oct 06, 2021

53485

Animals

1 Male C57BL/6J mice (The Jackson laboratory), n= 8, age 12-16 weeks were used in this study. Animals were kept in the animal room at which the dark/light cycle is set to 12/12 hours and water and food were supplied ad libitum. All procedures were carried out under the ethical guidelines of University of Central Florida and approved by the Animal Care and Use Committee of University of Central Florida.

Preparation of tissue

2 In order to facilitate the stomach being full and relaxed in accommodation, animals had food available ad libitum until they were anesthetized. Animals were euthanized by a lethal dose (i.p., 100 µg/g) of sodium phenobarbital. As the animals did not have hind paw pinch withdrawal reflex, the abdomen and chest cavity were opened with minimal incisions, and heparin (0.2 ml; 1,000 units/ml) was injected into the heart, followed by transcardial perfusion. Mice were perfused with at least 150 mL 40 °C phosphate-buffered saline (0.1 M PBS, pH = 7.4) via a blunt 18-ga needle inserted to the left ventricle, and blood was drained by cutting the inferior vena cava. The perfusion solution was switched to 150 mL ice cold Zamboni's fixative (15% picric acid, 2% paraformaldehyde in PBS, pH = 7.4) to fix the tissue. After the perfusion, the stomach was removed from the visceral cavity and trimmed to include the distal lower esophageal sphincter and the proximal pylorus. The entire stomach was then opened with a longitudinal cut along the lesser and greater curvature into two equal halves (dorsal and ventral parts) and any food or residue in the stomach was removed by rinsing thoroughly with DI H2O. The stomach was post-fixed in the same fixative for 8 hours.

After post-fixation, each part of the stomach was cleaned, and the mucosal and submucosal layer were dissected from the muscular wall of the stomach. The myenteric plexus together with the longitudinal muscle layer (facing the serosa of the stomach) and circular muscle layer (facing the submucosal layer) comprise the stomach muscular wall. The whole mount muscular wall of the stomach was processed with CGRP antibody.

Immunohistochemistry (IHC)

3 All IHC steps were performed using a 24-well plate on a shaker at room temperature (~24 °C) in dark environment. The IHC steps include washes, blocking, primary antibody, secondary antibody, and were carried out in different wells. Samples were washed 6x5 minutes in 0.1 M PBS (pH = 7.4) and immersed in blocking solution (0.1 M PBS containing 2% bovine serum albumin, 10% normal donkey serum, 2% Triton X-100, and 0.08% sodium azide) for 5 days. After that, the stomach was incubated in primary antibodies (CGRP Mouse Abcam, Cat#ab81887 at a concentration 1:170) for 5 days in a 0.1 M PBS solution contains 2% Bovine serum Albumin, 4% normal Donkey serum, 0.5% Triton X-100, and 0.08% Sodium azide. The tissue was extensively washed 6x5 minutes in 0.1 M PBS containing 0.5% Triton X-100 (PBS-T) to wash any unbound primary antibody, followed by incubation in fluorescent secondary antibodies (Alexa Fluor 488 Invitrogen, Cat#A21202 at a concentration 1:90) for 3 days in 0.5% PBS-T. Negative controls were prepared from stomach without the addition of primary antibodies to exclude secondary antibodies nonspecific binding.

The tissue was washed 6x5 minutes in 0.1 M PBS and mounted serosal side facing up on slide. The tissue was flattened by applying pressure from lead blocks weights (6.75 kg) for 4 hours and dried under the fume hood for 1 hour. The tissue was dehydrated using four increasing concentrations of ethanol (75%, 95%, 100%, and 100%), 4 minutes each, followed by 20 minutes of xylene to render the tissue transparent. DEPEX mounting media was applied over the tissue and covered with a cover slip. The tissue was air-dried in the fume hood overnight.

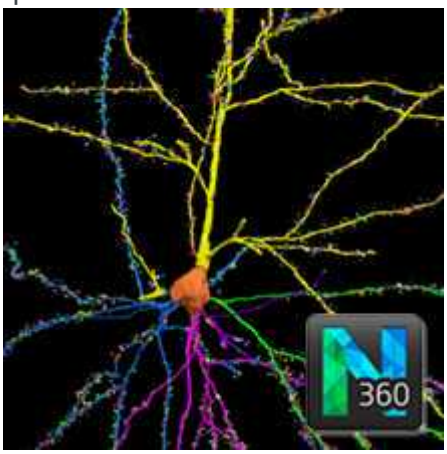
Citation: Duyen Nguyen, Jichao Ma Mapping CGRP-IR innervation of male mice stomach with Neurolucida 360
https://dx.doi.org/10.17504/protocols.io.bygmptu6

Tissue scanning and Montage assembly

4 The samples were scanned with two microscopes: one is Leica TCS SP5 Confocal Laser Scanning Microscope. Argon-krypton laser was excited at 488 nm and emitted at 500-550 nm to detect CGRP-IR axons. Image stacks of 1.5 µm z-acquisition were saved as .lif and fully projected confocal images were saved as .tif. All image tiles were stitched together using Adobe Photoshop to assemble the montage of the whole stomach. Another microscope is ZEISS Axio Imager M2.

Software installation setup

5 Have Neurolucida 360 installed and licensed on your workstation through an MBF Bioscience representative.



[https://www.mbfbioscience.com/neurolucida360]

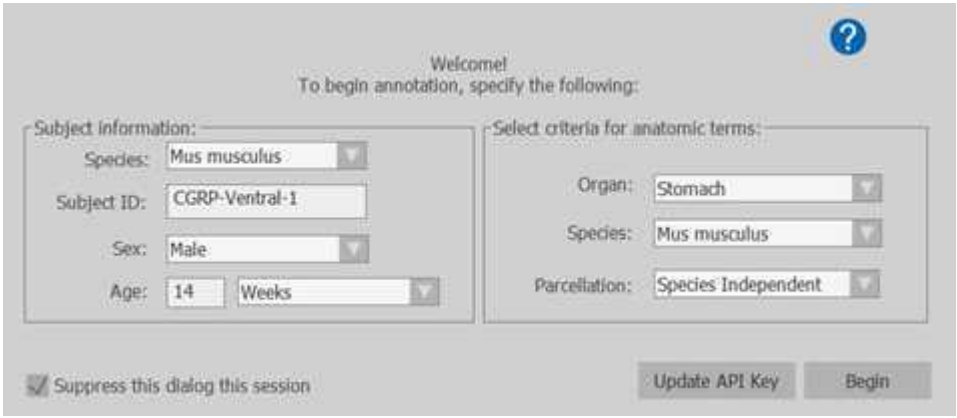
After launching Neurolucida 360, set an API key to your profile to pull the ontology list from SciCrunch to annotate your organ of choice with curated SPARC anatomy terms (you might have to register an account on scricrunch.org in order to obtain an API key).

Image annotation and Nerve tracing

6 Opening and configuring the image file: In the "File" ribbon and under the "Open" menu, select "Image," and select the image file (.jpg or .tif) of interest. A dialog will appear to verify or adjust the XY scaling properties of the image. If the scaling properties are correct and do not need adjustment, click "OK" to load the image.

Citation: Duyen Nguyen, Jichao Ma Mapping CGRP-IR innervation of male mice stomach with Neurolucida 360
https://dx.doi.org/10.17504/protocols.io.bygmptu6

6.1 Metadata setup: Create a profile for your sample by filling in the subject information of your sample with the species, sample ID, sex, and age on the left side of the dialog. Then specify the species and organ in which you are annotating on the right side of the dialog to access the curated SPARC anatomy terms and click "Begin" when you are done. For the purposes of our tracing, the organ is the "Stomach" and the species is "Mus musculus".



Manually inputting information about your sample only occurs once when you first create a document to annotate your sample. After you save your work as an .xml file and load it into the program, the metadata dialog will autopopulate and you can resume your work.

6.2 Workspace setup: For ease of annotation, it is recommended to set up your workspace with dockable windows. Under the "Workspace" ribbon, enable the "AutoMove" function and dock the "Macro view" and "Image adjustment". The AutoMove feature will define a working area and automatically reposition your image to the center when your move goes beyond the bounds of the working area. The "Macro view" acts as a mini-map in a separate window to show your current location on the image and to also take you to any region of interest. The "Image adjustment" allows you to modify the image's brightness and

Citation: Duyen Nguyen, Jichao Ma Mapping CGRP-IR innervation of male mice stomach with Neurolucida 360
https://dx.doi.org/10.17504/protocols.io.bygmptu6

contrast and is especially helpful for displaying your image.

More information on setting up dockable windows can be found here: https://www.mbfbioscience.com/help/neurolucida360/Default.htm#About/DockWindowsSetup.htm?TocPath=Interface%2520overview%257C_____5

6.3 Contour the sample: To begin contouring or tracing anatomical features, go to the "Trace" ribbon and in the "CONTOURS" submenu, click "Contour selection" to display the list of curated SPARC anatomy terms. If an anatomical structure of interest is not on the list, it can be created by clicking the blue Gear button at the bottom right corner of submenu. Under the "Contour selection" button, set tracing options to "Freehand" to manually contour the anatomical structures of interest.

6.4 Sample annotation: Select the desired anatomical structure that you wish you annotate under the "Contour Selection" list. Zoom in on the image as needed to contour your desired structure by clicking along the structure to place points and draw lines. Alternatively, you can also continuously draw a contour by right clicking on the mouse and selecting the "Continuous tracing" option. This method of tracing works best when paired with a Wacom tablet or other digital tablet drawing device. When you are finished with annotating your structure, right click to select "Close contour" or "End open contour."

6.5 Nerve tracing: To enable neuronal tracing mode, go to the "Trace" ribbon and in the "NEURONS" submenu, click "Axon".

Citation: Duyen Nguyen, Jichao Ma Mapping CGRP-IR innervation of male mice stomach with Neurolucida 360
https://dx.doi.org/10.17504/protocols.io.bygmptu6

Similarly to making a contour, deposit a point at the origin of the axon and click along the structure to place points to create an axon. The diameter of the red circle cursor can be adjusted using the scroll button on the mouse and should correspond to the diameter of the axon being traced. The current diameter value of the cursor is displayed at the bottom left corner of the interface (coordinates of the cursor (x,y,z) and [cursor diameter]). At a branching point, right click and select “Bifurcating node”. When you have finished tracing one branch of the axon, right click to select “Ending”, the cursor will renavigate you to the branching node to continue tracing the other branch.

For more information on how to trace all branches of the axons, visit: https://www.mbfbioscience.com/help/neurolucida360/Default.htm#Tracing-Editing/traceSingleSection.htm?TocPath=Working%2520in%2520the%2520main%2520window%2520(2D)%257CModel%2520neurons%2520in%25202D%257C_____1

Saving your work

7 To preserve your progress in annotating and tracing, go to the "File" ribbon and under the tab "Save as," select "Data File" and save your work as an XML document file (.xml). The associated metadata from the initial dialog box will autopopulate when you load the file to resume your work.

Visualization of contoured structure and traced axons and terminals

8 You can view the completely traced stomach by unclicking “AutoMove” from the “Workspace” ribbon, the white dashed line rectangle will disappear. The image can be saved either by selecting Publish ◊ Snapshot or using a snipping tool from your computer. 3D Environment is also available for visualizing files that consist of image stacks.

For more information on how to interact with 3D Environment elements, visit: https://www.mbfbioscience.com/help/neurolucida360/Default.htm#Ribbons/Workspace/3Denvt.htm?TocPath=Interface%2520overview%257C_____2

Citation: Duyen Nguyen, Jichao Ma Mapping CGRP-IR innervation of male mice stomach with Neurolucida 360 https://dx.doi.org/10.17504/protocols.io.bygmptu6