

Apr 12, 2023

Version 1

A single guide to impregnate samples with Golgi-Cox solution within 24hr and represent results with a set of algorithm V.1

DOI

<https://dx.doi.org/10.17504/protocols.io.3byl4jdrolo5/v1>

Avishek Roy^{1,2}, Binney Sharma¹

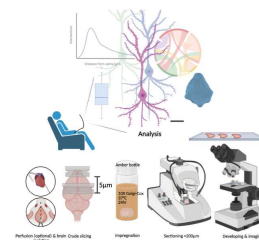
¹All India Institute of medical Sciences, New Delhi-110029, India;

²Interdisciplinary Institute for Neuroscience, UMR-5297, University of Bordeaux, Bordeaux Cedex, France



Avishek Roy

Interdisciplinary Institute for Neuroscience (UMR 5297)



Create & collaborate more with a free account

Edit and publish protocols, collaborate in communities, share insights through comments, and track progress with run records.

Create free account

OPEN  ACCESS



DOI: <https://dx.doi.org/10.17504/protocols.io.3byl4jdrolo5/v1>

Protocol Citation: Avishek Roy, Binney Sharma 2023. A single guide to impregnate samples with Golgi-Cox solution within 24hr and represent results with a set of algorithm. **protocols.io** <https://dx.doi.org/10.17504/protocols.io.3byl4jdrolo5/v1>

Manuscript citation:

Roy, A., Sharma, S., Nag, T.C.*et al.* Cognitive Dysfunction and Anxiety Resulting from Synaptic Downscaling, Hippocampal Atrophy, and Ventricular Enlargement with Intracerebroventricular Streptozotocin Injection in Male Wistar Rats. *Neurotox Res* 40, 2179–2202 (2022). <https://doi.org/10.1007/s12640-022-00563-x>

License: This is an open access protocol distributed under the terms of the **Creative Commons Attribution License**, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Protocol status: Working

We use this protocol and it's working

Created: February 23, 2023

Last Modified: April 12, 2023

Protocol Integer ID: 77517

Keywords: Golgi-Cox staining, One day impregnation, Neuronal morphometry, cox solution into deep brain structure, diffusion of the stain, samples with golgi, sample with golgi, cox staining, lipophilic nature of the central nervous system, brain sample, thickness of the brain sample, algorithm golgi, golgi, impregnation of the stain, deep brain structure, neurons in health, diffusion process at physiological temperature, goal of this staining, neuroscience, cox solution, rat brain, cox solution within 24hr, central nervous system, cell

Funders Acknowledgements:

Indian Council for Medical Research

Grant ID: IR-594

Abstract

Golgi-Cox staining is one of the old but relevant histological technique to identify neurons in superficial/ deep brain structures. The goal of this staining is to accurately perform morphometric analysis on the desired neurons in health and diseases. Due to the origin of its own there are different variations of the protocol itself. Majority of them take at least one to two week to have impregnation of the stain into the tissue/ cells. This is due to the physical property of the stain and lipophilic nature of the central nervous system. Therefore to enhance the diffusion of the stain into the brain samples, we have came up with a modification of only one protocol out there by Ranjan and his colleagues. Where we have decreased the thickness of the brain sample to 5µm from 25±1µm (for rat brain) and incubated the sample with Golgi-Cox solution at 37°C in order to reduce the path of travelling and simultaneously help the diffusion process at physiological temperature. The results showed a significant amount of impregnation of the Golgi-Cox solution into deep brain structures viz. hypothalamus, hippocampus within a timespan of 24 hour. This reduces the labour, time and enhance the efficiency of the impregnation process enabling a better image to analyse further. The overall goal of this protocol is to help experimenter focusing on the analysis of the morphometric data as well as complementing it with other relevant functional data by reducing the time to stain samples enhancing efficacy. Finally, we hope that this modified protocol will not only help researchers in field of neuroscience to perform the technique with ease but also help them to represent their result in the best/ unique way using different algorithm and softwares mentioned in the protocol.

Attachments



Attachment to the pr...

32.6MB

Guidelines

This protocol is meant to reduce the time and increase the efficiency of the Golgi-Cox staining in central nervous tissue. Due to the reason that this protocol is modified from the available literatures keeping in mind the basic principles of diffusion of a metal i.e. physical properties viz. temperature, thickness etc. This protocol may vary a little in sample preparation steps. We have got robust staining in every batch of samples we have stained using the same protocol. There is also few literature where use of penetrating agents viz. SDZ, triton X has been used alongside the same protocol and showed no further improvement. However, we have not tried ,manipulating that, however comparison with the regular 07 day protocol with change of solution at one week interval showed less efficiency in staining the deep structures. Availability this protocol will help researcher to focus on more critical analysis and presentation of the morphological data one can get from this technique.

We recommend to use fresh solutions with a filtration at least at an interval of 3-4weeks. And use of glassware/ plasticware during the whole procedure to avoid any kind metallic reaction. Further, keep the sections in dark especially in the slicing, humid chamber, and even during developing step as possible.

Materials

Animals step 9

Vibratome step 19

MethanolMerck MilliporeSigma (Sigma-Aldrich)Catalog #M3641Step 5

Mercury(II) chlorideMerck MilliporeSigma (Sigma-Aldrich)Catalog #M1136-100GStep 1

Agar agarMerck MilliporeSigma (Sigma-Aldrich)Catalog #05038-500GStep 18

Sodium thiosulfateMerck MilliporeSigma (Sigma-Aldrich)Catalog #72049-250GStep 2

Ammonium hydroxideMerck MilliporeSigma (Sigma-Aldrich)Catalog #05002-1LStep 2

Ethanol Merck Millipore (EMD Millipore)Catalog #100983Step 2

Hydrogen chloride solution (HCl 1M)Merck MilliporeSigma (Sigma-Aldrich)Step 3

Ethanol PureMerck MilliporeSigma (Sigma-Aldrich)Catalog #493511Step 5

500g Gelatin (Reagent Grade)G-BiosciencesCatalog #RC-053Step 8

Protocol materials

-  Ethanol **Merck Millipore (EMD Millipore) Catalog #100983**
-  Sodium thiosulfate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #72049-250G**
-  Ammonium hydroxide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #05002-1L**
-  Hydrogen chloride solution (HCl 1M) **Merck MilliporeSigma (Sigma-Aldrich)**
-  Ethanol Pure **Merck MilliporeSigma (Sigma-Aldrich) Catalog #493511**
-  Methanol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #M3641**
-  500g Gelatin (Reagent Grade) **G-Biosciences Catalog #RC-053**
-  Potassium chromate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #12249-100G**
-  Potassium di-chromate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #207802-100G**
-  Mercury(II) chloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog #M1136-100G**
-  Agar agar **Merck MilliporeSigma (Sigma-Aldrich) Catalog #05038-500G**

Safety warnings

-  This protocol includes use of heavy metals, paraformaldehyde and other potential carcinogens hence the researcher is suggested to use proper safety gears, and use of biosafety hood whenever possible.

Ethics statement

This study was performed under ethical clearance from Institutional Animal Ethical Committee (IAEC), All India Institute of Medical Sciences, New Delhi, which is under Committee for the Purpose of Control and Supervision of Animal experiments CPSCEA, India. Vide no. 937/IAEC/PhD-2016.

Before start

Before start one should have basic knowledge of central nervous system and have experience on microscopic experiments and histological experiments. However, we have tried to prepare this protocol keeping a larger readers in mind, and the motto of this protocol is to get sample stained on the first trial with just a knowledge/ experience in wet lab. Further, as the main criticality of the technique lies on the analysis of the results one can extract from the morphometry of the stained neurons, therefore a knowledge on the neuronal architecture specifically to the brain region you are interested with is an advantage. We have kept the minimum duration of the incubation/ impregnation to be 24 hr keeping in mind that once you go to >2days you might have more stained neurons and their branches, but you will loose the branch of a specific cell you are tracing. This problem happens when one neurite is being masked by another neighbouring neuron/ neurite.



Preparation of Golgi-Cox solution

25m

1 Golgi-Cox solution was prepared using

25m

⊗ Mercury(II) chloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog #M1136-100G**

⊗ Potassium chromate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #12249-100G**

⊗ Potassium di-chromate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #207802-100G**



Safety information

All these reagents are toxic/ carcinogenic therefore use of these reagents should be conducted with proper safety gears:

1. Prepare solution under safety hood
2. Use gloves +/- glasses as possible
3. Due to the metal in this stain avoid using any other equipment except it is made of plastic/ glass. Not even metal forceps

1.1 First, we have prepared **⚗ 5 % W/V** solution of all three aforementioned salts from 15gm of salts dissolving into 300ml of MiliQ water

15m

1.2 Next, mercury(II) chloride and potassium-di-chromate was mixed at **[M] 1:1 ratio (:) V/V**

5m

1.3 Potassium chromate was added at **[M] 4 % (v/v)** to the previous mixture

5m

Preparation of developing solutions

17m

2 For developing the impregnation colour we have used following reagents:

17m

⊗ Sodium thiosulfate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #72049-250G**

⊗ Ammonium hydroxide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #05002-1L**

⊗ Ethanol **Merck Millipore (EMD Millipore) Catalog #100983**





- 2.1 A 5% (W/V) solution of sodium thiosulfate was prepared mixing 100ml of MiliQ water in 5gm of sodium thiosulfate 5m
- 2.2 2 part of Ammonium hydroxyde was mixe with 1 part of MiliQ water to prepare 3:1 (V/V) ammonium hydroxyde 2m
- 2.3 In order to make ascending order of alcohol concentration (50%, 75%, 95%, 100%) we have mixed MiliQ water with Ethanol in the aforementioned ratio (V/V) 10m
- 3 In order to make the section stick to the slides first we have kept frosted micro slides (Bluestar, 75mm x 25mm) in 12h
 - Hydrogen chloride solution (HCl 1M) **Merck MilliporeSigma (Sigma-Aldrich)**
 - 18.5 % volume in MiliQ water for Overnight in the staining trough

STEP CASE

Etching process 38 steps

This step is done to increase the surface area, which will help sections to stick to the slide while staining

- 4 HCl was discarded and replaced with running tap water for 2-3hr 3h
- 5 Running tap water was discarded and replaced with 50 % (v/v) of admixture of
 - Ethanol Pure **Merck MilliporeSigma (Sigma-Aldrich) Catalog #493511**
 - Methanol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #M3641** and kept for 2-3hr3h
- 6 Again the admixture was replaced with running tap water and slides were kept for 1-2hr 2h

STEP CASE

Removal of any lipophilic substances 35 steps

This step is important to remove any oily substances/ grease from the surfaces of the slides

- 7 Finally tap water was replaced with MiliQ water and slides were dried in slide racks inside incubator (BIOOCN India, India) at warmer 60-70 °C 30m



Now the slides are ready to coat

- 8 A solution of [M] 3 % (v/v)  500g Gelatin (Reagent Grade) **G-Biosciences Catalog #RC-053** was prepared mixing gelatin in MiliQ water (eg. 3gm in 100ml of MiliQ)

- 8.1 Cleaned slides were incubated in the 3% Gelatin solution at  40 °C inside incubator (BIOOCN India, India) for  00:10:00

10m

Anaesthesia

5m 50s

- 9 Rats were treated with lethal dose of sodium thiopentone (150mg/kg of BW) through intra-peritoneal route
- 10 Level of anaesthesia was checked though paw-pressor test

STEP CASE

CO2 Asphyxiation 30 steps

One can opt for euthanasia in CO₂ chamber by treatment of >60% CO₂. And at the end level of anaesthesia could be tested as in step 3

- 11 Perfusion setup was filled with  0.9 % W/V NaCl (saline)  2-4 °C and the flow rate was set at a rate of 3ml/min

5m



STEP CASE

Minimum volume of saline to be perfused 29 steps

25-35 ml of ice cold saline can be perfused or else one can perfuse till the lungs and kidneys get white. This indicates saturation of the fluid at pulmonary as well as aortic circuit respectively.

- 12 Rats were placed onto their back and heart was made visible by opening cardiac envelop followed by an access to plural cavity through incisions through diaphragm

3m

- 13 Finally, the saline needle was introduced to the left ventricle and then the right auricle was incised to break the close loop

40s

Brain isolation

2m 20s

- 14 After the completion of perfusion process, animals can be decapitated to isolate the complete brain

20s

- 14.1 Lateral incision was made by occipital bone, followed by I-incision through sagittal suture

1m

- 14.2 Finally, the nasal bone was broken to peel off the skull bone in order to isolate brain

1m

Preparation of coronal chunks

50s

- 15  5 mm coronal chunk of the brain from anterior to posterior was cut using the brain matrice (51388, Stoelting Co., USA) (see fig. 1.C-D in attachment)

50s

Equipment

Brain Matrices

NAME

Crude tissue slicer

TYPE

Stoelting CO. (USA)

BRAND

51388

SKU

<https://stoeltingco.com/Neuroscience/Stainless-Steel-Brain-Matrices-10mm~9995>

LINK

1.0 mm thickness coronal slices

SPECIFICATIONS



Impregnation step

1d

16 Immerse your sample (brain chunks viz. frontal lobe, cerebellum, spinal cord etc.) in the filtered Golgi-Cox solution as prepared in step 1. Keep in mind to use Golgi-Cox at 10X the volume of the sample.



16.1 Keep the preparation in amber colour bottle (or use aluminium foil to wrap in any glass/plastic bottle available) at 37 ± 2 °C for minimum 24:00:00 (see fig. 1.E-F in attachment)

1d

Block preparation

10m

17 Wash brain chunks incubated with Golgi-Cox solution in 30 Mass / % volume sucrose in MiliQ at Room temperature for 00:10:00

10m

18 Prepare 3 % Agar agar Merck MilliporeSigma (Sigma-Aldrich) Catalog #05038-500G in MiliQ and replace the 30 % W/V sucrose solution with the same pouring gently into a customized mold from 50ml falcon tubes (see attachment; Fig. 3B in attachment)

2w

Equipment

Corning® 50 mL centrifuge tubes

NAME

conical bottom tube

TYPE

Corning 430829

BRAND

CLS430829-500EA

SKU

These can be preserved at 4 °C for 336:00:00 when sealed with parafilm till vibrotomy

Sectioning and transferring them to slides

1d 0h 36m



19  300 μm thick coronal sections were prepared with vibratome in a solution of 30m

 6 % sucrose made in MiliQ

Equipment

Vibrotome

NAME

Slicing sections at higher thickness

TYPE

Leica

BRAND

VT1000 S

SKU

20 Once sections are prepared they were immediately transferred onto the pre-coated frosted glass slides 5m

21 Finally, extra solutions were wiped with a gentle pressure of palm with a tissue wet in 1m
 6 % sucrose



Note

Put gentle pressure at a specific angle (90 degree) with the wet tissue paper. This step not only help you get rid of extra sucrose/ cutting solution but also stable the sections which will help you mounting them at the last step of staining.

Keep in mind not to put pressure at different angle at a same time by moving the palm over the slide. If you do so, then there is a risk of losing the orientation of the sections as well as making an irreversible impression on the section

22 These slides were then kept in a humidified chamber and again placed into a incubator at 1d
 37 $^{\circ}\text{C}$ for  24:00:00



**Note**

This step is required to fix the sections onto the slides. If not carried out then there is a high risk of losing the sections during developing step

Developing and mounting**1h 5m**

- 23 Sections were hydrated with MiliQ water at Room temperature for 00:05:00 5m
- 24 Transferred to 50 % ethanol at Room temperature for 00:05:00 5m
- 25 Transferred to 3:1 ratio (:) ammonium solution at Room temperature for 00:10:00 10m
- 26 Rinsed in MiliQ water at Room temperature for 00:05:00 5m
- 27 Transferred to 5 % sodium thiosulfate solution at Room temperature for 00:12:00 12m
- 28 Rinsed again with MiliQ water at Room temperature for 00:02:00 2m
- 29 Sections were dehydrated with graded alcohol (70, 95, 100: 5 min each) 15m
- 30 Transferred to Xylene at Room temperature for 00:10:00 10m

Note

Sections can stay in the xylene solution until they are mounted however more than 10-12 minute in our hand causes a significant tissue brittle, breakage at spaces



31 Now sections can be mounted in a coverslip with DPX

1m

Imaging and tracing

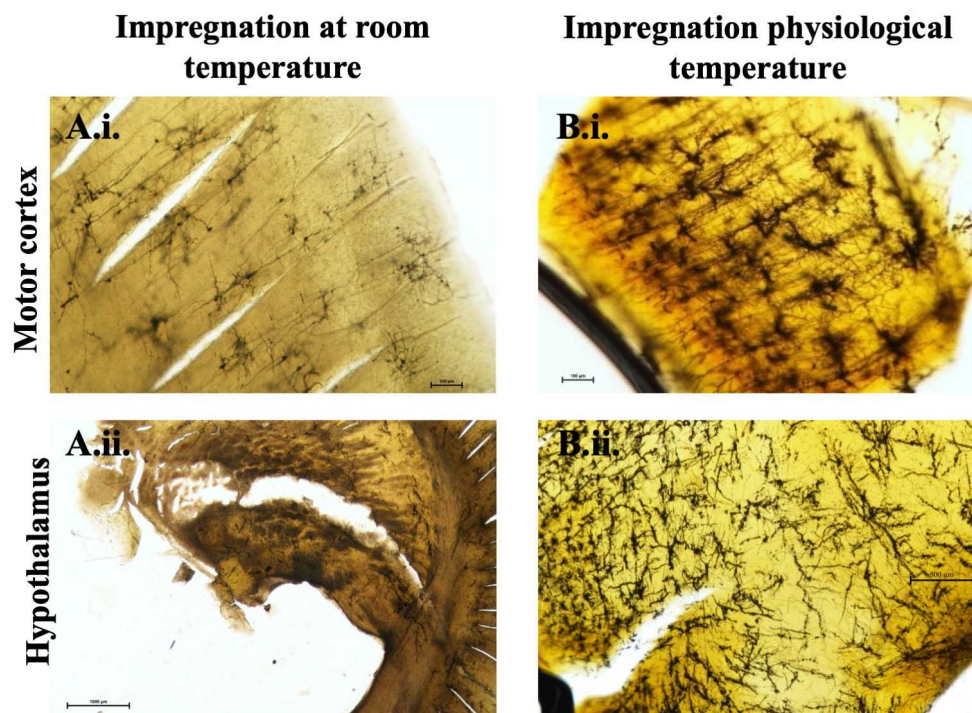
2h

32 After one day at DPX slides can be imaged in bright-field microscope.



32.1 While imaging at higher magnification (preferably at 100X with oil-immersion) you can trace your neuron with the specific segmentation annotation i.e. giving name to the traces viz. soma, basal dendrite, apical dendrite etc. Further, in the same time you can also tag your neurites with specific type of spines (viz. mushroom, thin, stubby etc.) this process can take  02:00:00 for a complete tracing of neurons like pyramidal cells in hippocampus.

2h



Comparative images with regular 7 days incubation at room temperature (Ai-ii); and at physiological temperature with thin slices (Bi-ii).

Note

There are different softwares available for neurite tracing (see fig. 4 A-B in attachment)

Software

Neurolucida

NAME

Windows 10, 64-bit

OS

MBF BioSciences

DEVELOPER

<https://www.mbfbioscience.com/wp-content/uploads/2022/11/Neurolucida-product-sheet.pdf>

SOURCE
LINK

Software

Imaris

NAME

Bitplane

DEVELOPER

<http://www.bitplane.com/Default.aspx>

REPOSITORY

- 32.2 The same thing can be achieved by making a stack out of single plane images at $\leq$  1 μm thickness and then tracing using Matlab or Image J plugins or even with some other standalone softwares:



Software

Simple Neurite tracer

NAME

Tiago Ferreira

DEVELOPER

<https://github.com/morphonets/SNT.git>

REPOSITORY

You should see this window

You should see

This should appear

So you click so e where and end some where in the branches like this:

Now when you repeat step 6 you should see this:

Turns this

Short cut keys:
Y= to keep the path
N= not to keep the path
F= to finish the path
G= select nearest path
Ctrl+Alt= to make a fork point

You should see this window

You can play with this scale a bit

Start clicking on the branches you want to trace while scrolling the mouse wheel

Don't forget to tag your tree using the path manager

Tracing with SNT plugin in Image J/ FIJI; in this scheme of images you can follow directly to perform tracing using SNT (neuroanatomy plugin) from step1-12. This is also quite easy protocol we have used a premade stack of image kindly provided by Mr. Ignacio Javier Novoa, Brain Plasticity and Neurorehabilitation Laboratory (BPNL, <https://www.muthaiahlab.com/>).



Software

ShuTu

NAME

Windows/ Mac/ Ubuntu

OS

Dezhe Jin

DEVELOPER

<http://personal.psu.edu/dzj2/ShuTu/>

SOURCE LINK

Software

Neurite Tracing With Object Process

NAME

Matlab

OS

Shreetama Basu

DEVELOPER

<https://ieeexplore.ieee.org/document/7373641>

SOURCE LINK

Data extraction

5m

- 33 Once the neurons are traced it is ready to get the data out of it. Traced neurons can be saved in different file formats:

5m

1. dat
2. ASCII
3. SWC

However, we can change the format at anytime with a NLMorphology converter/
Neuroland viewer



Software

NLMorphology Converter

NAME

<http://neuronland.org/NLMorphologyConverter/NLMorphologyConverter.html>SOURCE
LINK

Next, data can be directly extracted from SNT plugin/ neuroanatomy package by doing Sholl analysis function or you can go for Neurolucida Explorer for the same (MBF Biosciences, USA)

Even we can do the same in Shutu/ NeuTube

Software

ShuTU/NeuTube

NAME

Dezhe Jin

DEVELOPER

<http://personal.psu.edu/dzj2/ShuTu/>

SOURCE LINK

Or we can upload ASC file to the "Patchview" / NeuroM software and can perform Sholl analysis

Software

Patchview

NAME

<https://github.com/ZeitgeberH/patchview.git>

REPOSITORY

<https://patchview.readthedocs.io/en/latest/>

SOURCE LINK

Or even in NeuromorphoVis



Software

NeuroMorphoVis

BlueBrain Project

<https://github.com/BlueBrain/NeuroMorphoVis.git>

https://scholar.google.com/scholar_url?url=https://academic.oup.com/bioinformatics/a/pdf/34/13/i574/25098456/bty231.pdf&hl=en&sa=T&oi=ucasa&ct=ufr&ei=wNYZZPOpJZGgBnWWd_w3a-EY1dQmoaxang

This process should not take more than  00:05:00 per neuron traced

Visualization of neuron

1m

- 34 Visualization of the neuron is secondary to the analysis of data extracted from the tracings. However, this is important in the sense that you can represent and compare between treatments/ cases. For the same Neurolucida/ Imaris already will do the job however if you want work with free/ open source softwares then it can be done in following softwares:

1m

Software

NeuroMorphoVis

BlueBrain Project

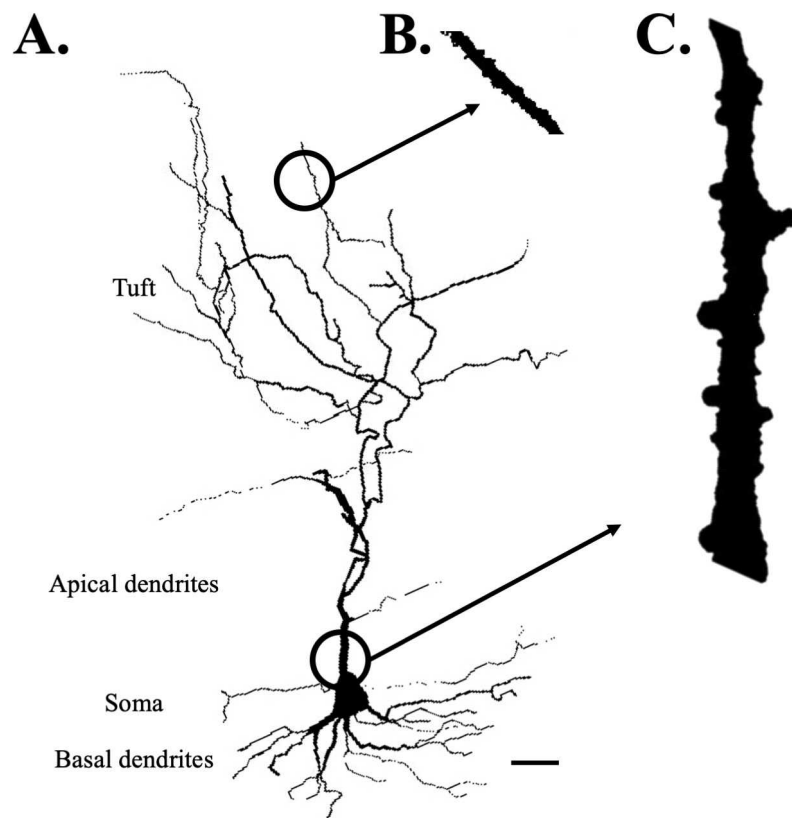
<https://github.com/BlueBrain/NeuroMorphoVis.git>

https://scholar.google.com/scholar_url?url=https://academic.oup.com/bioinformatics/a/pdf/34/13/i574/25098456/bty231.pdf&hl=en&sa=T&oi=ucasa&ct=ufr&ei=wNYZZPOpJZGgBnWWd_w3a-EY1dQmoaxang

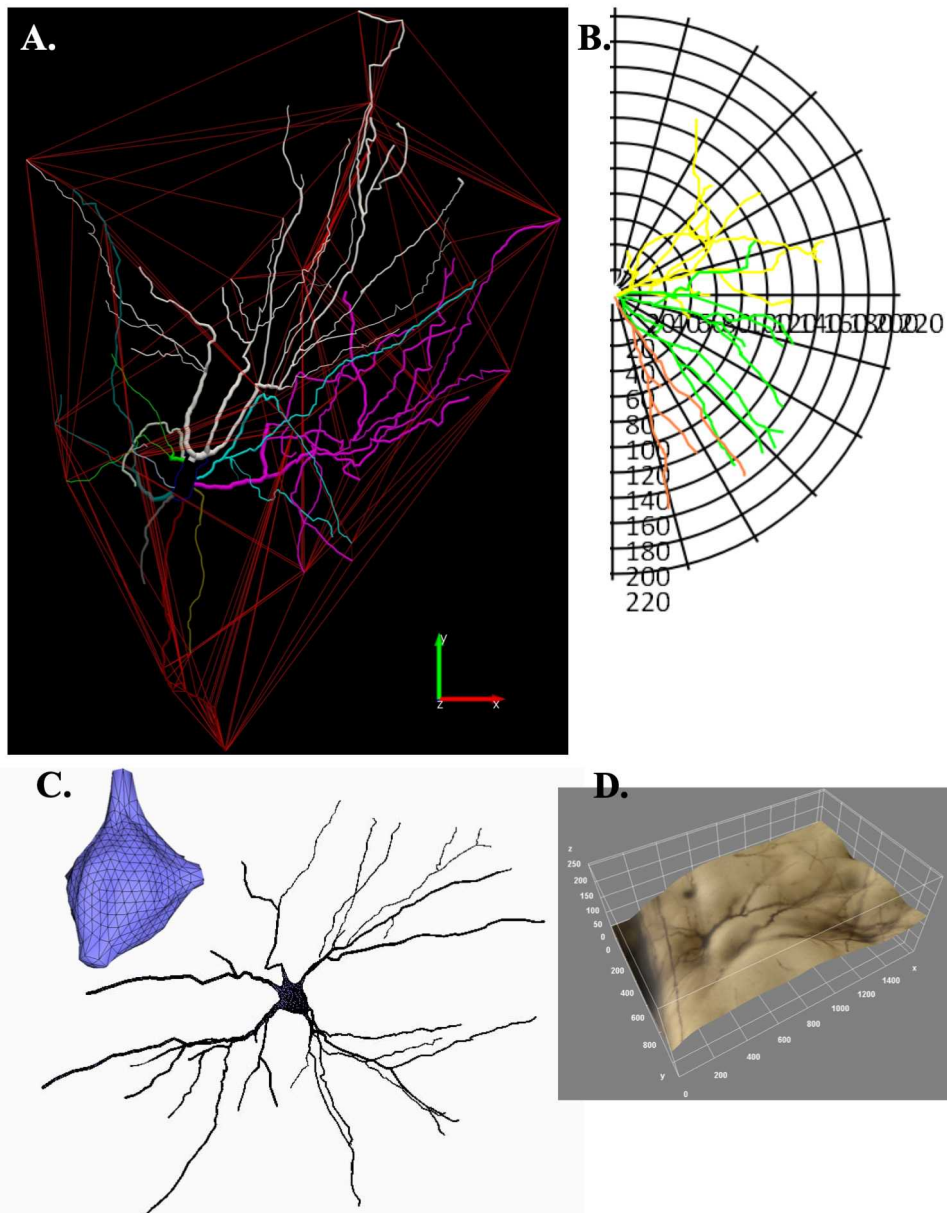
Software

Neuronize	NAME
Windows	OS
Visualization & Graphics Lab	DEVELOPER
https://vg-lab.es/neuronizev2/	SOURCE LINK

This can be done with few clicks in the software GUI



Reconstructed and processed images of 200um thick sections from control animal hippocampus *in regular ways*. CA1 pyramidal neuron; 400X magnified image of dendritic complexity (a); 1000X magnified image of tuft dendrite spines ((b); 1000X magnified image of apical dendrite spines (c). Scale bar=50µm



Ways to represent results apart from morphometric results; convex hull representation of a CA3 neuron (A); fan diagram of a CA1 neuron (B); hypothalamic neuron traced and rendered with Neuronize(C); 3D surface plot of a CA1 neuron in 3D view tool in Image J (D).

Analysis and its types

- 35 For morphometric analysis of neurons we generally perform following type of analysis:
1. Spine density calculation (which can be extracted from the tagged spine during tracing)
 2. Type of spine (this approach is good when your research question is more restricted to the types of spine viz. mushroom-shaped spine as this is site for glutamatergic synapse)
 3. Sholl analysis where you perform one variable (length/ intersection) versus the distance from soma
 4. Branch structure analysis where you mainly perform the various parameters related to branch viz. number of terminal branches, tortuosity, branch order etc.
 5. Convex hull analysis where one can measure the volume of the neurite or soma

Protocol references

Rutledge LT, Duncan J, Beatty N. A study of pyramidal cell axon collaterals in intact and partially isolated adult cerebral cortex. *Brain Res.* 1969 Nov;16(1):15-22. doi: 10.1016/0006-8993(69)90082-1. PMID: 4186864.

Ranjan, A., and Mallick, B. N. (2010). A modified method for consistent and reliable Golgi-cox staining in significantly reduced time. *Front. Neurol.* 1:157. doi: 10.3389/fneur.2010.00157

Zaqout S and Kaindl AM (2016) Golgi-Cox Staining Step by Step. *Front. Neuroanat.* 10:38. doi: 10.3389/fnana.2016.00038