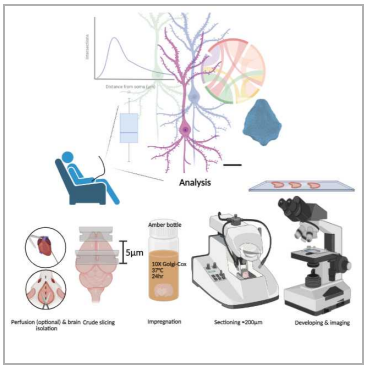

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Protocol status: Working
We use this protocol and it's working

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

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Avishek Roy

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 protocol.pdf

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PROTOCOL integer ID: 77517

Keywords: Golgi-Cox staining, One day impregnation, Neuronal morphometry

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

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 INSTRUCTIONS

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

25m

Golgi-Cox solution was prepared using

- 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 #207802-100**

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

Slide cleaning and coating 20h 40m

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)**

[M] 18.5 % volume in MiliQ water for Overnight in the staining trough

Step 3 includes a Step case.

+ing surface

step case 3h

+ing surface

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

5 Running tap water was discarded and replaced with [M] 50 % (v/v) of admixture of 3h

Ethanol Pure **Merck MilliporeSigma (Sigma-Aldrich) Catalog #493511**

Methanol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #M3641** and kept for 2-3hr

6 Again the admixture was replaced with running tap water and slides were kept for 1-2hr 2h

Step 6 includes a Step case.

Grease free

step case 30m

Grease free

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

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 10 includes a Step case.

Alternative

Transcardial perfusion

step case 5m

Alternative

One can opt for euthanasia in CO_2 chamber by treatment of >60% CO_2. And at the end level of anaesthsia 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

Step 11 includes a Step case.

Perfusant Vol.

step case 3m

Perfusant Vol.

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

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 [M] 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 6 % sucrose made in MiliQ 30m

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 6 % sucrose 1m

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 p[ut 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 loosing 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 37 °C for 24:00:00 1d

Note

This step is required to fix the sections onto the slides. If not carried out then there is a high risk of loosing 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 [M] 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

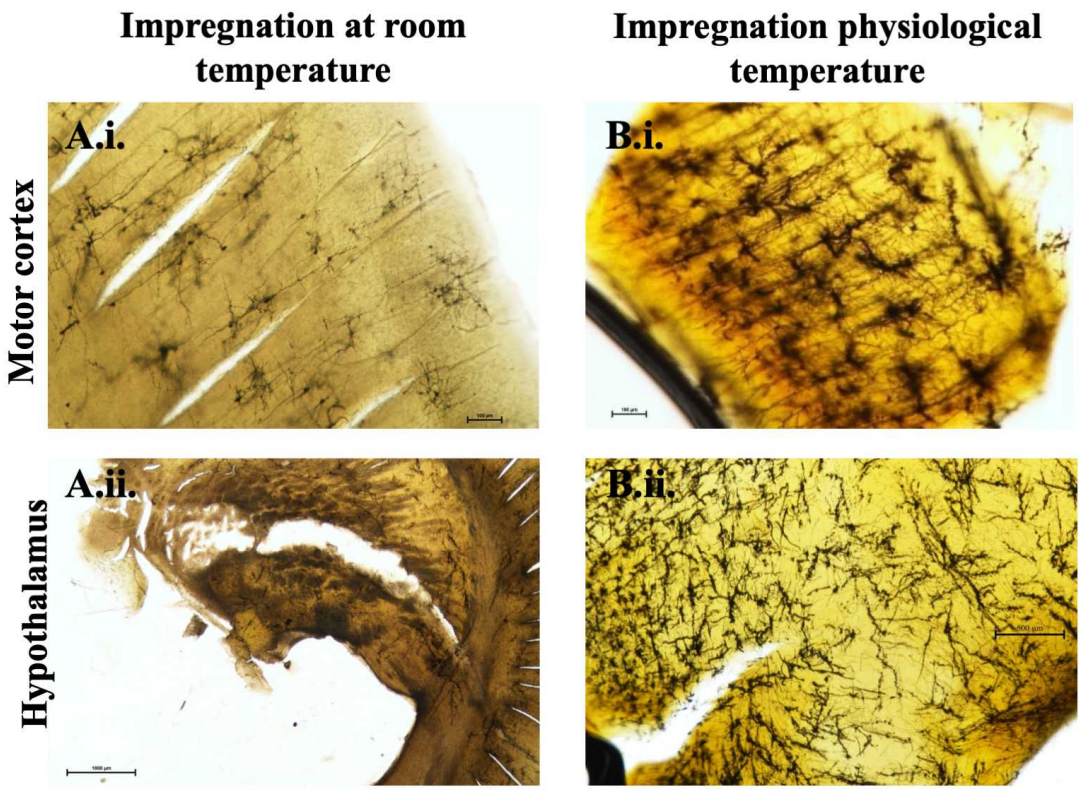
31 Now sections can be mounted in a coverslip with DPX 1m

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 2h

02:00:00 for a complete tracing of neurons like pyramidal cells in hippocampus.



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

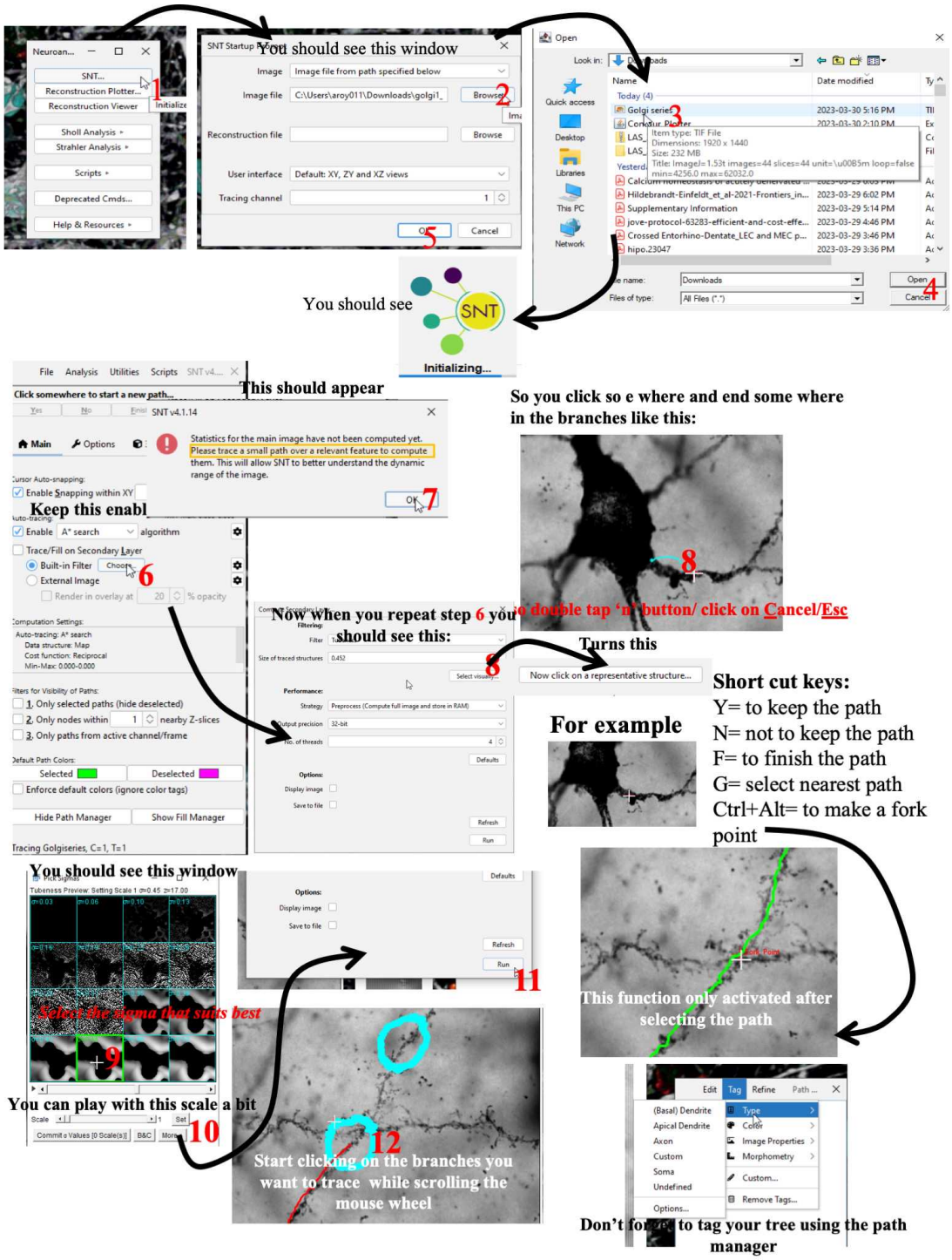
Software

Imaris	NAME
Bitplane	DEVELOPER
http://www.bitplane.com/Default.aspx	SOURCE LINK

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

Software

Simple Neurite tracer	NAME
Tiago Ferreira	DEVELOPER
https://github.com/morphonets/SNT.git	SOURCE LINK



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

Software

Neurite Tracing With Object Process	NAME
Matlab	OS
Shreetama Basu	DEVELOPER

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:
1. dat
2. ASCII
3. SWC
However, we can change the format at anytime with a NLMorphology converter/ Neuroland viewer

5m

Software

NLMorphology Converter	NAME

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

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	SOURCE LINK

Or even in NeuromorphoVis

Software	
NeuroMorphoVis	NAME
BlueBrain Project	DEVELOPER
https://github.com/BlueBrain/NeuroMorphoVis.git	SOURCE LINK

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

Visualization of neuron

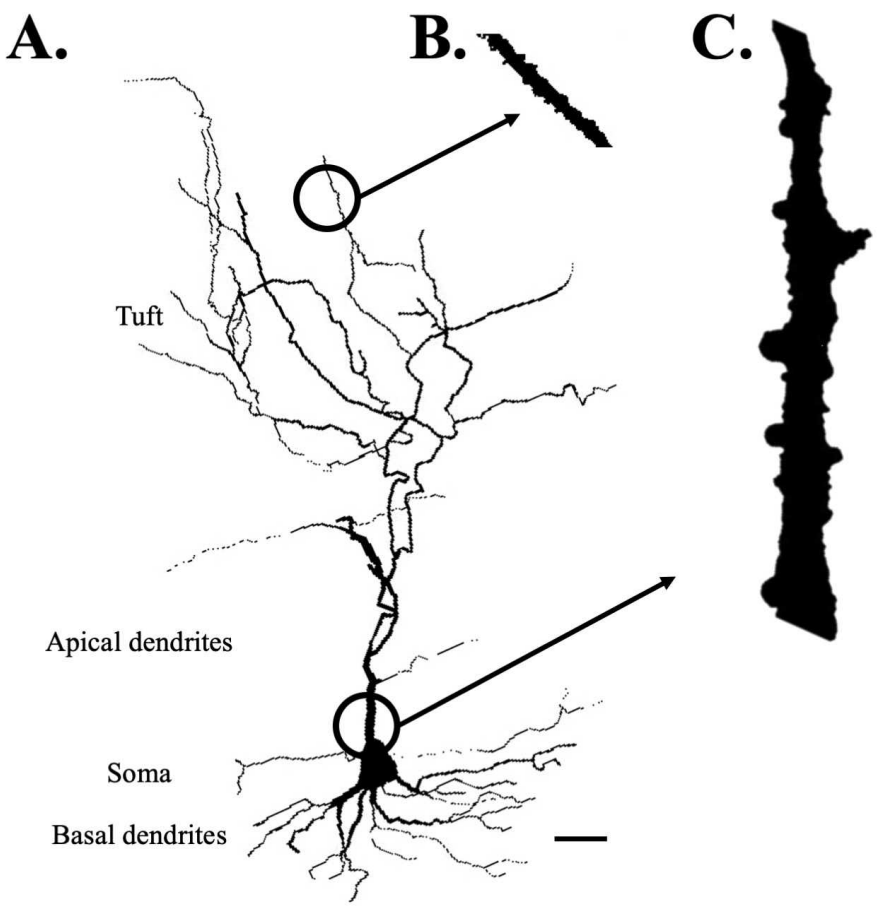
1m

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

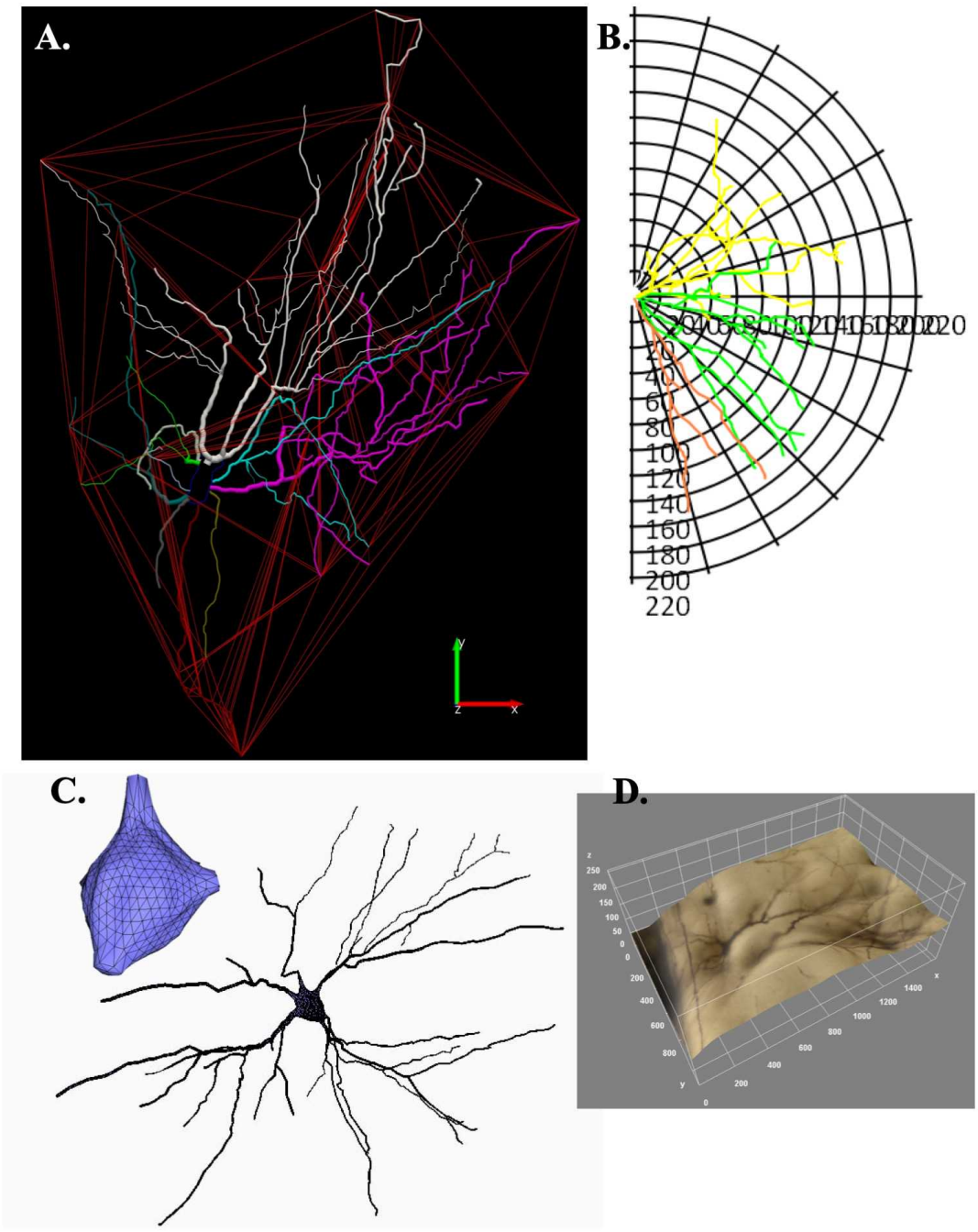
Software	
NeuroMorphoVis	NAME
BlueBrain Project	DEVELOPER
https://github.com/BlueBrain/NeuroMorphoVis.git	SOURCE LINK

Software	
Neuronize	NAME
Windows	OS
Visualization & Graphics Lab	DEVELOPER

This can be done with few clicks in the software GUI



Reconstructed and processed images of 200um thick sections from control animal hippocampus n]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, turtuosity, branch order etc.
5. Convex hull analysis where one can measure the volume of the neurite or soma