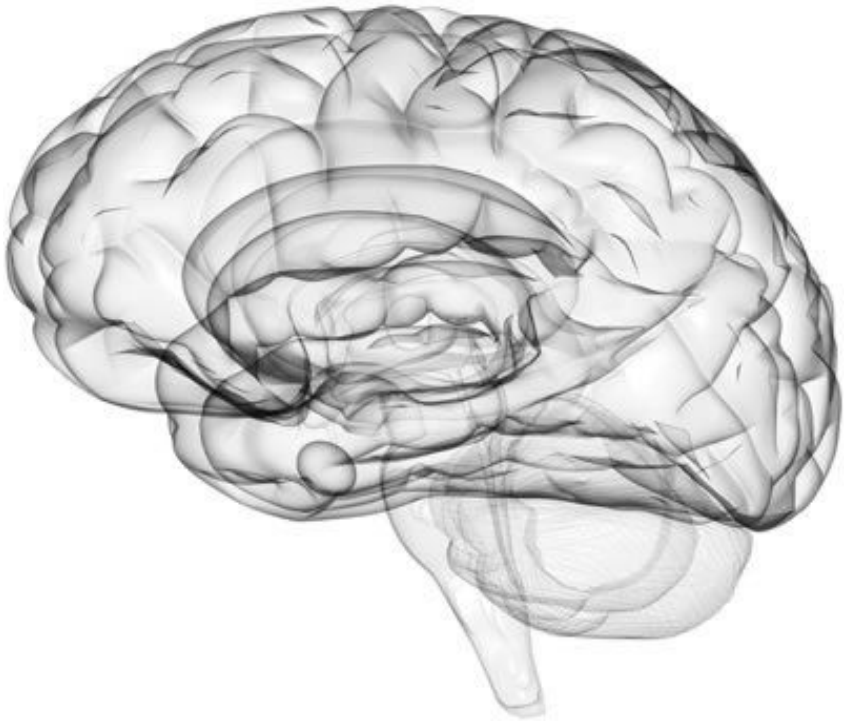
# Transparent Brain: New Approaches to Study the Anatomy of Neurons Throughout the Brain.



Bachelor of Life Sciences – Graduation Internship

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## Abstract

It is appealing to study anatomical features of neurons and protein localization in thick sections in order to maintain optimal network connectivity. However the tissue thickness hinders the quality of immunohistochemistry due to the difficulties regarding antibody penetration. Since the study of neuronal network distribution through immunolabeling in thick slices would cause data loss, I adopted a high-throughput strategy that allows the visualization of neurons while maintaining cell morphology in thick slices by combining the labeling of (biocytin filled and Golgi-cox stained) neurons with a recently discovered tissue clearing technique (iDISCO). The practical approach of replacing mechanical sectioning with chemical clearing is a big step towards advancing our understanding of the brain. This report provides two detailed protocols that are established in order to efficiently perform single cell analysis and high-throughput analysis. These functional, reproducible and robust methods are suitable for neuronal 3D reconstruction, linking a neuron's anatomy with its physiological data, axonal density distribution, and revealing morphological features of a neuron upon genetic or pharmacological manipulations.


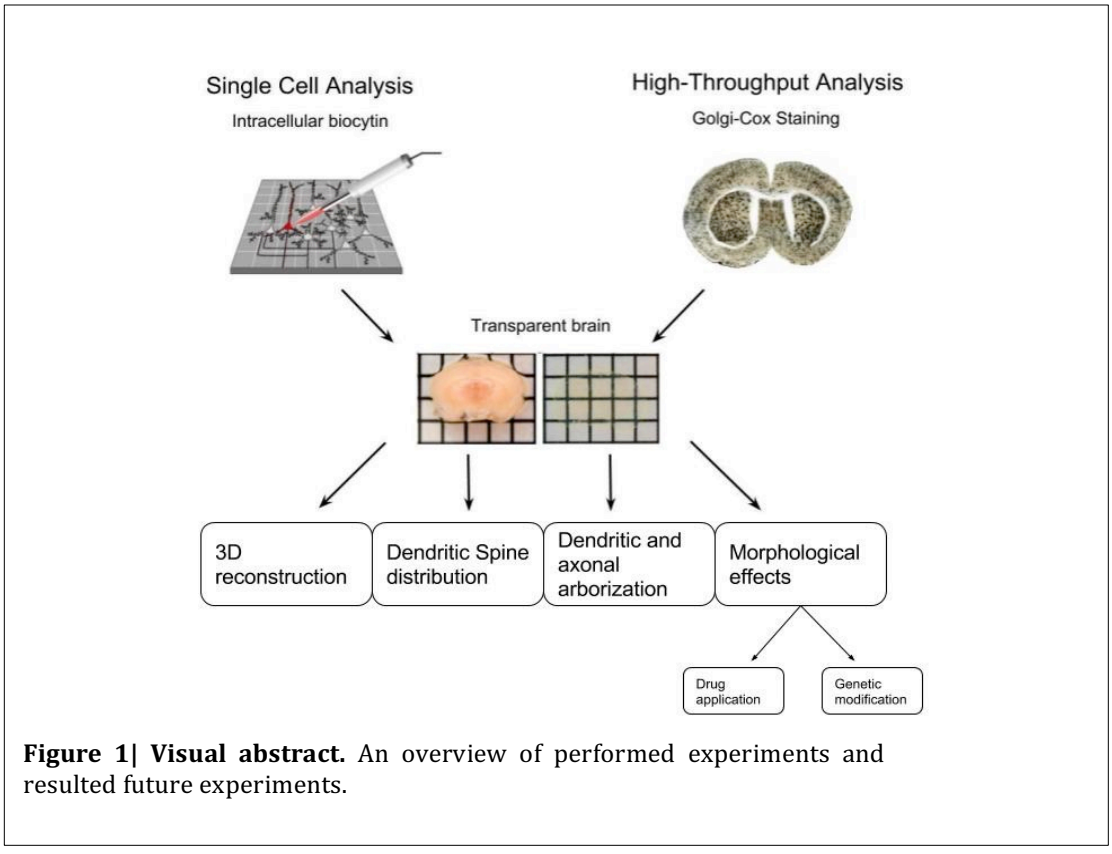


**Figure 1| Visual abstract.** An overview of performed experiments and resulted future experiments.

## Introduction

Visualization of the neurons and their molecular building block require thin mechanical sectioning of the brain tissue before histological staining (Zaqout & Kaindl, 2016). Aligning multitudinous amount of thinly stained slices to map neuronal projections is time-consuming and error-prone, rendering visualization and quantification of full dendritic trees and dendrite spines throughout the brain unattainable. This problem could be potentially overcome by imaging thick sections. However, light scattering caused by the variation of refractive indexes within the tissue interferes with the visualization (Richardson & Lichtman, 2015). The heterogeneity in the amount of scattering, as the light travels across the different planes of the tissue, gives rise to a scattering appearance, making the data acquisition in thick sections impossible (Richardson & Lichtman, 2015). Thus, despite the numerous existing staining techniques such as silver reaction (e.g. traditional Golgi), intracellular labeling (e.g. by biocytin), or immunohistochemical (IHC) staining (Ranjan & Mallick, 2010; Swietek, et al. 2016; Ertürk et al. 2012; Renier et al. 2014) virtually all structural analysis of histological samples are performed after mechanical sectioning.

In recent years, to overcome the obstacle of imaging thicker tissue sections and eliminate the diffractive boundaries in the samples, various tissue-clearing techniques have been developed. Tissue clearing includes dehydration steps because water contains a low refractive index compared to cellular structures with protein and lipids (Ertürk et al., 2012). Thereafter, the dehydrated tissue undergoes lipid extraction and then cleared by insinuating an organic solvent such as dibenzyl ether (DBE) homogeneously throughout the sample by matching the higher refractive index of the defatted and dehydrated tissue (see figure 2). (eg. Ertürk et al. 2012; Renier et al. 2014). There are many types of tissue clearing techniques. Clearing methods such as iDISCO allow high visualization depth and preserves the 3D structure of neuronal cells by eliminating the need to section the brain into thin sections (Renier et al. 2014), however so far the only staining that has been published that successfully performed with this clearing technique is with a few IHC antibodies.

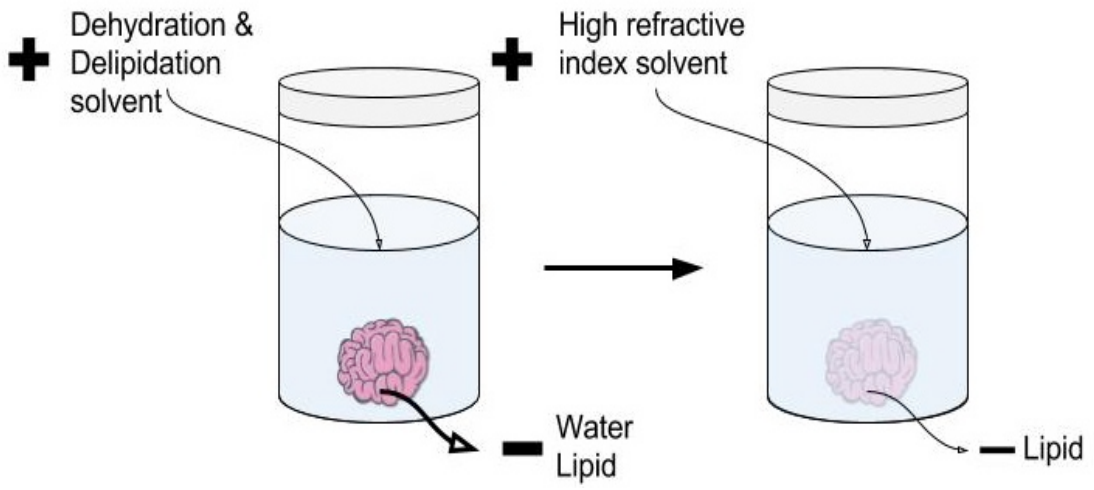


**Figure 2| Method of solvent-based clearing.** Left, the tissue is dehydrated and lipid is removed. Right, the tissue is moved to a high refractive index solvent where additional lipid solvation and clearing occurs.

IHC techniques are advantageous because of their ability to bind to specific proteins; however light scattering is not the only problem when applying this principle into thicker sections (Horikawa & Armstrong, 1988). Next to the issue of light scattering due to reflection and refraction, labeling thick brain sections with antibodies is not practical giving the limited depth of antibody penetration (Chen et al. 2010). Additionally, the usage of various chemicals in attempt to reduce the light scattering is limited in this case due to the high probability of fluorescent signal quenching (Chen et al. 2010). It would therefore be a great advantage to obtain the ability for alternative labeling for the transparent brain. For this reason, in this internship I have developed novel staining methods, named bDISCO and goDISCO, and applied the methods to visualize single neurons individually filled with a contrast agent, biocytin, and populations of neurons stochastically labeled using a silver-staining technique in thicker brain sections termed Golgi-Cox.

Biocytin is an intracellular marker introduced in 1988 (Horikawa & Armstrong, 1988). The labeling of biocytin allows the axonal and dendritic projection patterns to be visualized, which can reveal different neuronal classes as long as neurons can be individually filled with biocytin. This could be achieved by including the dye in the patch pipette solution used during whole cell recording experiments. Thus, the very same neuron can be studied electrophysiologically while its anatomy can be revealed using intracellular injections of biocytin.

Combining the chemoenzymatic staining of biocytin and a tissue clearing technique, allows the visualization of intact neuronal projections in thick slices.

Biocytin is a molecule that is built up from biotin and L-lysine (see figure 3A). To visualize biocytin, numerous kits are available one of them being the ABC kit. The ABC vector staining kit Vectastain ® contains two reagents named reagent A and B. Reagent A consists of avidin-DH, which is a molecule that contains four high affinity Biotin binding sites. Reagent B is horseradish peroxidase (HRP) that is bound to biotin. The bindings of these molecules create a macromolecular complex that remains very stable. 3,3'-Diaminobenzidine (DAB) is then added which is a substrate that becomes oxidized by the HRP with the help of the oxidizing agent $H_2O_2$ (see figure 3B). DAB reaction with HRP in the presence of peroxidase yields a brown-colored signal at the place where biocytin is present. I use the ABC kit to label the biocytin filled neuron, which in the end should allow obtaining a high-resolution image of the detailed projections of the full neuron that has been electrophysiological recorded.

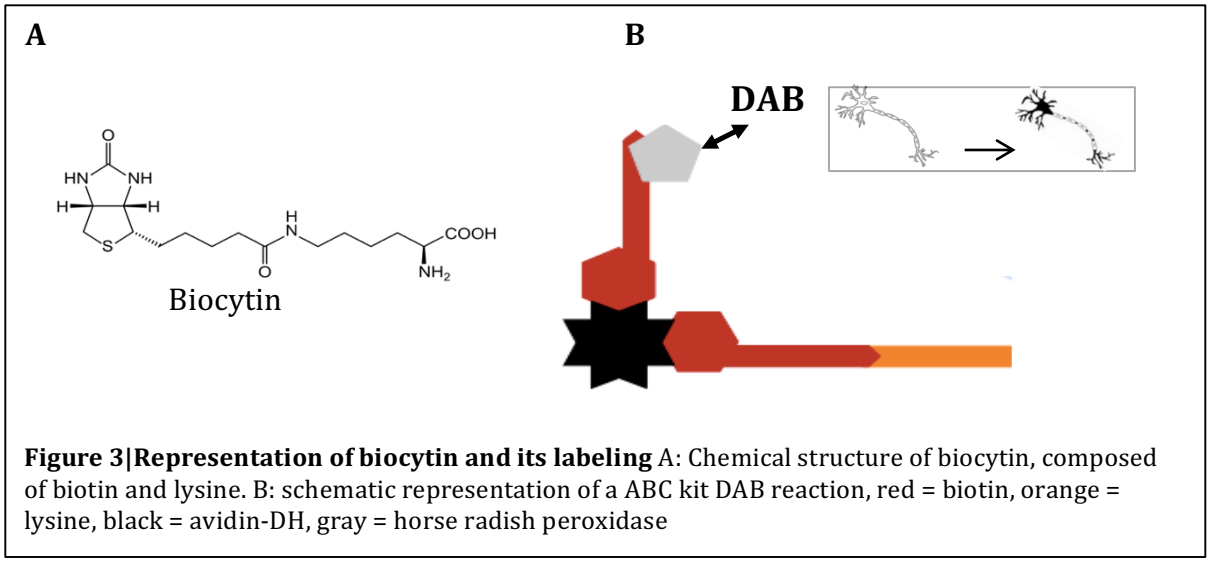


**Figure 3|Representation of biocytin and its labeling** A: Chemical structure of biocytin, composed of biotin and lysine. B: schematic representation of a ABC kit DAB reaction, red = biotin, orange = lysine, black = avidin-DH, gray = horse radish peroxidase

The single neuron labeling by biocytin is crucial to understand how the neuronal anatomy and function is related; however it is a low yield method. Only one cell in a given locus can be studied, as overlapping projections originating from different neurons cannot be differentiated with ease. Visualization of networks of neurons, thus, requires alternative approaches.

Together with the glial cells, neurons form intricate networks (Nakae et al. 2014). The Golgi method, originally established in 1873 (Golgi, 1873), has allowed visualization of the full neuron, glia and neural circuits in great detail in order to acquire more knowledge of such a complex network. Moreover, the Golgi method is a powerful staining technique for providing a thorough representation of an individual neuron as the neuronal cells are stained only sparsely (Koyama Y 2013). Numerous variations of Golgi staining are currently available. Each variant

has its own advantages and disadvantages. One specific drawback has been the crystallization artifacts, thought to be due to the nonselective reaction of silver nitrate with potassium dichromate, which in turn causes a higher level background noise (Levine et al. 2013). Therefore I used a variation of the original Golgi staining in which silver nitrate is replaced with mercury chloride to aid the neuron impregnation, termed Golgi-Cox (Zaqout and Kaindl 2016).

A major technical issue for visualizing the Golgi-stained neurons is the maximum slice thickness. The maximal thickness of an imaged Golgi-Cox sample thus far has been approximately 200 µm, limited primarily by the light scattering in the opaque tissue (Louth et al. 2017). To make a 3D reconstruction and to analyze the long projections of Golgi-stained nerve cells throughout the brain, researchers must align thousands of these (relatively) thin imaged tissues. Not only is this a laborious task, sectioning also damages the tissue and network connection, providing an insufficient view of neuronal branching and variations in spine densities. And again, light scattering is present in analyzing the data, even in thin tissue, limiting the resolution.

To overcome the shortcomings of the methods mentioned above, I focused on establishing a simple, rapid, inexpensive, and robust method for cellular labeling in thick brain slices (≥3 mm) with volume imaging, using the building blocks of the Golgi-Cox staining and iDISCO tissue clearing technique (Chen et al. 2012; Zaqout & Kaindl, 2016). The resulting technique, called goDISCO, gives stained neurons within ≥3 mm thick brain slices and allows visualization of neuronal morphology, their axonal and dendritic arborization, and spines with a bright field setting of the confocal microscope, allowing z-stack images for 3D reconstruction, and continue on with future experiments. Altogether, this efficient staining technique is a powerful high-throughput approach that gives structural information of the neurons, which in turn offers more understanding in the influences that neuronal morphology and distribution may undergo by neurological diseases or environmental factors.

In short, the following two issues will be addressed in this report: 1. Generation of a method that allows the visualization of intracellular labeled single neurons in 300 µm thick mouse brain slices. 2. Establishing a high-throughput protocol for visualizing Golgi-Cox staining in 3 mm thick mouse brain slices.

## Material and Methods

### Mice

The Pvalb-cre line, 3 months old female mice were used for these experiments according to local regulations. The animals were sacrificed by authorized personnel, and thick tissue slices were prepared without prior fixation. For short term storage the tissue was placed in ACSF (artificial cerebral spinal fluid). The slices that were used for biocytin visualization were fixated in 4% PFA for an hour, then stored in antifreeze and were placed in the -20°C freezer until further processing. All other slices were processed according to the goDISCO protocol introduced below.

### Biocytin DISCO (bDISCO) Protocol

After sacrification, the brain slices were incubated in an ACSF bath of 37°C, for an hour. The slices were obtained after the completion of electrophysiological recordings. Voltage-patch clamping has been performed on 300 µm fresh mouse brain slices. The patch-clamp biocytin containing electrodes were used for injection of the biocytin during recording of the cells that were recorded for 15-20 minutes. The slices were then fixed in 4% PFA for an hour, then they were stored with antifreeze in the refrigerator in a plastic 24-well plate until processing (see figure 5).

*Pre-Treatment*

The pre-treatment of the tissue includes washing the slices in Ptx.2 solution twice; please refer to Table 1 in Supplemental Material for the recipes. Each slice is then incubated in $H_2O_2$ for 30 minutes. For tissue permeabilization, the slices were then transferred into brain jars and incubated in a solution of 0.2% Triton and 20% DMSO in 1xPBS at 37°C overnight.

*Staining with the ABC Kit*

The next day, the following two solutions were freshly made at the time of use: the ABC vector, and Solution Step 5. The Vectastain ® ABC vector kit contains reagent A and reagent B, each in dropper bottles. 30 minutes prior to the experiment, 4 drops of each reagent was added in 5 mL $PBS^{+}$. ($PBS^{+}$ = 0.5% Triton in $PBS^{TSA}$). Please refer to table 2 in Supplemental Material for the recipe of Solution Step 5. In each brain jar, half of each solution was filled and then the brain slice was added to incubate at 37°C overnight.

*DAB Staining*

The following day the samples were washed in PBS for 15 minutes at room temperature (RT) and then in Tris buffer (TB) for 15 minutes at RT. Thereafter, they undergo DAB staining giving them a brown-like color. The DAB staining solution contains 10% DAB powder in 20 mL TB. 15mg of ammonium-nickel-sulfate was added in order to increase the reaction rate, thus intensifying the signal. Right before staining, 3 µL $H_2O_2$ was added in order to activate the reaction. Whilst staining, staining intensity was observed to stop the reaction by transferring the slice into TB when necessary (see figure 4A and 4B). After washing the slices in TB for 10 minutes, the procedure continued with a dehydration series.

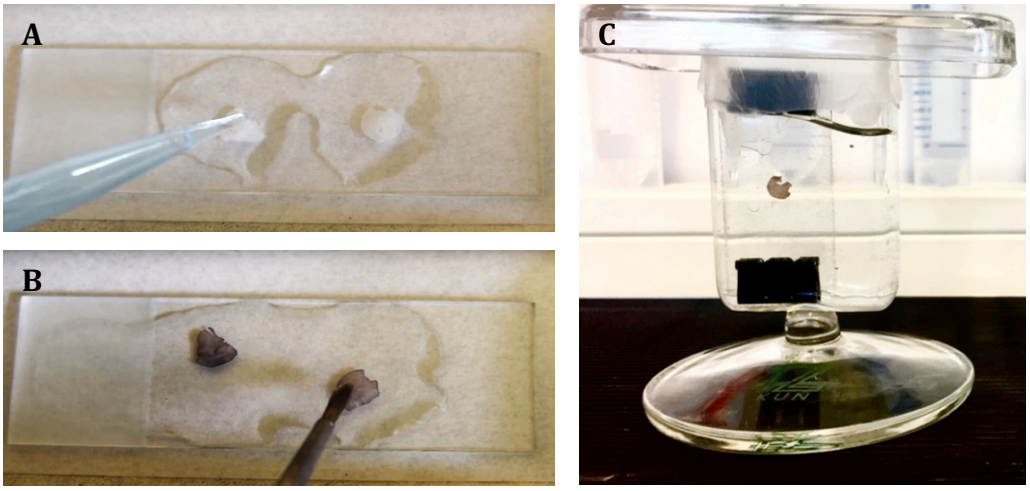


**Figure 4l DAB Staining and Dehydration.** A: The slices are incubated in DAB staining solution . B: The slices are taken out when it appears to have a dark brown color formation. C: Thereafter, dehydration series are done in glass constructs to prevent tissue curling.

*Methanol Dehydration Series*

Dehydration of the tissue encompasses gradual steps of increased methanol concentrations. The tissue was incubated in 20%, 40%, 60%, 80%, and 100% for one hour each at RT on a shaking plate. Due to tissue curling, constructs for the slices were made in order to prevent the curling. A construct contains two microscopic glasses sandwiching the slice. In order to prevent the tissue from being squished and giving room for solution to penetrate, a stack of two cover slips on each side was added in between the two microscopic glass slides. The set up was held together with metal binder clips (see figure 4C). The dehydration step of 100% was done free floating (without glass-construct).

*Tissue Clearing with DCM and DBE*

After dehydration, the slices undergo lipid removal as described before (Renier et al., 2014). From 100% methanol, the slices were transferred into brain jars containing a solution of 66% dichloromethane (DCM) and 33% methanol, subsequently incubating them in the dark at RT. The next day, the tissue underwent secondary lipid removal by being incubated in 100% DCM twice for 15 minutes each. Then the sample went into a glass jar of DBE, which is a high refractive index solution, stored in the dark without shaking until imaging.

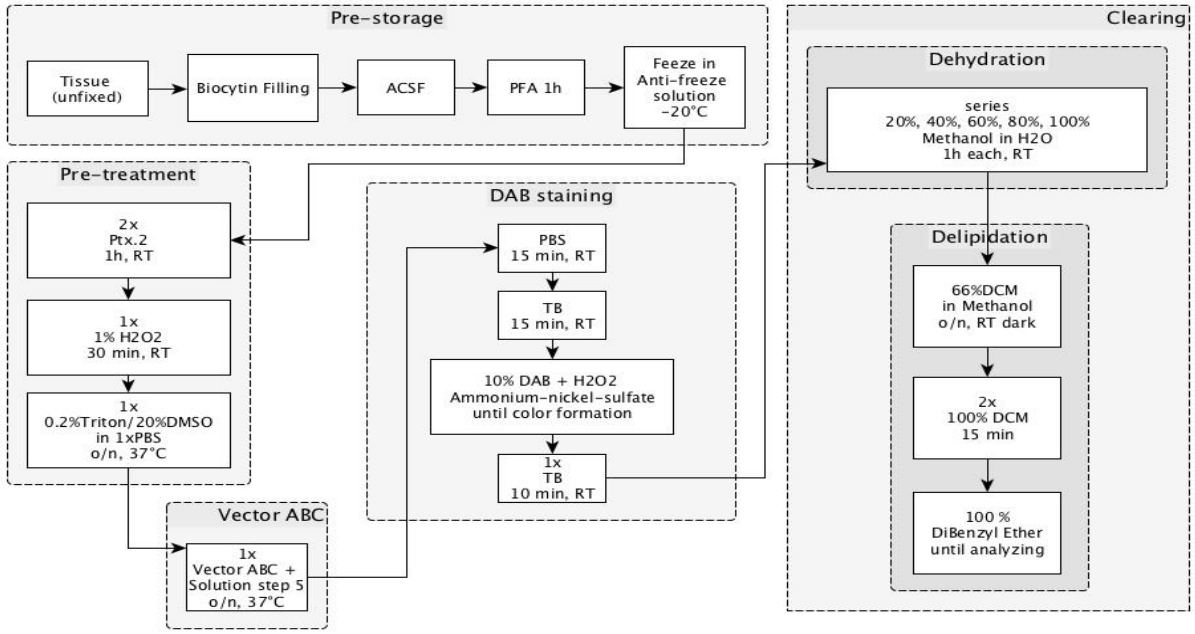


**Figure 5| An overview of the steps in the bDISCO method.**

**Golgi-Cox DISCO (goDISCO) protocol**

The following is an extensively modified protocol based on the three method based papers "Golgi-Cox Staining Step by Step." by Zaqout 2016, "Differential staining of glia and neurons by modified Golgi-Cox method" by Ranjan 2012 and "iDISCO: A Simple, Rapid Method to Immunolabel Large Tissue Samples for Volume Imaging" by Renier 2014. The resulting protocol can achieve visualization of stochastically targeted network of neurons in thick tissue sections that could be imaged using standard microscopic methods.

*Golgi-Cox impregnation Solution*

Stock solutions for the impregnation step were prepared in advance and were stored in bottles at room temperature in the dark. They are for long-term usage to prepare the Golgi-Cox

impregnation solution (see Table 3 of Supplemental Material). Using the stock solutions, the Golgi-Cox impregnation solution was made. 50 mL potassium dichromate, 50 mL mercuric chloride, and 40 mL potassium chromate were mixed with 100 dd-$H_2O$. The Golgi-Cox Impregnation solution was left in the dark at room temperature for 48 hours allowing formation of precipitation. For tissue impregnation only the upper clear part of the solution was used.

*Solutions for the Developing Step*

For the developing step, the following solutions were prepared according to the recipe in Table 4 of the Supplemental Material.

*Golgi-Cox Impregnation Step*

After perfusion with ACSF the unfixed brain tissue was sliced into 3 mm coronal and sagittal sections. Each brain section was washed with dd-$H_2O$, and transferred into a small glass bottle containing the Golgi-Cox impregnation solution lacking the precipitation. After incubating them overnight in the dark at 37°C, the brain sample proceeds to the developing steps.

*Developing*

Using a well plate, the slices were developed on a mechanical shaking platform. Color development includes the slices to first be incubated in dd-$H_2O$ for five minutes twice. Thereafter, the slices were incubated in 50% ethanol for five minutes. For the actual color reaction, the slices were incubated in 35% ammonia for 15 minutes in the dark. The slices were then washed in dd-$H_2O$ six times for five minutes each time. Thereafter, they were transferred to 25% sodium thiosulfate and were incubated at room temperature for one hour in dark. The slices were then washed again in dd-$H_2O$ for 5 minutes, six times.

*Clearing*

After the development of the color, the sample undergoes the dehydration series of 70%, 80% and 100% methanol for one hour each during gentle mixing. The tissue was then left overnight in a solution containing 33% methanol and 66% DCM. The following day the tissue was incubated in 100% DCM twice for 5 minutes each. Finally the slices were placed and stored in glass bottles wrapped in aluminum foil and filled almost completely with DBE preventing oxidization of the samples.

*Imaging + Data Analysis*

For imaging of the biocytin and Golgi stained samples, the Leica Sp8x confocal microscope with the LasX acquiring software was used. This microscope allowed the acquiring of images through the xyz plane. By combining the z-stack images and tile images with automatic stitching, it was possible to image the sections completely throughout its whole thickness. After imaging, the program ImageJ (Schindelin et al. 2012) was used in order to process and merge the images and the plugin "Simple Neurite Tracer" was used for making 3D reconstructions (Longair et al. 2011).

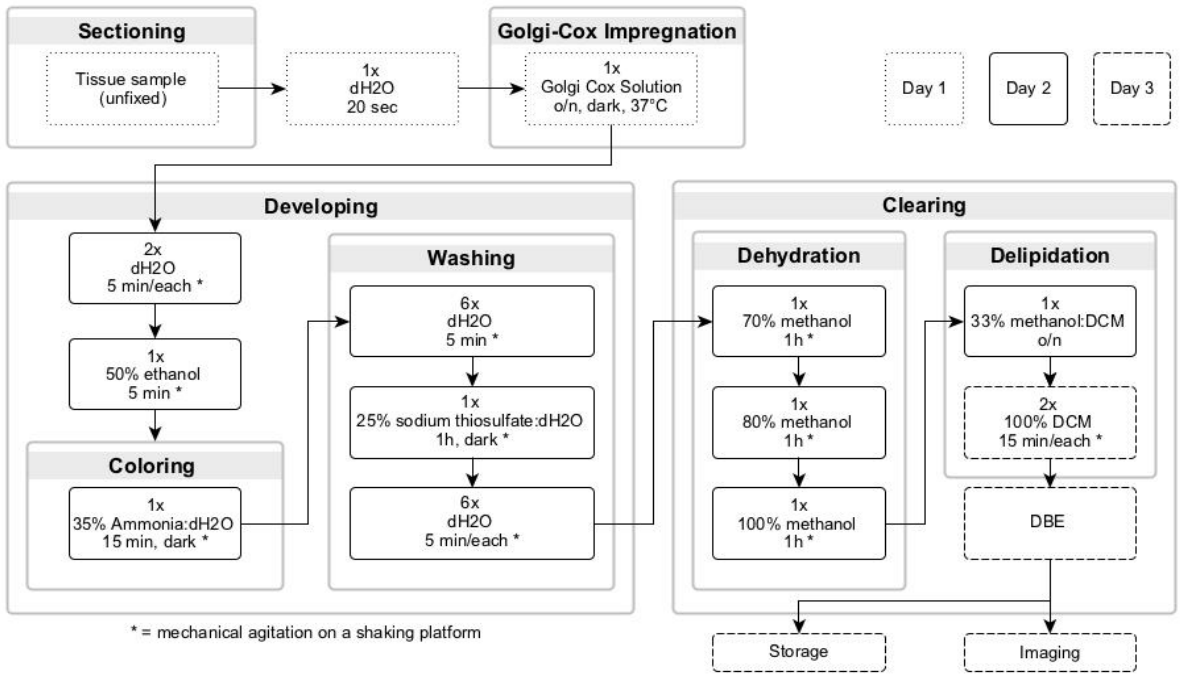


**Figure 6| The goDISCO protocol.**

## Representative results

### Biocytin DISCO (bDISCO) Results

The first attempts of clearing the stained biocytin labeled neuron did not show a visible neuron (see figure 7). This first obtained image shows a local area on the brain slice that appears to be darker than the rest of the slice. Transparency of the slices was sufficient for light to pass through, however the slice was curled up. Therefore, there was a need to adjust some parameters in the protocol in order to solve the problem of curling eventually.

The second attempt resulted in a clearly visible stained pyramidal neuron that had been labeled with biocytin. The transparency of the sample was good; therefore in all the images throughout the z-plane the neuron was clearly visible. With the z-stack imaging I was able to make a montage of all the images throughout the z-plane in order to indicate that in each plane the neuron was clearly visible thanks to the high transparency of the slice (see figure 8).

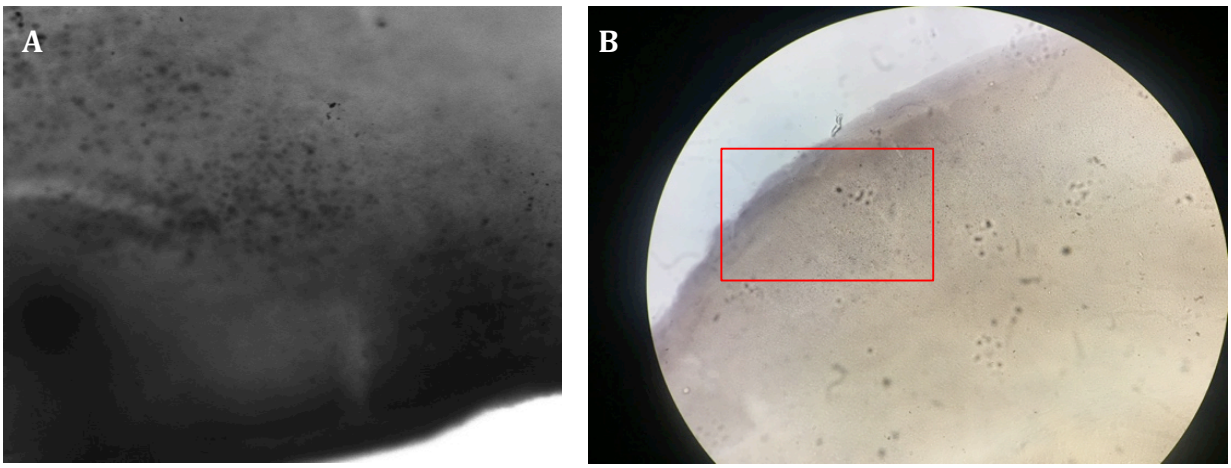


**Figure 7| First try biocytin staining in 300um slice.** A: a microscopic image of the unsuccessful attempt of staining the biocytin filled neuron. B: Representation of how the local dark area appears on the slice whilst looking underneath the binoculars.

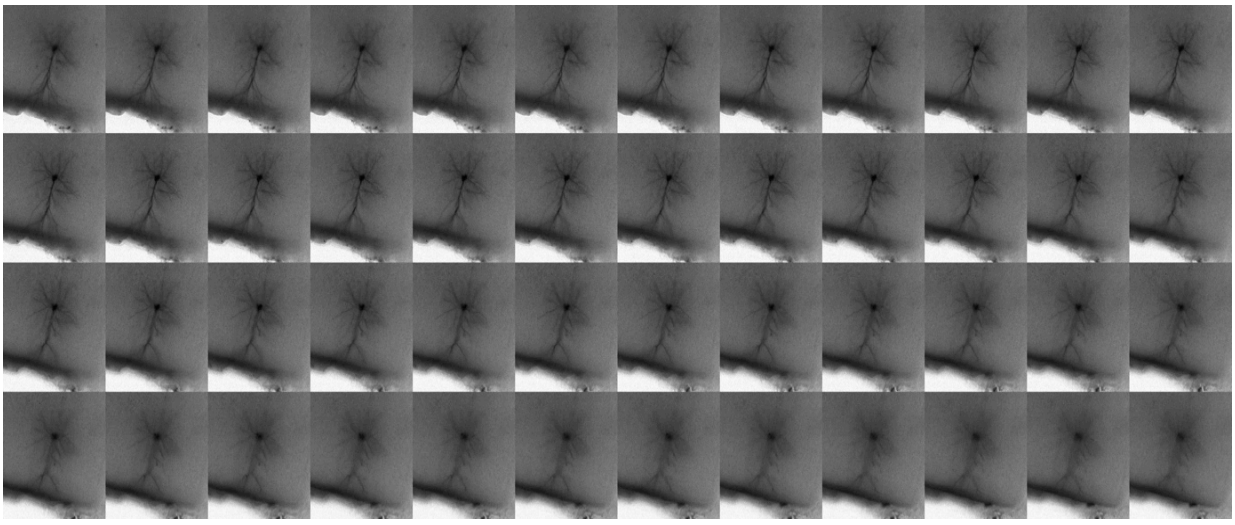

**Figure 8| Montage of z-stack images of biocytin filled neuron, 20x magnification.**

Using the multiple images throughout the z-plane, an image of the minimum projection and a skeletal 3D reconstruction was made (see figure 9A and 9B). A minimum projection means that it takes the darkest spots on the multiple images in order to make a merged image, showing all the projections throughout the slice.

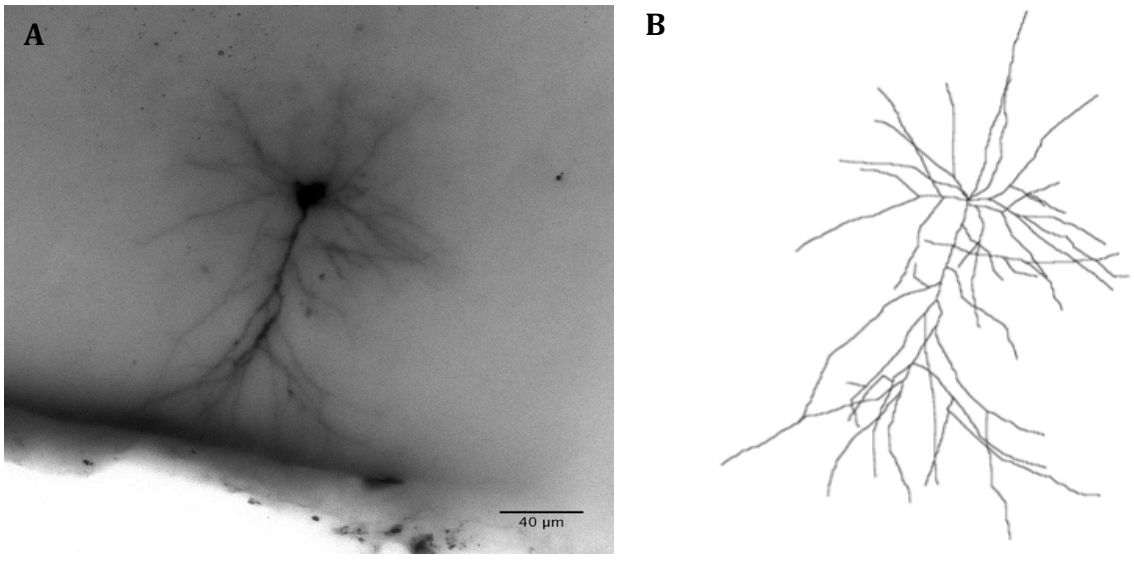


**Figure 9| Successfully biocytin filled neuron.** A: 20x objective minimum projection image of the biocytin filled pyramidal neuron in the layer 2/3. B: corresponding skeletal 3D reconstruction.

Although the signal was present and the visualization had a high contrast, the slices still curled up like mentioned before. After adjusting the protocol by introducing the glass-construct into the dehydration series (see figure 4C), the protocol was optimal and many clear visible neurons appeared (see figure 10).

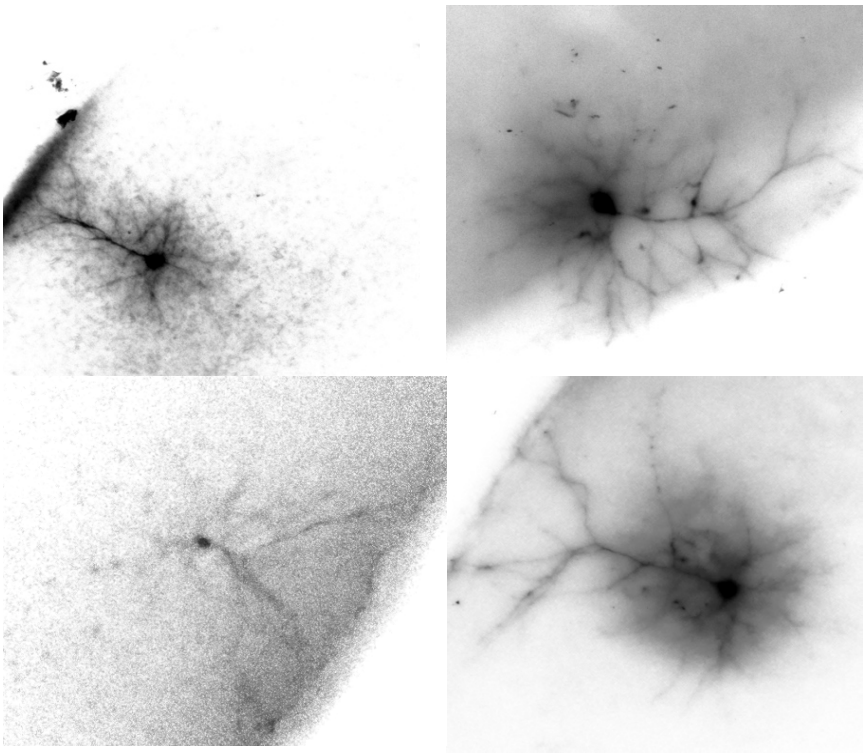

**Figure 10| Collection of the positive results.**

Even after the final protocol was established, there were still many slices that lacked a single visual neuron (similar to figure 7). In this case, what was most probable to have occurred was the cell was not successfully filled with biocytin during the electrophysiological recordings. Either the cell did not survive during recording or the injection time was not long enough.

**Golgi-Cox DISCO (goDISCO) Results**

The goDISCO protocol was attempted multiple times in order to achieve perfect results (see Table 5 of the Supplemental Material.) The images in figure 11 were obtained at 10x magnification and indicated that the chemicals of staining and clearing were compatible with each other. The brain in figure 11 was not completely transparent, therefore another experiment was done with a few incubation time adjustments. After adjusting the incubation time of sodium thiosulfate and ammonia, the brain was completely transparent (see figure 12).

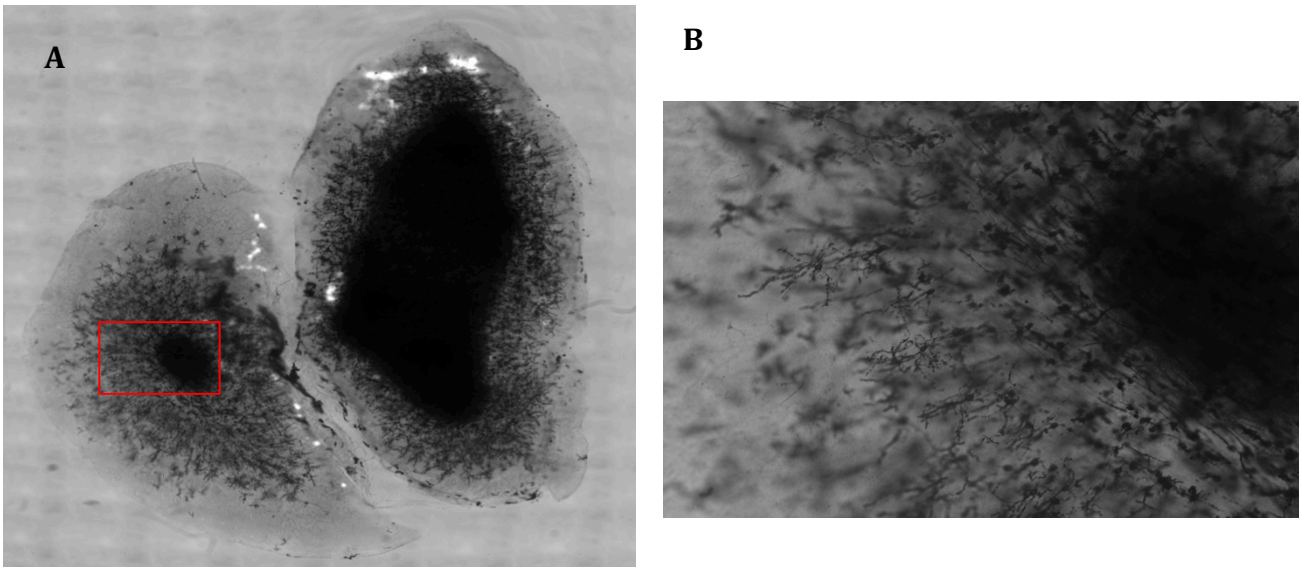


**Figure 11| Golgi-Cox staining in 3 mm thick mouse brain slice.** A: Stitched mosaic image a 10 times magnification. B: 10x magnification image zoomed in.

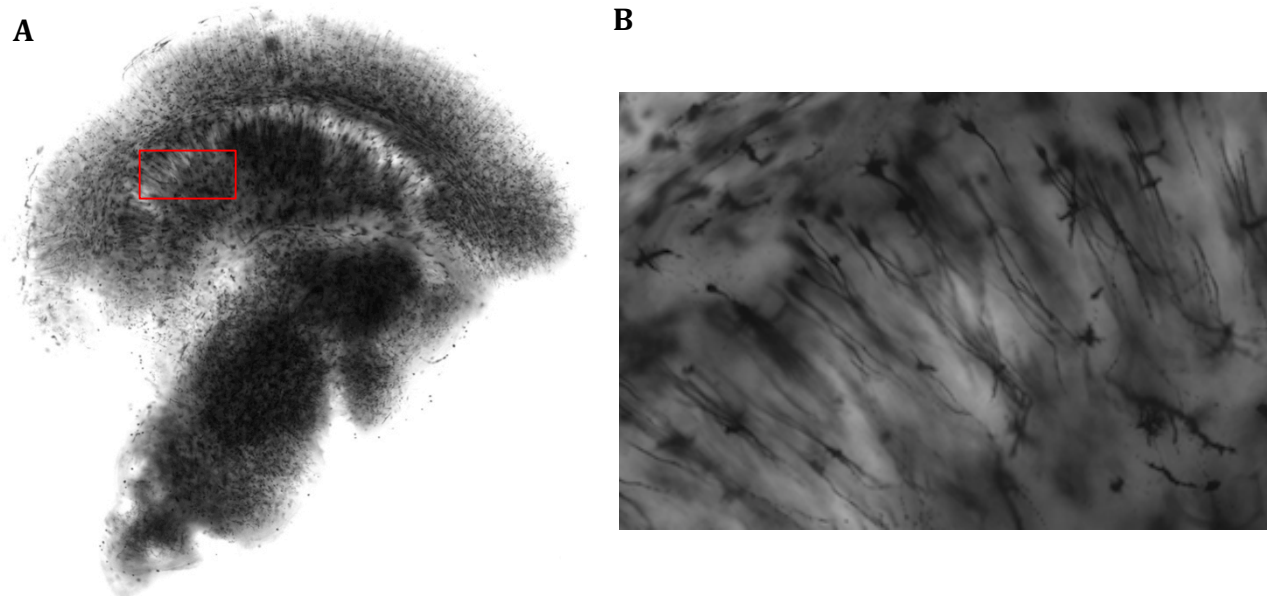


**Figure 12| Golgi-Cox staining in a 3 mm thick mouse brain slice with longer incubation in sodium thiosulfate.** A: Stitched mosaic image a 10x magnification. B: 10x magnification image zoomed in.

The quality of the staining is high enough to visualize individual neurons, neuronal projections and their dendritic spines. Therefore images were also obtained at 40x magnification in order to visualize the spine heads and spines (see figure 13B-F). The images are color inverted in order to visualize the spines with a higher contrast, making it easier to detect. Next to being able to visualize the dendritic spines, again it was also possible to make images throughout the z-plane (see figure 14A), allowing 3D network reconstruction (see figure 14B). The 3D reconstruction allowed each distinctive neuron to have their own identity, and therefore it was possible to give each one of them a color (see figure 14C).

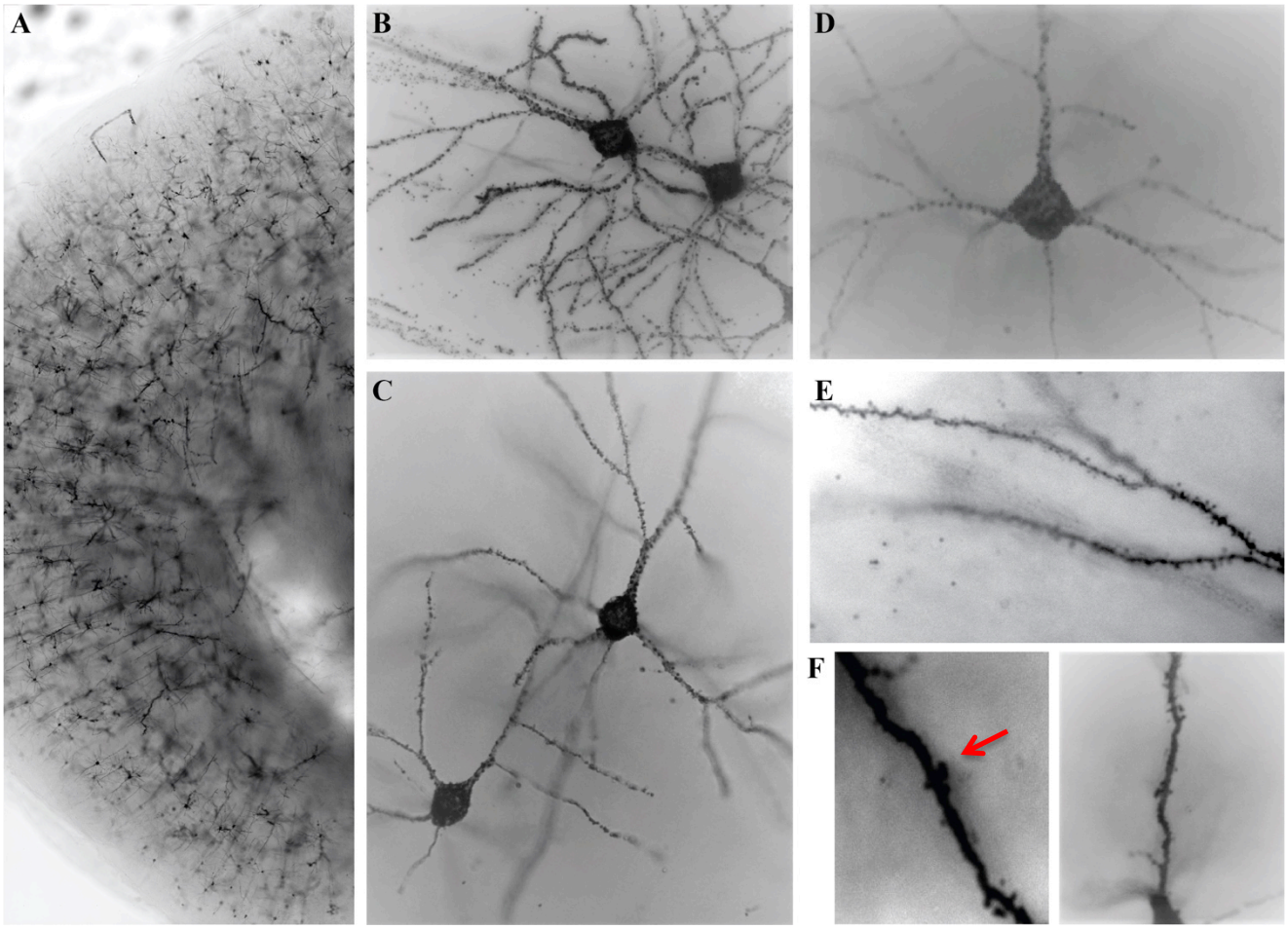


**Figure 13| Images of goDISCO stained cells in the layer 2/3 neocortex of 3 mm brain slices.** A: Birdseye view of the neocortex layer 2/3 made with mosaic scanning with a 10x objective. B – D: Pyramidal cells of the layer 2/3 neocortex imaged at 40x magnification. E – F: Zoomed images of the dendritic spines; a mushroom spine is detected (see red arrow).

## Discussion

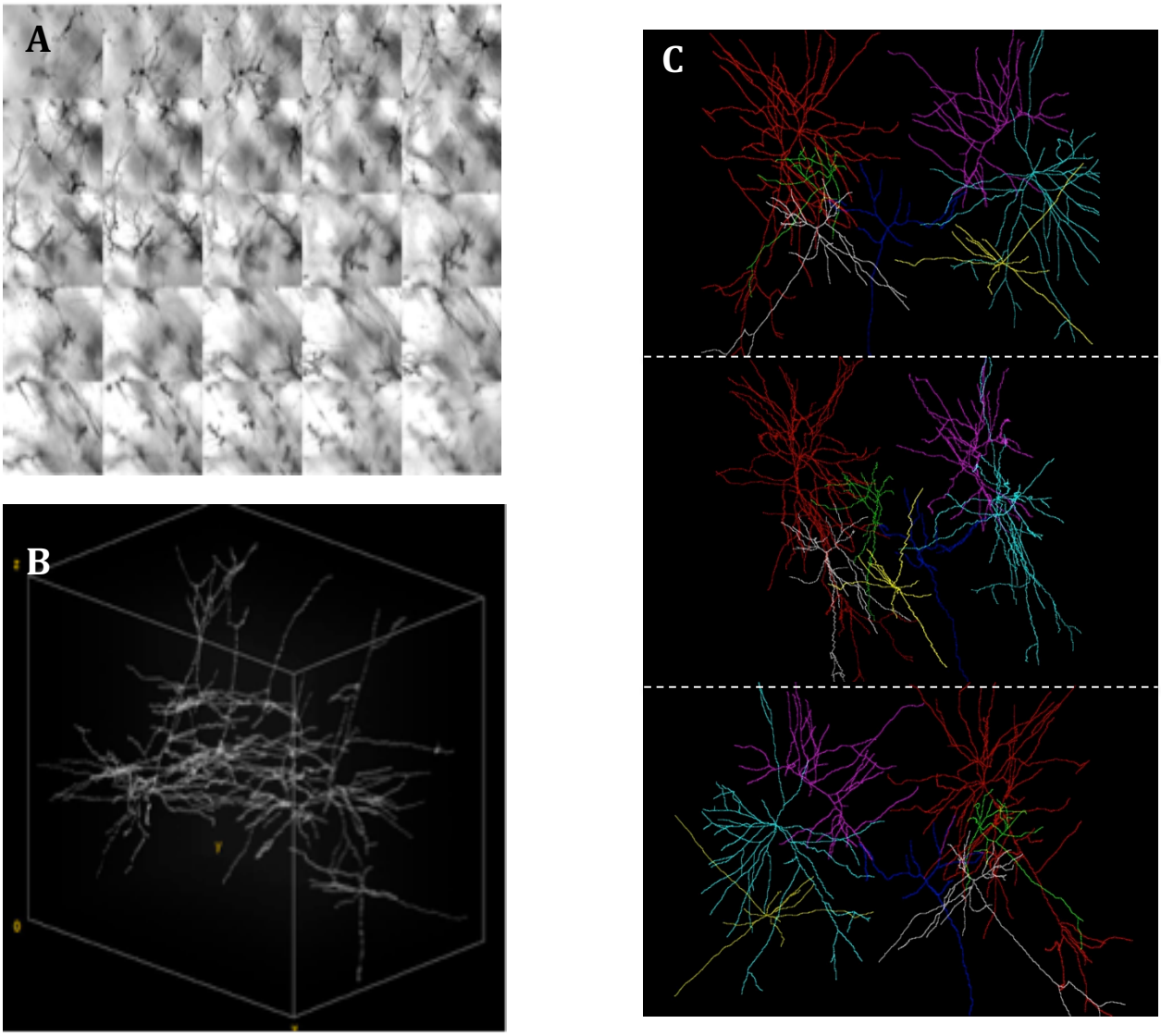


**Figure 14| ImageJ processed images.** A: Montage of the 890 z-stacks, every three images merged as minimum projections and montage is made with increments of 8. B: 3D reconstruction of neuronal network made from the z-stacks. C: Three different perspectives of the 3D reconstruction.

This report introduces advanced biocytin and Golgi-Cox staining protocols that are established for high quality 3D visualization of neurons. These two protocols allow morphological studies of neurons in thick brain slices. Staining and visualizing in thick slices is of high priority in anatomical studies of the brain, because it allows the maintenance of the network connectivity of the neurons by eliminating the need for slicing. During the process of optimizing these protocols, many difficulties were encountered mainly because these experiments have never been performed before.

The concept of tissue clearing is used for enhancing the visualization of the neurons by making the brain transparent. Because achieving a transparent brain is dependent on dehydration series and lipid removal solvents, it is not compatible with every type of staining, hence it is rather challenging. Regardless, two protocols for the ideal results of Golgi-staining and biocytin staining are established.

The slices that contained biocytin filled cells from electrophysiological recordings are 300 µm thick. In this experiment, they were used for staining and clearing. A few conditions were changed before obtaining the optimal results. The pilot run showed very dark staining, which seems to be caused by incubating the slices in the DAB solution for too long. The second run was done with a shorter DAB incubation time, which ended up with a nice visible stained neuron. However, the tissue was completely curled making it difficult for imaging. This led to the incorporation of the glass-construct allowing the slices to remain flat. Also to reduce time and increase transparency, the incubation time of 66% DCM/ 33% Methanol is changed from 3h to an overnight step. This allowed the reaction to continue overnight and gave ideal images for 3D reconstruction.

The images of the biocytin filled neurons show excellent neuronal projections, which are all intact because it is visualized in a thick slice. Since a high-resolution confocal microscope (sp8x) was used, the images are high quality, suitable for quantitative analysis of the single neuron. Because the neuron that has been patched-clamped is being visualized, this protocol allows revealing the morphological features of the patched neurons and links its anatomy with the physiological data.

Next to the single cell analysis, it is appealing to find an approach to study multiple neurons in a high-throughput manner. Therefore, the Golgi staining was implemented because it is a silver-based technique that stains neurons completely allowing the studying of neuronal morphology with axonal and dendritic arborization and spines through staining neurons only sparsely (Zaqout & Kaindl, 2016). Specifically in Golgi-Cox, mercury chloride replaces silver nitrate as coloring agent. This is thought to reduce background staining due to crystallization artifacts, yielding higher contrast images (Zaqout & Kaindl, 2016).

The first experiment of combining Golgi-Cox and iDISCO, performed on the 300 µm fresh (unfixed) slices, resulted in severe tissue damage with no signal. When it was assumed that the damage was due to the delicate texture of the fresh slices, some slices were then fixed prior to processing. It was then found that fixing interfered with the Golgi staining so there were no results. I then assumed the tissue would more likely stay intact if it was even thicker than 300 µm. I started to process 3mm thick slices and that is when I obtained the image in figure

11. From there on I knew the protocol could be performed, however I needed to achieve a more even transparency throughout the brain slice. Increasing dehydration and delipidation incubation times did not create a difference. But because literature states that sodium thiosulfate can be used for removing iodine when treating mercury treated samples, and acts as a stop bath for silver stains (Kopriwa & Huckins, 1972), I tried increasing the time of sodium thiosulfate incubation anticipating improved transparency. This indeed was a correct hypothesis, increasing the timing and concentration of sodium thiosulfate resulted in a more even transparency throughout the brain as in figure 12. The success of this experiment allowed a neuronal network 3D reconstruction (figure 14B and 14C), which is very useful for creating a virtual representation of the brain.

Altogether, this project has provided the opportunity for imaging biocytin and Golgi-Cox labeling in extremely thick slices that can be visualized in any type of animal, under any type of light microscope. This also allows many opportunities for future experiments. Ideally, the next step is to stain the entire brain without slicing. The protocol is already optimized for 3 mm, which allows segmenting the entire mouse brain into 3 sections before visualization (as opposed to 2250 slices using the standard Golgi-Cox staining). Regardless of whether the protocol can be performed on whole brain or not, there are still many advantages already to this current protocol. The established methods allow experimenting with drugs to test the anatomy of neuronal structure under the influence of different types of drugs. Also, neuronal network reconstruction of the developing brain, adult brain, or diseased brain would be a very useful tool in brain research.

## Conclusion

I have established new protocols in order to visualize intracellular labeled and silver-based stained neurons in extremely thick slices. When compared to other methods in literature, these are very simple, rapid, robust, and promising techniques mainly because the brain samples do not have to be thinly sliced, maintaining the network connections, the slices do not have to derive from a transgenic mouse, nor is there a special microscope needed in order to perform data acquisition. This work is being submitted for publication to Nature Methods (attachment follows).

## Acknowledgements

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## Supplementary Material

*Supplementary Table 1: Solutions for Ptx.2 solution for bDISCO.*

| *Compound* | *Amount* | *Dissolve in:* |
|---|---|---|
| *TritonX-100* | *2 mL* | *100 mL PBS 10x* |

*Supplementary Table 2: Solutions for Solution Step 5 for bDISCO.*

| *Compound* | *Concentration* | *Dissolve in:* |
|---|---|---|
| *Tween-20* | *0.2%* | *1X PBS* |
| *TritonX-100* | *0.2%* | *1X PBS* |
| *Deoxycholate* | *0.2%* | *1X PBS* |
| *NP40* | *0.2%* | *1X PBS* |
| *DMSO* | *40%* | *1X PBS* |

*Supplementary Table 3: Chemicals for the Golgi-Cox Impregnation Stock Solution.*

| Chemical | Amount | Dissolve in: |
|---|---|---|
| Potassium Dichromate $K_2Cr_2O_7$ | 15 g | 300 mL dd-$H_2O$ |
| Mercuric Chloride $HgCl_2$ | 15 g | 300 mL dd-$H_2O$ |
| Potassium chromate $K_2CrO_4$ | 15 g | 300 mL dd-$H_2O$ |

*Supplementary Table 4: Chemicals for the Developing solutions.*

| Chemical | Concentration | Dissolve in: |
|---|---|---|
| Ethanol | 50% | dd-$H_2O$ |
| Ammonia | 35% | dd-$H_2O$ |
| Sodium Thiosulfate | 25% | dd-$H_2O$ |

*Supplementary Table 5: Incubation variations for goDISCO.*

| *Date* | *4% PFA* | *2% Triton* | *Sect-ion* | *Thick-ness* | *Tissue protect-ion* | *Impreg. Time & temp* | *A Time (min)* | *ST Time (min)* | *ST Con. (%)* | *Signal* |
|---|---|---|---|---|---|---|---|---|---|---|
| 3-6-17 | No | - | Co | 300 um | 1-2h, 4C | 1-2h, RT | 5 | 5 | 5 | No |
| 7-6-17 | Yes | - | Co | 300 um | 1-2h, 4C | 1-2h, RT | 5 | 5 | 5 | No |
| 10-6-17 | No | - | Co | 300 um | No | O/N, 37C | 5 | 5 | 5 | No |
| 10-6-17 | Yes | - | Co | 300 um | No | O/N, 37C | 5 | 5 | 5 | No |
| 16-6-17 | No | - | Ce | 2 x2 mm | No | O/N, 37C | 8 | 10 | 5 | Slightly |
| 16-6-17 | No | + | Ce | 2 x2 mm | No | O/N, 37C | 8 | 10 | 5 | Slightly |
| 19-6-17 | No | - | Co | 2 mm | No | O/N, 37C | 8 | 10 | 5 | Slightly |
| 24-6-17 | No | + | Co | ~2 mm | No | O/N, 37C | 12 | 30 | 5 | Yes |
| 24-6-17 | No | - | Co | ~2 mm | No | O/N, 37C | 12 | 30 | 5 | Yes |
| **5-7-17 *** | **No** | **-** | **Co** | **3 mm** | **No** | **O/N, 37C** | **12** | **60** | **25** | **Yes** |
| **5-7-17 *** | **No** | **+** | **Sa** | **< 3 mm** | **No** | **O/N, 37C** | **15** | **120** | **25** | **Yes** |
| 5-7-17 | No | - | Ce | 2 x2 mm | No | O/N, 37C | 30 | 120 | 50 | Yes |
| 5-7-17 | No | - | Co | 3 mm | No | O/N, 37C | 30 | 120 | 50 | Yes |
| 5-7-17 | No | + | Ce | 2 x2 mm | No | O/N, 37C | 45 | 120 | 50 | Yes |
| 5-7-17 | No | + | Co | < 3 mm | No | O/N, 37C | 45 | 120 | 50 | Yes |
| 5-7-17 | No | - | Sa | 3 mm | No | O/N, 37C | 15 | 180 | 25 | Yes |
| 5-7-17 | No | - | Sa | 3 mm | No | O/N, 37C | 30 | 180 | 50 | Yes |
| 5-7-17 | No | - | Co | 3 mm | No | O/N, 37C | 30 | 180 | 25 | Yes |
| 5-7-17 | No | + | Co | < 3 mm | No | O/N, 37C | 60 | 180 | 50 | Yes |
| 5-7-17 | No | - | Co | 3 mm | No | O/N, 37C | 60 | 180 | 50 | Yes |

*GI= Golgi Impregnation*

*A = Ammonia*

*ST = Sidium Thiosulfate*

*Co = Coronal*

*Ce=cerebellum*

*Sa = Sagittal*

** = Most successful result, used for images*