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## A hydrophobic tissue clearing method for rat brain tissue

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July 6, 2020

Dr. Ronald Myers,

Senior Science Editor

Journal of Visualized Experiments

One Alewife Center, Suite 200,

Cambridge, MA 02140

Dear Dr. Myers,

Thank you very much for your kind invitation to submit a manuscript for publication in *Journal of Visualized Experiments*. Now, we submit our manuscript entitled "**A hydrophobic tissue clearing method for rat brain tissue**" by Dr. Kirchner and co-authors for consideration for publication in *JoVE*. In this manuscript, we described the procedures of hydrophobic tissue clearing method allows for the viewing of target molecules as part of intact brain structures. Viewing the brain as a whole system rather than a series of individual pieces is the biggest advantage of the whole-brain tissue clearing method. The manuscript or parts of it have not been, and will not be submitted elsewhere for publication.

Sincerely,

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**TITLE:**

# A Hydrophobic Tissue Clearing Method for Rat Brain Tissue

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**KEYWORDS**

tissue clearing, immunohistochemistry, iDISCO, brain, rat, confocal

**SUMMARY:**

Here we present a hydrophobic tissue clearing method that allows for the viewing of target molecules as part of intact brain structures. This technique has now been validated for F344/N control and HIV-1 transgenic rats of both sexes.

**ABSTRACT:**

Hydrophobic tissue clearing methods are easily adjustable, fast, and low-cost procedures that allows for the study of a molecule of interest in unaltered tissue samples. Traditional immunolabeling procedures require cutting the sample into thin sections, which restricts the ability to label and examine intact structures. However, if brain tissue can remain intact during processing, structures and circuits can remain intact for the analysis. Previously established clearing methods take significant time to completely clear the tissue, and the harsh chemicals can often damage sensitive antibodies. The iDISCO method quickly and completely clears tissue, is compatible with many antibodies, and requires no special lab equipment. This technique was initially validated for the use in mice tissue, but the current protocol adapts this method to image hemispheres of control and transgenic rat brains. In addition to this, the present protocol also makes several adjustments to preexisting protocol to provide clearer images with less background staining. Antibodies for Iba-1 and tyrosine hydroxylase were validated in the HIV-1 transgenic rat and in F344/N control rats using the present hydrophobic tissue clearing method. The brain is an interwoven network, where structures work together more often than separately of one another. Analyzing the brain as a whole system as opposed to a combination of individual

pieces is the greatest benefit of this whole brain clearing method.

**INTRODUCTION:**

Several tissue clearing techniques have been validated for the use in the brain: hydrogel, hydrophilic, and hydrophobic. These techniques aim to turn a tissue transparent through delipidation, decolorization, and decalcification via the administration of solvents. Once the refractive index of the tissue sample matches the refractive index of the chosen imaging medium, a clear image of the sample can be obtained. Hydrogel based techniques, such as CLARITY, secure biomolecules in the tissue by linking them to acryl-based hydrogels, which prevents structural damage and loss of proteins[1]. However, hydrogel techniques utilize harsh chemicals than have the potential to damage more fragile tissues, and denser tissue samples may not be compatible with hydrogel protocol. Certain hydrogel techniques may require expensive equipment or lead to tissue expansion. Hydrophilic techniques, like CUBIC, preserve 3D structure through the formation of hydrogen bonds within the tissue[2]. Tissue expansion can also occur in certain hydrophilic protocol. The clearing ability of hydrophilic techniques often does not match the ability that hydrophobic techniques have, which is important for denser and thicker tissues[3].

Hydrophobic techniques are usually fast, do not require special equipment, and produce a sample that is easy to handle and store. iDISCO is a hydrophobic technique that eliminates the shrinkage that can occur in other hydrophobic protocol. Originally, the iDISCO tissue clearing technique was described by Renier et al. [4] for the embryos and dense, adult organs of mice. This technique removes water from the tissue in the initial dehydration step, thereby reducing the light scatter. Tissue is permeabilized during pretreatment to allow deep antibody penetration. Alexa Fluor dyes in the far-red spectrum are used for immunolabeling to avoid autofluorescence of the tissue at lower wavelengths[5]. Tissue that has undergone hydrophobic clearing protocols are able to be handled easily and can be imaged several times due to the longevity of the Alexa Fluor dyes. If properly stored, tissues can provide images for months to a year after initial processing.

In the present protocol, tyrosine hydroxylase (TH) antibody, Iba1 antibody, and Cholera Toxin Subunit B (CTB) were used on both male and female control and HIV-1 transgenic (Tg) rat brains. The HIV-1 Tg rat possesses seven of the nine genes that comprise the HIV-1 viral genome, which leads to a non-infectious model of long-term HIV-1 protein exposure[6-8]. Dopaminergic alterations have previously been demonstrated in the HIV-1 Tg rat, and HIV-1 itself is an inflammatory disease, so the antibody choice was relevant to the experimental design[9-12]. CTB is a tracer that attaches to neurons via ganglioside binding and can be used to trace afferent projections in the brain. CTB has been previously used to study projections from the nucleus accumbens to the substantia nigra area, two areas of the brain heavily involved with dopaminergic pathways[13-15].

In this protocol, TH will serve as a marker for dopamine production, and Iba1 will mark activated microglia. The TH antibody used selectively labels a single band at approximately 62 kDa that corresponds to tyrosine hydroxylase. The Iba1 antibody corresponds to the Iba1 carboxy-terminal sequence. Both antibodies were raised in rabbit and were polyclonal. CTB will be used for retrograde tracing of neurons from the nucleus accumbens area to the substantia nigra area.

The current protocol also offers a guideline for adjustment of the original iDISCO protocol in two distinct ways: 1) overall reduction of the background staining by changing the serum reagents in the blocking steps, and 2) scaling of the incubation time to be suitable for larger tissue samples. Overall, the present protocol provides continued evidence that hydrophobic clearing techniques are feasible in brain tissues of the rat, have no discernible detrimental interactions with HIV-1 viral proteins, and are compatible with TH antibody, Iba1 antibody, and CTB.

**PROTOCOL:**

All animal protocols were reviewed and approved by the Animal Care and Use Committee at the University of South Carolina.

## 1. Stock solution preparation

1.1. Solution 1 (1 L): To 900 mL of deionized $H_2O$ add 100 mL of 10x phosphate buffered saline (PBS) and 2 mL of Triton X-100.

1.2. Solution 2 (1 L): To 900 mL of deionized $H_2O$, add 100 mL of 10x PBS, 2mL of Tween-20 and 1 mL of 10 mg/mL Heparin stock solution.

1.3. Solution 3 (500 mL): To 400 mL of Solution 1, add 11.5 g of glycine and 100 mL of dimethylsulfoxide (DMSO).

1.4. Solution 4 (50 mL): To 42 mL of Solution 1, add 3 mL of corresponding serum and 5 mL of DMSO.

1.5. Primary antibody solution (50 mL): To 46 mL of Solution 2, add 2.5 mL of DMSO and 1.5 mL of corresponding serum.

1.6. Secondary antibody solution (50 mL): To 48.5 mL of Solution 2, add 1.5 mL of corresponding serum.

NOTE: For Solution 4, primary solution, and secondary solution, use a serum that corresponds with the secondary antibody (e.g., goat, horse, bovine).

## 2. Sample preparation

NOTE: Perform steps 2.1 through 2.7 in a fume hood due to limit the exposure to PFA.

2.1. Deeply anesthetize young (3-6 week) rat using sevoflurane; proceed to 2.2 when rat is not responsive and fails to respond to toe and tail pinch.

2.2. Lay the rat in a supine position and make an incision through the abdominal wall using a pair of Iris scissors.

2.3. Cut up through the bottom of the rib cage to the collarbone on both sides of the rib cage using Mayo scissors.

2.4. Affix the sternum and ribs away from the heart and lungs with a hemostat.

2.5. Pierce the left ventricle with a 23 G needle attached to a perfusion pump and cut the right atrium with Iris scissors.

2.6. Perform transcardial perfusion with approximately 75 mL of 100 mM (1x) PBS.

2.7. Continue transcardial perfusion with approximately 100 mL of 4% paraformaldehyde (PFA) buffered in 1x PBS.

2.8. Remove the brain from the skull using forceps.

2.9. Place the brain in sagittal position and slice into 4 equal sections (approximately 3 mm in width) using a razor blade and a rat brain matrix.

2.10. Fix each section in 4% PFA in 1x PBS in a sealed, 5mL plastic tube overnight at 4 °C on a shaker.

2.11. Continue to fix in 4% PFA in 1x PBS at room temperature (RT) on a shaker for 1 h.

2.12. Wash each section with 1x PBS at RT for 30 min, 3 times.

**3. Dehydration and depigmentation**

3.1. Incubate the sample in a 20% methanol/80% deionized water solution for 1 h at RT.

3.2. Incubate the sample in a 40% methanol/60% deionized water solution for 1 h at RT.

3.3. Incubate the sample in a 60% methanol/40% deionized water solution for 1 h at RT.

3.4. Incubate the sample in an 80% methanol/20% deionized water solution for 1 h at RT.

3.5. Incubate the sample in 100% methanol for 1 h at RT. Repeat with more 100% methanol, incubating for 1 h at RT.

3.6. Chill the sample in 100% methanol at 4° C for approximately 10 min.

3.7. Prepare a 66% dichloromethane (DCM)/33% methanol solution.

3.8. Incubate the sample overnight in the DCM/methanol solution at RT, with shaking.

3.9. Wash with methanol at RT for 30 min, 2 times.

3.10. Chill the sample in 100% methanol at 4° C for approximately 10 min.

3.11. Bleach in chilled, freshly prepared 5% hydrogen peroxide in methanol overnight at 4° C.

3.12. Incubate the sample in an 80% methanol/20% deionized water solution for 1 h at RT.

3.13. Incubate the sample in a 60% methanol/40% deionized water solution for 1 h at RT.

3.14. Incubate the sample in a 40% methanol/60% deionized water solution for 1 h at RT.

3.15. Incubate the sample in a 20% methanol/80% deionized water solution for 1 h at RT.

3.16. Incubate the sample in 1x PBS for 1 h at RT.

3.17. Wash in Solution 1 for 1 h at RT, 2 times.

**4. Cholera Toxin Subunit B labeling**

4.1. Insert needle tip of 1 mL syringe into nucleus accumbens site.

4.2. Slowly inject 2.5 µL of 1% biotin-CTB over 20 s.

4.3. Leave the needle tip in place for 1 min and remove slowly over 10 s to prevent leakage.

**5. Antibody application**

5.1. Incubate the sample in Solution 3 for 2 days at 37 °C in a water bath.

5.2. Incubate the sample in Solution 4 for 2 days at 37 °C in a water bath.

5.3. Incubate with primary antibody for 7 days at 37 °C in a water bath.

5.4. Wash in Solution 2 for 5 times, incubating 1 h per wash. Store in Solution 2 at RT overnight.

5.5. Incubate with secondary antibody for 7 days at 37 °C in water bath.

5.6. Wash in Solution 2 for 5 times, incubating 1 h per wash; store in Solution 2 at RT overnight.

NOTE: All steps from 5.6 onwards, including final storage, should occur in low light. The sample tubes can be shielded with aluminum foil. The concentrations used for the antibodies in this protocol were: TH at 1:100, Iba1 at 1:200, and the secondary antibody at 1:100. Add additional Solution 2 to steps 5.1-5.6 to ensure tubes are completely filled.

## 6. Tissue clearing

6.1. Incubate the sample in a 20% methanol/80% deionized water solution for 1 h at RT.

6.2. Incubate the sample in a 40% methanol/60% deionized water solution for 1 h at RT.

6.3. Incubate the sample in a 60% methanol/40% deionized water solution for 1 h at RT.

6.4. Incubate the sample in an 80% methanol/20% deionized water solution for 1 h at RT.

6.5. Incubate the sample in 100% methanol for 1 h at RT.

6.6. Incubate in fresh 100% methanol overnight at RT.

6.7. Incubate in 66% DCM/33% methanol for 3 h, with shaking, at room temperature.

6.8. Incubate in dibenzyl ether (DBE) without shaking. Leave the sample in DBE until clear and store in DBE until imaging.

## 7. Mounting

7.1. Obtain a 3D printed chamber made of Visijet M3 Crystal resin (templates available on the idisco.info website).

7.2. Secure the chamber to a microscope slide using an epoxy.

7.3. Place the sample in the square space in the middle of the chamber.

7.4. Fill the chamber completely with DBE.

7.5. Place a 0.17 mm thick coverslip over the chamber and apply pressure until the coverslip has full contact with the chamber walls.

7.6. Seal the edges of the coverslip to the chamber using the epoxy.

7.7. Rotate the chamber to allow air bubbles to escape from the filling inlet.

7.8. Fill the chamber with additional DBE.

7.9. Seal the filling inlet with epoxy and allow to cure.

NOTE: Excess DBE will spill out during mounting.

## 8. Imaging

8.1. Use a confocal microscope system to perform z-scan at 4x magnification (see the attached **3D video**).

8.2. Achieve fluorescent excitation of the sample using a laser set to emit at a wavelength similar to the wavelength specified by the secondary antibody. TH excitation was achieved utilizing a HeNe laser set to emit at 632 nm.

8.3. Set the detector to a wavelength close to the laser emission. For the present protocol, the detector for TH was set to 650 nm.

8.4. Raise the gain of the detector until there is a visible signal. The 650LP gain for images was set to 7.50 B.

8.5. Using the **Scan** option in the confocal imaging software, move the microscope stage until the sample is brought into focus.

8.6. Navigate around the tissue in all plains and obtain either single images or z-stack images using the confocal imaging software.

8.7. Once imaging is completed, remove the sample from the chamber. Place it back into a tube completely filled with DBE and store at RT in a dark place, preferably covered in aluminum foil.

NOTE: Samples can be remounted and imaged as long as the florescence signal is still strong.

**REPRESENTATIVE RESULTS:**

Full clearing of large sections of both F344/N and HIV-1 rat brain tissue was achieved using this modified hydrophobic tissue clearing protocol. **Figure 1** displays a typical confocal image for TH in the substantia nigra region. **Figure 1A** represents dense, positive staining. Dense areas such as these can be parsed out by focusing through the "Z" plane to confirm positive staining and proper cell morphology. **Figure 1B** represents sparse positive staining, which can be identified by the TH neurons' distinct morphology. **Figure 1C** represents tissue that is not positively stained but is still dark to light blue due to background signal. Areas of the image that appear as completely black indicate an absence of tissue in that area. Completely black areas in the image can be due to being on the edge of the sample, a hole that is in the sample, or tissue that is out of focus.

**Figure 2A** shows typical morphology of a TH positive neuron at 20x magnification. Properly stained and focused confocal images will ideally show bright and crisp fluorescence against a dark background. A TH positive neuron has a large soma (cell body) and several branching processes[16]. **Figure 2B** shows Iba1 stained microglia, which have small cell bodies and shorter extending processes[17]. Confirmation of proper staining and expected cell morphology is the first step in imaging.

**Figure 3** compares an ideal image (**Figure 3A**) to three undesirable images (**Figure 3B-D**). **Figure 3A** shows positive, bright staining against a clear, dark background. Positive staining is easily distinguishable from the background. **Figure 3B** is an example of an improperly focused image with too much fluorescent gain. **Figure 3C** shows a false positive signal. Bright spots that are not in focus with the rest of the tissue are artefacts and should not be considered as a positive signal. **Figure 3D** is an example of using a blocking serum that does not match the secondary antibody, as suggested in the original iDISCO protocol[4]. In this image, use of donkey serum for a secondary antibody made in goat produced an image with high background staining, which obscures the ability to properly identify positive staining.

**Figure 4A** shows positive CTB staining in the rat brain. The long, thin fibers are afferent projections along the dopaminergic pathway. **Figure 4B** and **Figure 4C** show colocalization of TH and CTB. In **Figure 4B**, the injection can be seen in the nucleus accumbens area, which appears as a densely fluorescent green circle. **Figure 4C** displays colocalization of TH and CTB in the substantia nigra area.

**FIGURE LEGENDS:**

**Figure 1: Confocal image of a hydrophobically cleared tissue sample with markers, 4x magnification.** (**A**) Dense TH staining in the substantia nigra area. (**B**) Sparse TH neurons adjacent to the substantia nigra. (**C**) Tissue lacking positive TH neurons.

**Figure 2: Typical morphology of fluorescently labeled TH positive neurons (20x magnification) and Iba1 positive microglia (10x magnification).** (**A**) Two representative TH positive neurons. (**B**) Iba1 positive microglia.

**Figure 3: Ideal and poor confocal images of hydrophobically cleared tissue, 4x magnification.** (**A**) Dense TH staining in the substantia nigra area, properly in focus with a suitable fluorescence gain. (**B**) An improperly focused and overly exposed image. (**C**) False positive staining. (**D**) High background staining as a result of following the unaltered iDISCO protocol.

**Figure 4: Cholera Toxin B labeling along the dopaminergic pathway, 4x magnification.** (**A**) Confocal image of positive CTB staining. (**B**) Overlapped image of TH and CTB and nucleus accumbens injection site. (**C**) Colocalization of TH and CTB in the substantia nigra area.

**Movie 1: 3D movie of z-scan of the sample at 4x magnification.**

**DISCUSSION:**

Tissue clearing offers a solution to the limitations of traditional IHC protocol. A sample that is transparent minimizes the scattering and absorption of light, which provides cellular level optical access to intact tissues[18-19]. Tissue clearing techniques turn a tissue transparent through delipidation, decolorization, and decalcification with the administration of solvents. Once the refractive index of the tissue sample matches the refractive index of the chosen imaging medium, the sample will appear completely transparent and can be properly imaged[3]. The present

hydrophobic protocol removes water from the tissue in the initial dehydration steps, thus reducing light scatter. Tissue is permeabilized during pretreatment to allow deep antibody penetration. Tissue that has undergone the protocol is able to be handled easily and can be imaged several times. If properly stored, tissues can still provide images for up to a year after initial processing.

In the present protocol, we apply a hydrophobic tissue clearing technique to F344/N and HIV-1 Tg rat brain tissue samples to stain for TH, Iba1, and CTB. After clearing, the tissue samples were imaged using a confocal microscope. The present protocol has several advantages over other hydrophobic protocol, like iDISCO[4]. First, the original iDISCO protocol was validated for the use in mice tissue, while the present protocol is viable in rat brain tissue. Second, the adjusted incubation time on each step allows the technique to be used for larger tissue sections. Third, including the corresponding serum during antibody incubation (as opposed to use of a general serum) provides clearer images for analysis.

While the present protocol is easily adaptable to suit the needs of individual research questions, there are several important considerations. The protocol takes, at minimum, 26 consecutive days from beginning to end. Do not leave the sample in a solution for longer than stated above. The sample can, however, be stored in DBE at the end of the protocol for at least 6 months safely until imaging, although it is advised to image as soon as possible. Order a 3D printed chamber that will fit the sample snugly between the microscope slide and coverslip. All stock solutions, aside from Solution 1, were mixed day-of in an amount that was appropriate for that day's use. The sample should be moved to a new tube whenever a new solution is introduced to prevent cross contamination. If microbial growth is of concern, sodium azide can be added to Solution 2, Solution 3, Solution 4, the primary antibody solution, and the secondary antibody solution at a concentration of 0.02% in total solution.

Overall, the present technique is an extremely versatile, easy to implement, and low-cost procedure that is compatible with many antibodies. With this protocol that is validated to work in both F344/N control and HIV-1 Tg rats, many new investigative research questions can be answered.

**ACKNOWLEDGMENTS:**

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**DISCLOSURES:**

None of the authors have conflicts of interest to declare.

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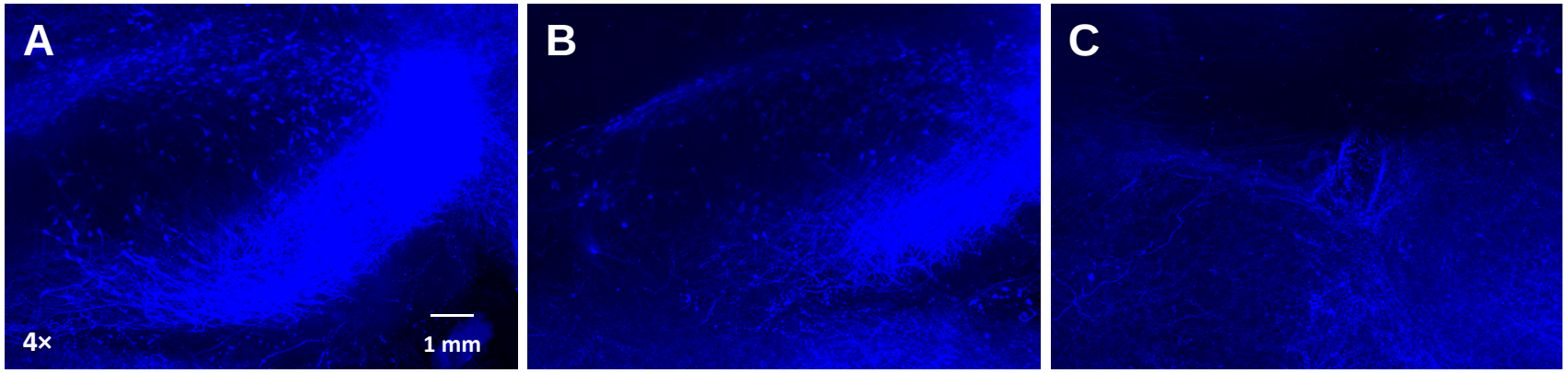
# Figure 1



# Figure 2

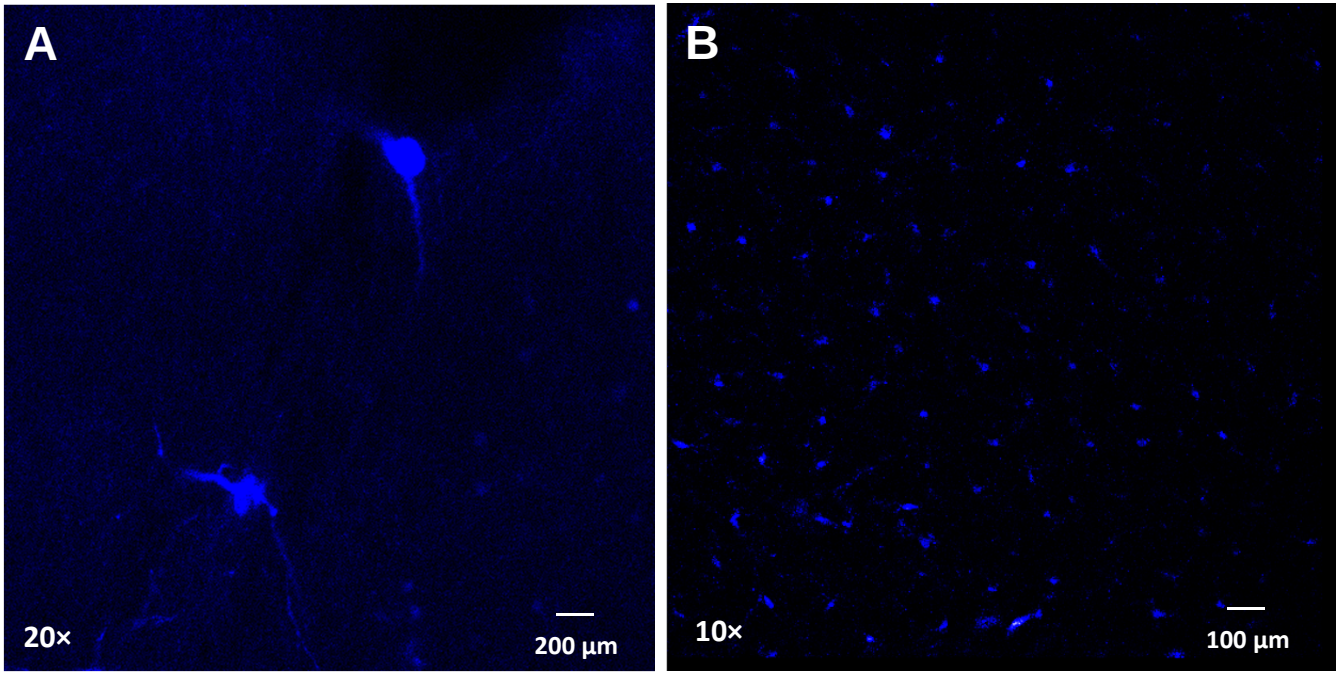
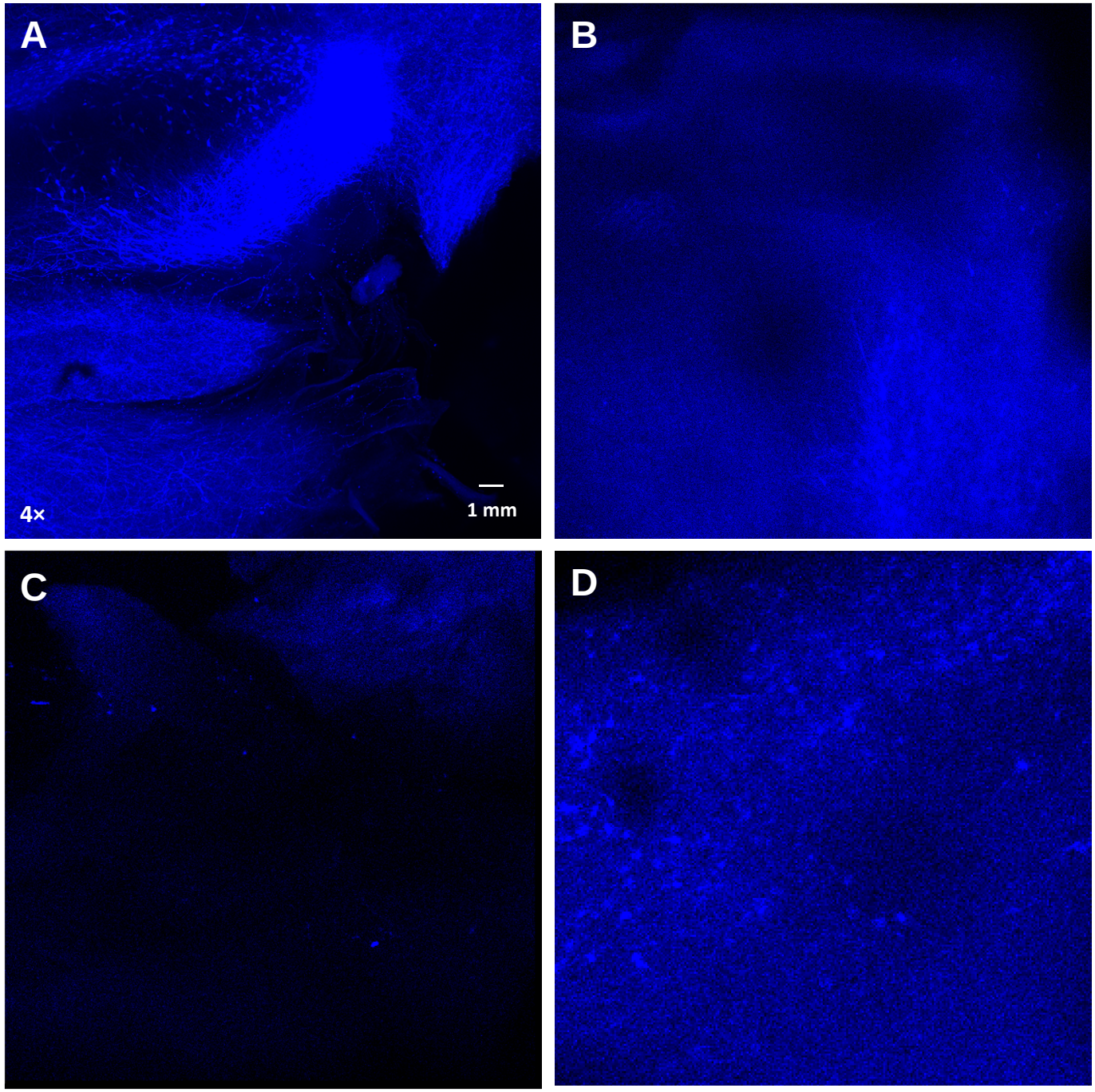
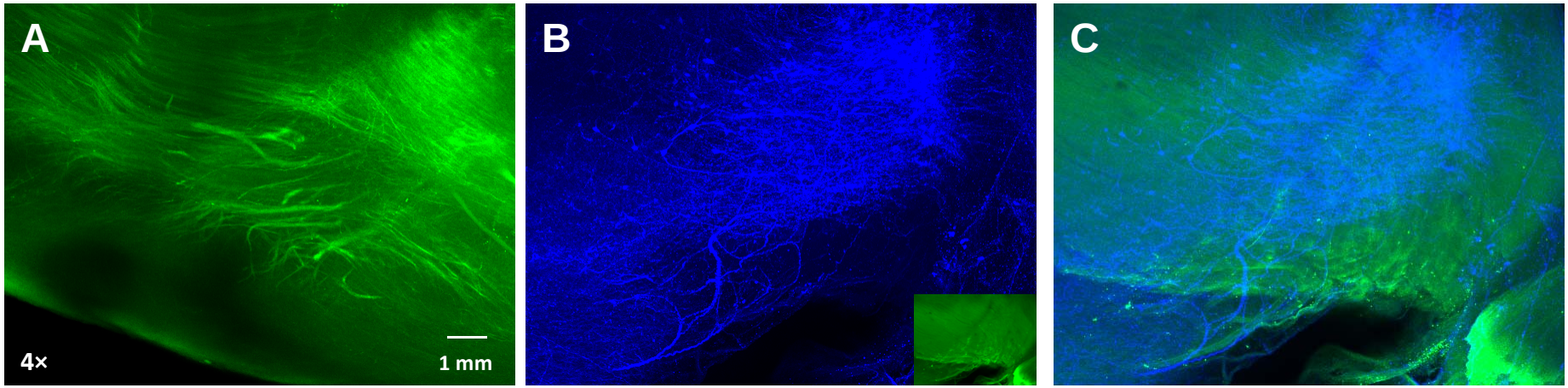


Figure 3



# Figure 4



Video or Animated Figure

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3-D video.mp4

| Name of Material/ Equipment | Company | Catalog Number |
|---|---|---|
| Cholera Toxin Subunit B (Recombinant), Alexa Fluor 488 | Invitrogen | C34775 |
| DBE | Sigma-Aldrich | 108014-1KG |
| DCM | Sigma-Aldrich | 270997-100mL |
| DMSO | Sigma-Aldrich | 472301-1L |
| Glycine | Fisher Chemical | G46-500 |
| Goat anti-rabbit Alexa Fluor Plus 647 | Invitrogen | A32733 |
| Goat serum | Sigma Life Science | G9023-10mL |
| Heparin | Acros Organics | 41121-0010 |
| Iba1 primary antibody | FUJIFILM Wako | 019-19741 |
| Kwik-sil epoxy | VWR | 70730-062 |
| Methanol | Sigma-Aldrich | 34860-1l-R |
| PBS | Fisher Bioreagents | BP2944-100 |
| Perfusion machine | VWR | 70730-062 |
| PFA | Sigma-Aldrich | 158127-3KG |
| TH primary antibody | Millipore Sigma | AB152 |
| TritonX-100 | Fisher Bioreagents | BP151-500 |
| Tween-20 | Fisher Bioreagents | BP337-500 |
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| Comments/Description |
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Oct 10, 2017

Dr. Nam Nguyen

Manager of Review

Journal of Visualized Experiments

One Alewife Center, Suite 200,

Cambridge, MA 02140

Dear Dr. Nam Nguyen,

Thank you very much for your kind consideration of our manuscript entitled “A hydrophobic tissue clearing method for rat brain tissue” by Dr. Kirchner and co-authors for consideration for publication in *JoVE*. We really appreciate your suggestions regarding the revision. According to the comments, we organized distinct representative images in our manuscript now. We would like to resubmit this revised manuscript to JoVE. Please feel free to let us know if you have any question.

Sincerely,

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**Editorial comments:**

We thank the editor for their consideration, review, and helpful criticisms to our manuscript.

*1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.*
**Answer:** The manuscript has been thoroughly proofread, and spelling or grammar issues have been revised.

*2. Unfortunately, there are sections of the manuscript that show overlap with previously published work. Please use original language throughout the manuscript. Please revise the Long Abstract after reviewing the attached iThenticate report.*
**Answer:** It has been changed.

*3. Please add scale bars to the Figures.*
**Answer:** Scale bars have been added to the figures.

*4. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s).*
**Answer:** In-text citations have been formatted as numbered superscripts.

*5. Please specify all surgical tools used throughout the protocol.*
**Answer:** Surgical tools have been specified, specifically in section 2 of the protocol.

*6. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.*
**Answer:** Approximately 3 pages of the protocol are highlighted.

*7. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.*
**Answer:** Full sentences have been highlighted and each step reflects an imperative mood.

**Reviewer #1:**
*Major Concerns:*
*The imaging quality of all the figures are very low, including the "ideal" confocal images.*
**Answer:** New images have been added which are of higher quality, and should better reflect ideal confocal images.

*The parameters of confocal imaging, including the parameters of the objectives need to be specified.*
**Answer:** Parameters for excitation, detection, and gain have been added to the "Imaging" section of the protocol, which will hopefully clarify the protocol.

We thank the reviewer for their suggestions, which have helped to clarify the manuscript and better reflect the efficacy of this clearing technique.

**Reviewer #2:**
*Major Concerns:*
*None*

*Minor Concerns:*
*Well written and elaborately detailed. No concerns noted.*
We thank the reviewer for their time and review of our manuscript.

**Reviewer #3:**
*However it is applied only to 3 mm thick slices and the recording is done with confocal microscopy. As objectives compatible with organic clearing and 3 mm working distance have a rather low NA such recordings will have a very poor z resolution. Furthermore 3 mm thick slices make not much sense as preparations for 3D imaging.*
**Answer:** 3D chambers for this technique were available in limited sizes. An appropriately sized chamber must fit the tissue snugly within, but still be thin enough to be properly mounted and imaged on the microscope without compromising the resolution of the images. Thus, 3 mm was chosen. New images have been added to better highlight the technique.

*Here rather whole rat brains would be of interest.*
**Answer:** While we agree with this suggestion, unaltered rat brains do not fully clear as reliably as hemispheric sections. Thus, for the present protocol, we hope to provide a technique that will work consistently, while still providing more circuit-level information than traditional thin slices would.

*For publication I thus suggest that the authors supply recordings of whole rat brains with light sheet microscopy demonstrating the versatility of their methods. Otherwise I see no practical use for the scientific community.*
**Answer:** The value of light-sheet microscopy techniques cannot be understated; however, it is a technique that is currently not within the scope of the present manuscript. We thank the reviewer for their suggestion for future applications of the present protocol.

We thank the reviewer for their valuable criticism and consideration of our manuscript.