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TITLE

Histological-Based Stainings Using Free-Floating Tissue Sections

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SUMMARY

The free-floating technique allows researchers to perform histological-based stainings including immunohistochemistry on fixed tissue sections to visualize biological structures, cell type, and protein expression and localization. This is an efficient and reliable histochemical technique that can be useful for investigating a multitude of tissues, such as brain, heart, and liver.

ABSTRACT

Immunohistochemistry is a widely used technique to visualize specific tissue structures as well as protein expression and localization. Two alternative approaches are widely used to handle the tissue sections during the staining procedure, one approach consists of mounting the sections directly on glass slides, while a second approach, the free-floating, allows for fixed sections to be maintained and stained while suspended in solution. Although slide-mounted and free-floating approaches may yield similar results, the free-floating technique allows for better antibody penetration and thus should be the method of choice when thicker sections are to be used for 3D reconstruction of the tissues, for example when the focus of the experiment is to gain information on dendritic and axonal projections in brain regions. In addition, since the sections are kept in solution, a single aliquot can easily accommodate 30 to 40 sections, handling of which is less laborious, particularly in large-scale biomedical studies. Here, we illustrate how to apply the free-floating method to fluorescent immunohistochemistry staining, with a major focus on brain sections. We will also discuss how the free-floating technique can easily be modified to fit the individual needs of researchers and adapted to other tissues as well as other histochemical-based stainings, such as hematoxylin and eosin and cresyl violet, as long as tissue samples are properly fixed, typically with paraformaldehyde or formalin.

INTRODUCTION

Immunostaining is a popular research practice that began 130 years ago with the discovery of serum antibodies in 1890 by Von Behring[1]. During the early 20th century, dyes were attached to antigens and later to antibodies as a way to quantify and visualize reactions[1], and in 1941 Albert Coons developed the first fluorescent antibody labels, a discovery that revolutionized light microscopy[2,3]. The term "immunostaining" encompasses many techniques that have been developed using this principle, including Western blot, flow cytometry, ELISA, immunocytochemistry, and immunohistochemistry[3,4]. Western blot detects the presence of specific proteins from tissue or cell extracts[5]. Proteins are separated by size using gel electrophoresis, transferred to a membrane, and probed using antibodies. This technique indicates the presence of protein and how much protein is present; however, it does not reveal any information on the localization of the protein within cells or tissues. Another method, immunocytochemistry (ICC), labels proteins within cells, typically cells cultivated in vitro. ICC shows both protein expression and localization within cellular compartments[6]. To detect and visualize a specific protein at the tissue level, immunohistochemistry (IHC) is utilized.

IHC is a method that researchers use to target specific antigens within tissue, taking advantage of chemical properties of the immune system[7,8]. By generating specific primary and secondary antibodies linked to either an enzyme or a fluorescent dye, antigens of interest can be labelled and revealed in most tissues (as reviewed in Mepham and Britten)[9]. The term "immunohistochemistry" by itself does not specify the labeling method that is used to reveal the antigen of interest; thus, this terminology is often combined with the detection technique to clearly delineate the labeling method: chromogenic immunohistochemistry (CIH) to indicate when the secondary antibody is conjugated to an enzyme, such as peroxidase; or fluorescent IHC to indicate when the secondary antibody is conjugated to a fluorophore, such as fluorescein isothiocyanate (FITC) or tetramethylrhodamine (TRITC). The selectivity of IHC allows clinicians and researchers to visualize protein expression and distribution throughout tissues, across various states of health and disease[10]. In the clinical realm, IHC is commonly used to diagnose cancer, as well as to determine differences in various types of cancer. IHC has also been used to confirm different types of microbial infections within the body, such as Hepatitis B or C[11]. In biomedical research, IHC is often used to map protein expression in tissues and is important in identifying abnormal proteins seen in disease states. For example, neurodegeneration is often accompanied by accumulation of abnormal proteins in the brain, such as A-β plaques and neurofibrillary tangles in Alzheimer's disease. Animal models are often then developed to mimic these pathological states, and IHC is one method that researchers use to locate and quantify the proteins of interest[10, 12, 13]. In turn, we can learn more about the causes of these diseases, and the complications that arise with them.

There are many steps involved in performing IHC. First, the tissue of interest is collected and prepared for staining. Arguably most researchers prepare fixed tissue samples, with perfusion of the fixative via the circulatory system being optimal as it preserves morphology[14,15]. Post-fixation of tissue samples may also be used but may yield less than ideal results[16]. Crosslinking fixatives, such as formaldehyde, act by creating chemical bonds between proteins in the tissue[17]. Fixed

tissue is then sliced into very thin layers or sections using a microtome, with many researchers preferring to collect frozen sections using a cryostat. From there the tissue is collected and either mounted directly onto a microscope slide (slide-mounted method), or suspended in a solution (free-floating method), such as phosphate buffered saline (PBS)[18]. The method of collection used is predetermined based on the needs of the researcher, with each of these two methods presenting its own advantages and disadvantages.

The slide-mounted method is by far the most commonly used, with an important benefit being that very thin sections (10-14 μm) can be prepared, which is important to investigate protein-protein interactions for example. There is also minimal handling of the specimen, which decreases potential damage to the structural integrity of the tissue[19]. Researchers often use this technique with fresh frozen tissue (tissue that is immediately frozen using dry ice, isopentane, etc.), which is very delicate as compared to fixed tissue and much care to prevent thawing of the sample needs to be taken. Another advantage of using slide-mounted sections is that large volumes of solutions for staining are usually not required[4]. Thus, researchers can use a smaller amount of expensive antibodies or other chemicals to complete the stain. Additionally, it is possible to mount sections from several different experimental groups on the same slide, which can be advantageous, especially during image acquisition. On the other hand, there are some disadvantages of using slide-mounted sections, most notably that the tissue section is adhered to the slide thus restricting antibody penetration to one side of the section, which limits the section thickness and the 3D representation of the tissue. It can also happen that during washings, the edges of the tissue and entire sections may detach from the slide, rendering useless the whole experiment. Moreover, IHC usually has to be performed relatively quickly when using the slide-mounted approach to avoid degradation of the antigen epitope[20,21] with unprocessed slides typically stored at -20 or -80 °C, often coverslipped and stored horizontally or in slide boxes, resulting in a relatively large storage footprint. Lastly, the slide-mounted technique can also be time consuming if researchers must handle large numbers of slides to process large numbers of tissue sections.

Due to some of these challenges using the slide-mounted method, a modification called the free-floating method has become a popular alternative. This technique came into the literature in the 1960-70s[22–24], gaining popularity in the 1980s[25–29], and is now a well-established method that involves performing the stain on the collected sections in suspension rather than adhered to a slide[12, 30, 31]. The free-floating method is not recommended when tissue sections are less than 20 μm; however, in our experience it is the approach of choice when thicker (40-50 μm) sections are to be stained. One distinct benefit is that antibodies can penetrate free-floating sections from all angles and generate less background staining due to more effective washing, all resulting in better signaling when imaging. Additionally, the sections are mounted onto the slides after processing, thus eliminating the possibility of tissue detachment as well as decreasing the time occupying the cryostat. The free-floating method can also be much less labor intensive, especially for large-scale biomedical studies. For instance, it is possible to stain many (18-40) sections from the same sample together in the same well, which saves time in performing both the wash and antibody incubation steps. Moreover, since a larger number (12-16) of sections can be mounted per slide using this approach, it is often more convenient and quicker for the researcher to view

and image sections. Notably, during the mounting of tissue slices on the slides, sections can be attached and detached until the desired orientation is obtained. Researchers also often use slightly lower concentrations of antibodies using the free-floating method, and since the incubations are performed in microcentrifuge tubes, the antibodies can be easily collected and preserved with sodium azide for reusage (see Step 5.1). Another advantage is that the sections can be directly stored at -80 °C in small microcentrifuge tubes with cryoprotectant solution[32], thereby minimizing storage space and maximizing longevity of the samples[33]. A down-side of using this technique is that the sections are handled a lot, and thus are apt to damage. This, however, can be mitigated by using low shaking and rotating speeds as well as properly training researchers how to transfer the samples and mount the sections onto the slides.

Taken together, IHC is an established, essential tool for visualizing and localizing protein expression in both the clinical and biomedical research fields. Free-floating IHC is an efficient, flexible, as well as economic method, especially when performing large-scale histological studies. Here, we present a reliable free-floating fluorescent IHC protocol for the scientific community that can be adapted accordingly for chromogenic IHC and other stainings such as hematoxylin and eosin or cresyl violet staining.

PROTOCOL

1. Tissue preparation for cryosectioning

1.1. Embed fixed tissues in an appropriate embedding mold (see **Table of Materials**) to create a specimen block using an appropriate specimen matrix (see **Table of Materials**) and freeze on dry ice. Store specimen blocks at -80 °C until ready to section.

NOTE: Fixed tissues are typically prepared by perfusing adult (approx. 2.5 – 30 months old) male or female rodents (mouse or rat)[34], in accordance with available ethical permit, with an appropriate fixative (e.g. 10% formalin), followed by post-fixing tissues in the same fixative for 12 h at 4 °C, washing tissues three times with 1x PBS, and placing tissues in 15% and then 30% sucrose in 1x PBS for overnight or until tissues sink[35]. Researchers may try to adapt this general protocol to different development stages.

2. Cryosectioning

2.1. When ready to section, acclimatize samples in the cryostat for at least 1-2 h prior to sectioning to prevent shattering of tissue.

2.2. Using a cryostat, cut tissue into sections (20-50 μm) and collect in 6 or 12-well inserts (see **Table of Materials**) filled with 1x PBS solution.

NOTE: Depending on the section thickness, how much tissue is to be collected, and the number of well inserts used, each well will contain a variable number of sections spanning from approximately 10 to 40 slices for well. For example, if an entire brain is sectioned at 40 μm,

approximately 18-24 sections will be collected in each well using 12-well inserts. Also, 20 µm sections can be somewhat challenging to handle, thus 40 µm is recommended for bulk staining (see Discussion).

3. Storing sections

3.1. Once collected, wash the sections with freshly prepared 1x PBS for 5 min. Repeat 3 times.

3.2. Transfer the sections into 2 mL microcentrifuge tubes filled with 1-1.5 mL of storage solution (for 250 mL, mix 70 g of sucrose, 75 mL of ethylene glycol, and bring to volume with 0.1 M phosphate buffer).

3.3. Store at -80 °C until ready for staining.

4. Staining Day I

4.1. Remove samples from freezer and equilibrate at room temperature (RT) for 10 - 20 min.

4.2. Pour sections into a well insert in a 6-well plate to separate storage solution from sections.

4.3. Move the well insert to another well containing approximately 6 mL of 1x TBS. Wash 3 times with 1x TBS for 5 min each on an orbital shaker using low speed at RT.

4.4. While sections are washing, prepare 7 mL (per sample) of a blocking- permeabilizing solution consisting of 1x TBS with 0.3% Triton X-100 and 3% normal serum (e.g. normal horse serum). Block sections for 30 min at room temperature on orbital shaker, using low speed.

NOTE: Blocking with sera prevents non-specific binding of antibodies to tissue or non-specific Fc-receptors – a serum matching the species of the species of the secondary antibody is recommended, but if not available, any normal serum from a species different from the primary antibody host animal can be used. The detergent Triton X-100 allows for better antibody penetration by permeabilizing the tissue.

4.5. Prepare 1 mL per sample of primary antibody solution consisting of selected primary antibody (diluted appropriately) in 1x TBS with 0.3% Triton X-100 and 1% normal serum (see Step 4.4). Transfer sections from well insert into a 2 mL microcentrifuge tube containing primary antibody solution to bind to the antigen(s) of interest.

NOTE: Multiple primary antibodies may be used (generated in different host species).

4.6. Place 2 mL microcentrifuge tube with sections on a rotating mixer using low speed (e.g., speed 7 rpm) and incubate overnight for 12-16 h at 4 °C.

5. Staining Day II

5.1. The following day, pour sections into a well insert in a 6-well plate to separate sections from primary antibody solution.

NOTE: Antibody solution can be collected and reused; add 0.02% (w/v) sodium azide to inhibit microbial growth.

5.2. Wash sections 3 times with 1x TBS at RT (30 s for the first 2 washes and 10 min for the final wash).

5.3. Prepare 1 mL per sample of secondary antibody solution consisting of appropriate secondary antibody (diluted accordingly) in 1x TBS with 0.3% Triton X-100 and 1% normal serum (shield solution from light).

NOTE: Indirect labelling with a conjugated secondary antibody amplifies the signal and allows for colorimetric or fluorescent visualization of the protein target.

5.4. Transfer sections into a 2 mL microcentrifuge tube containing secondary antibody solution. Incubate for 2 h at room temperature on orbital shaker using low speed (shield solution from light).

5.5. Pour sections into a well insert in a 6-well plate to separate sections from secondary antibody solution.

5.6. Continuing to shield samples from light, wash 2 times with 1x TBS for 30 s at RT. Then wash for 15 min in 1x TBS, add DAPI (1:10,000) if desired.

6. Mounting

6.1. Pour sections into a glass, rectangular histological chamber filled three-quarters with 1x TBS.

6.2. Submerge a glass slide into the 1x TBS and use a fine paintbrush to coax the sections towards the slide.

6.3. Gently tap the sections onto the slide, making sure there are no wrinkles or folds.

6.4. Repeat until all sections are mounted onto the slide(s).

NOTE: If an entire brain, for example, is sectioned at 40 μm, collected in 12 well inserts with one aliquot containing 18-24 sections, and then sections are typically mounted on 1-2 slides, but fewer sections can also be mounted per slide depending on the researcher's preference.

7. Coverslipping

7.1. After sections are dried onto the slide(s), about 10-15 minutes at RT or until sections look opaque (remember to shield slides from light), apply an appropriate aqueous mounting medium (hardening or non-hardening). Antifading is preferred if using a fluorescent conjugated secondary antibody.

NOTE: Fluorescence quality may be lesser when using a hardening mounting medium, but slides should last longer.

7.2. Using tweezers, place a coverslip on top of the medium. Cover with filter paper and press down firmly to remove excess mounting medium.

NOTE: If using a non-hardening mount, paint the edges of the coverslipped slide with clear nail polish to seal.

7.3. Image using an appropriate microscope. Store in a dark slide box at 4 °C.

NOTE: Sections can be imaged using a variety of microscopes, such as laser scanning confocal and inverted or upright widefield epifluorescent, at magnifications (e.g. 10x, 20x, 40x) based on researcher's needs.

REPRESENTATIVE RESULTS

The overall scheme of the using the free-floating method to perform a fluorescent immunohistochemical assay is illustrated in **Figure 1**. Representative example of fluorescent IHC using the free-floating method in mouse brain examining glial fibrillary acidic protein (GFAP) expression is shown in **Figure 2** at both lower and higher magnification to illustrate the overall quality of the staining. This approach is also appropriate for revealing low-expressing proteins, with an example from a GFP low-expressing transgenic mouse brain shown in **Figure 3**. The free-floating method can also be used in other histochemical staining protocols, such as cresyl violet, as shown in **Figure 4**, by following Steps 1 through 4.3 of the protocol and then mounting the sections as indicated in Step 6. Thereafter, sections can be processed using any staining that requires slide-mounted sections. When using this protocol for chromogenic IHC, follow the protocol from Steps 1 through 5.6 (do not add DAPI to the last wash), adjusting accordingly if using an amplification step (e.g., avidin-biotin complex). Replace the buffer with chromagen/substrate reagent, incubating 5-20 min until tissue turns the desired color. The reaction can be monitored by checking the tissue periodically with a low-power microscope. Terminate the reaction by moving the well insert with the sections to fresh buffer, washing them three times, at least 5 min each. Proceed with Step 6 to mount the sections onto glass slides, allowing the sections to dry on a slide warmer for at least 3-4 h. Dehydrate the slides with increasing ethanol concentrations (i.e., 70%, 90%, 95%, 99.5%, 2-5 min each) followed by xylene (5-10 min) and then coverslip with a hard-mounting medium (e.g., Entellan), allowing slides to dry at least 1-2 h in a ventilated area. If the background is too high, quench the endogenous peroxidase activity for 15 min at RT with 3% H_2O_2 in 1x TBS followed by three buffer washes, 15-20 min each, before blocking (Step 4.4). Several peripheral tissues are also amenable to using this

technique with no modifications of the protocol required, with an example of liver sections from a GFP-expressing mouse shown in **Figure 5**.

FIGURE AND TABLE LEGENDS

Figure 1. Flow chart of the free-floating fluorescent immunohistochemical assay. Dissect the organ of interest (preferably fixed tissue) and embed tissue in embedding molds (see **Table of Materials**) using a specimen matrix (see **Table of Materials**), and then freeze on dry ice and store at -80 °C. Section tissue using a cryostat at 20-50 μm and collect slices in well inserts (see table of materials) filled with 1x PBS. Using an orbital shaker on low speed, wash sections 3x for 5 min each in 1x PBS. At this point, store extra sections in storage buffer at -80 °C until needed. Wash remaining sections 3x for 5 min with 1x TBS. Block sections for 30 min at RT shaking at low speed. Prepare primary antibody solution and incubate sections overnight in microcentrifuge tube(s) at 4 °C (12-14 h). The following day, wash sections with 1x TBS 3x, first two washes for 30 s, with the third wash for 10 min. Incubate sections in secondary antibody solution in microcentrifuge tube(s) for 2h at RT, making sure to shield sections from light when possible from this step forward. Then wash 3x with 1x TBS, first two washes for 30 s, and third wash for 15 min. Add DAPI to last wash if desired and if not present in mounting medium. Pour sections into a chamber box, three-quarters full of 1x TBS, and use a paintbrush to adhere sections onto the slide(s). Allow slides (~10-15 min) to dry before coverslipping with mounting medium of choice. Image sections using an appropriate microscope.

Figure 2. Fluorescent immunohistochemistry using free-floating brain sections. Hippocampal brain regions examining GFAP expression in adult mouse are shown and were labeled using an anti-GFAP primary antibody raised in rabbit and an anti-rabbit Alexa568 secondary antibody raised in donkey. DAPI was used in the last wash to label nuclei. Tissue was sectioned at 40 μm using a cryostat. Images were taken at 10x (upper) and 40x (lower) magnification using a laser point scanning confocal microscope. 10x image scale bar = 400 μm. 40x image scale bar = 100 μm.

Figure 3. Fluorescent immunohistochemistry on lower-expressed proteins using the free-floating method. Hippocampal brain regions from a low expressing GFP transgenic adult mouse are shown. Neurons expressing GFP were labeled using an anti-GFP primary antibody raised in goat and an anti-goat Alexa488 secondary antibody raised in donkey. DAPI was used in the last wash to label nuclei. Tissue was sectioned at 40 μm using a cryostat. Images were taken at 40x magnification using a laser point scanning confocal microscope. Scale bar = 100 μm.

Figure 4. Cresyl violet staining using free-floating brain sections. Using a cryostat, 40 μm sections from adult mouse olfactory bulb to cerebellum were collected, washed, mounted onto slides, stained with cresyl violet, and coverslipped. Images were taken at 10x magnification using an inverted widefield microscope. Scale bar = 1 mm.

Figure 5. Fluorescent immunohistochemistry using free-floating liver sections. Sections of liver taken at 40 μm using a cryostat from a transgenic adult mouse expressing low levels of GFP are

shown. An anti-GFP primary antibody raised in goat with an anti-goat Alexa488 secondary antibody raised in donkey were used to label cells expressing GFP. DAPI was added to the last wash to label nuclei. Images were collected using a laser point scanning confocal microscope at 40x magnification. Scale bar = 100 μm.

DISCUSSION

Immunohistochemistry (IHC) is a versatile technique that has become crucial in identifying protein expression and localization within tissue sections. This assay is used throughout the scientific community to further understand characteristics of tissue across stages of normal function to disease-states. IHC is employed across a variety of fields from clinical diagnosis of diseases such as cancer to initial discoveries in preclinical research[10,36].

The technique most commonly used to perform IHC is the slide-mounted method in which the sections are immediately adhered to the slide after being sliced. Some advantages of using this technique is that researchers can handle very thin sections needed for protein colocalization studies and use little solution to stain the sections per slide. Antibodies are often expensive; therefore, this approach can be an economic option if few sections are to be processed. This is also the method of choice for researchers using fresh-frozen specimens because the handling of the tissue is minimal, thus the structural integrity of the tissue will be protected. Using the slide-mounted approach would also be appropriate if only a few sections are to be collected and immediately stained, as is the case in clinical pathology. On the other hand, there are some disadvantages, such as only the exposed side of the tissue is accessed during staining, thus limiting section thickness due to poor antibody penetration and effective washing. Another drawback is that once the tissue is sectioned and collected onto slides, IHC normally must be completed rather quickly with storage of unprocessed slides taking up much freezer space. Furthermore, when processing larger experiments (e.g., several brain regions with multiple, representative levels to be stained), this approach may actually use more reagents, be rather time consuming, and can often limit the number of slides to be processed per experiment.

Some limitations associated with the slide-mounted method can be overcome by the free-floating staining technique that has become an increasingly popular alternative when working with thicker sections. Although this method is not a novel approach, in our experience, it has been a reliable, reproducible, and flexible approach, especially for staining tissue samples in bulk, thus permitting the processing of larger-scale studies in an efficient manner. Researchers can also effectively run multiple, large IHC experiments at the same time with this method. Moreover, samples are stained in suspension, thus the solutions can penetrate the sections from all angles, particularly important for thicker sections, often leading to a higher quality stain (**Figure 2, Figure 3,** and **Figure 5**). Free-floating sections can be sliced anywhere from 20-50 μm in thickness[37], with thicker sections useful for researchers to see structures or cells in different plains of view. For example, in brain tissue, thicker sections allow researchers to see the structure of dendrites and axons throughout their samples. The ability to collect thinner slices (20 μm) broadens even more the spectrum of applications; however, thinner slices can be difficult to handle and often require more time and effort to minimize damage, thus sections should not be thinner than 40 μm for bulk staining. One key benefit of the free-floating method is that

researchers can quickly section entire brains (or other tissue), collecting all sections in small tubes with each aliquot having a representative slice for all different brain regions, thus allowing researchers to quickly stain the entire brain. Tubes containing slices can be stored in cryoprotectant at -80 °C for several years[33], with aliquots not taking up much freezer space, effectively allowing researchers to generate a "tissue library". This method also reduces the amount of wasted materials, including slides, coverslips, and especially antibodies, which can easily be recovered and preserved for reusage, as well as precious animal tissues since sections can be stored and saved for as long as users choose.

The free-floating approach and the protocol presented here also gives researchers the option to easily modify the protocol or repurpose resources. For example, the collected sections can be used for many different histochemical stains in addition to immunofluorescence with simple protocol modifications, such as chromogenic IHC, hematoxylin and eosin (H&E), cresyl violet (**Figure 4**), and RNAscope[38]. Chromogenic IHC allows the visualization of antigen expression when a soluble substrate is converted by an enzyme conjugated to a secondary antibody to an insoluble chromogenic product. The two enzymes most commonly used are the horseradish peroxidase (HRP), which converts the 3,3' diaminobenzidine (DAB) to a dark brown end-product, and the alkaline phosphatase (AP), which converts the 3-amino-9-ethylcarbazole (AEC) substrate to a red product[39]. We routinely perform cresyl violet staining and use free-floating sections in order to examine gross brain organization and morphology[40]. We have also successfully applied this protocol to many different tissues, including brain, liver, heart, kidney, and spleen (**Figure 5**). Other researchers have also successfully used this technique for peripheral tissues including liver, kidney, and ovary[22–24].

A major concern when using free-floating technique is the potential for structural damage to the tissue sections, especially to brain slices, because throughout the protocol, samples are on shakers and rotators during almost every step to ensure that they are evenly washed, blocked, and stained. Occasionally, specific brain regions can become detached, especially at the cerebellar levels; however, using a brain atlas and a form of magnification, such as a jeweler's lamp, during the mounting process can be helpful for piecing together sections. This challenge can usually be prevented through gentle handling of the samples and by keeping rotating machines on the correct, low setting.

In conclusion, we present an established free-floating IHC technique that has proven to be an indispensable, dependable, flexible, and efficient modality that we regularly use to visualize protein expression and localization as well as tissue structure in a variety of tissues. The protocol herein can easily be modified to fit individual research needs making it valuable for the scientific community.

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DISCLOSURES

Nothing to disclose

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Figure 1 revised

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Fig 1

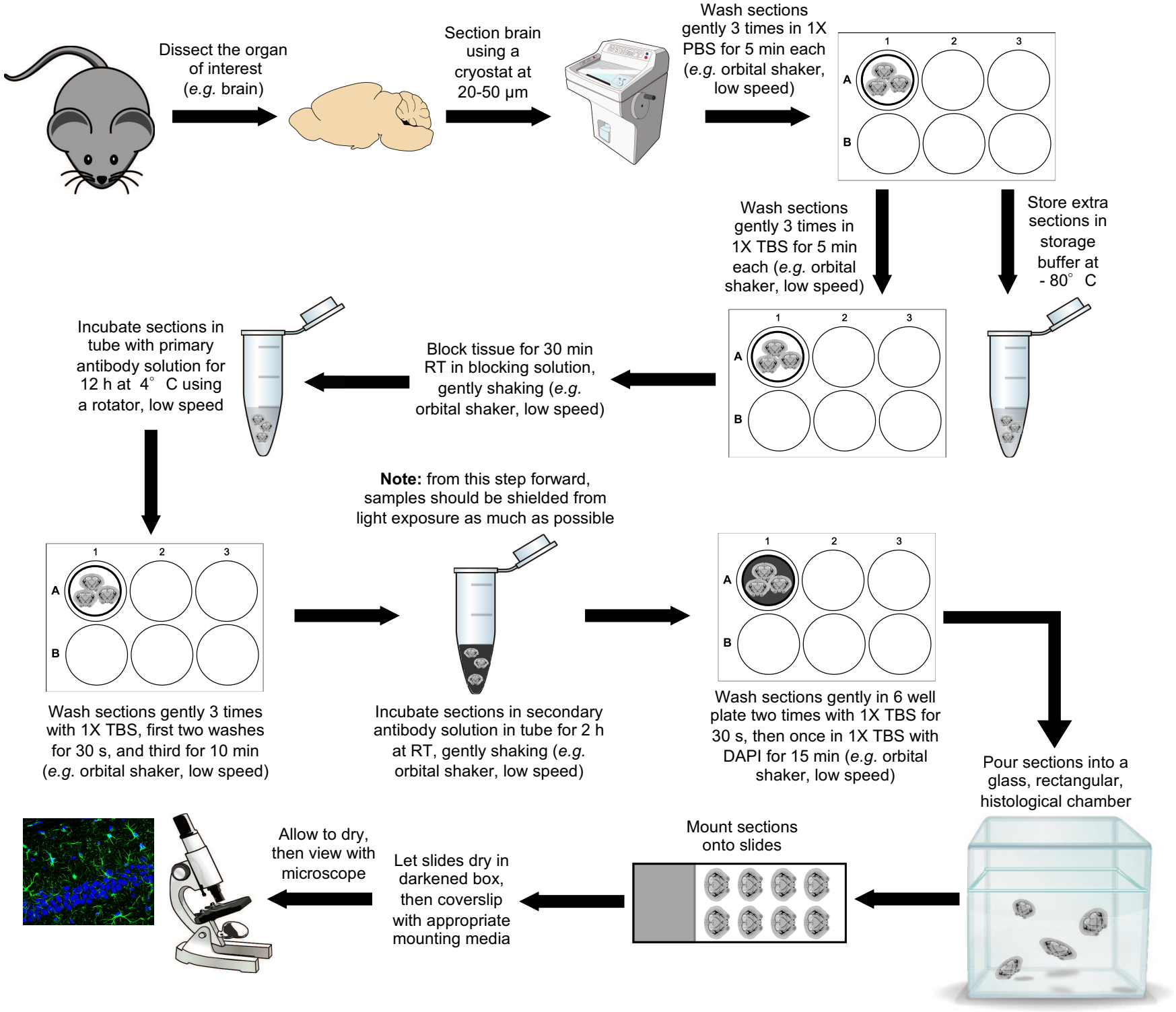


Fig 2

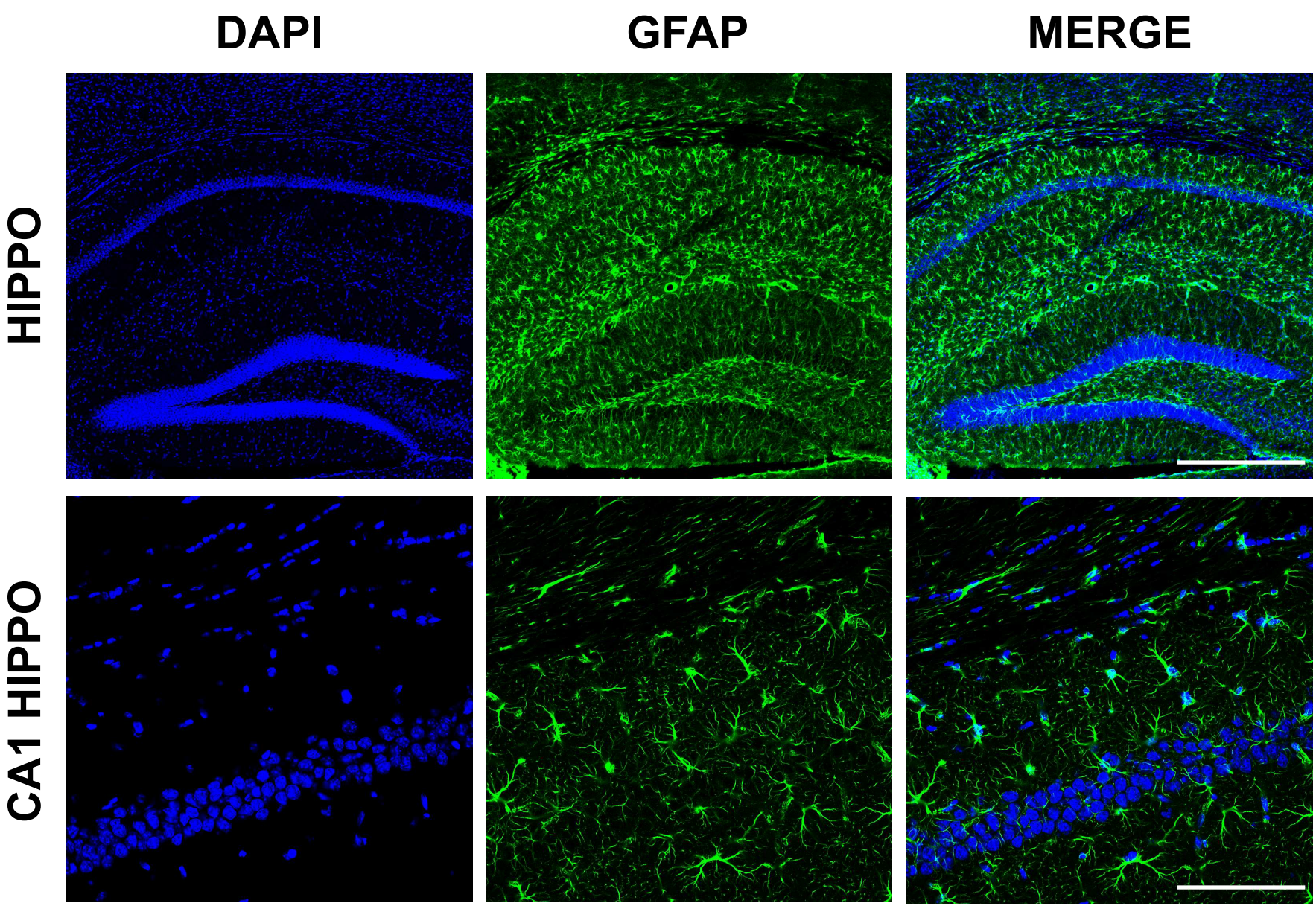


Fig 3

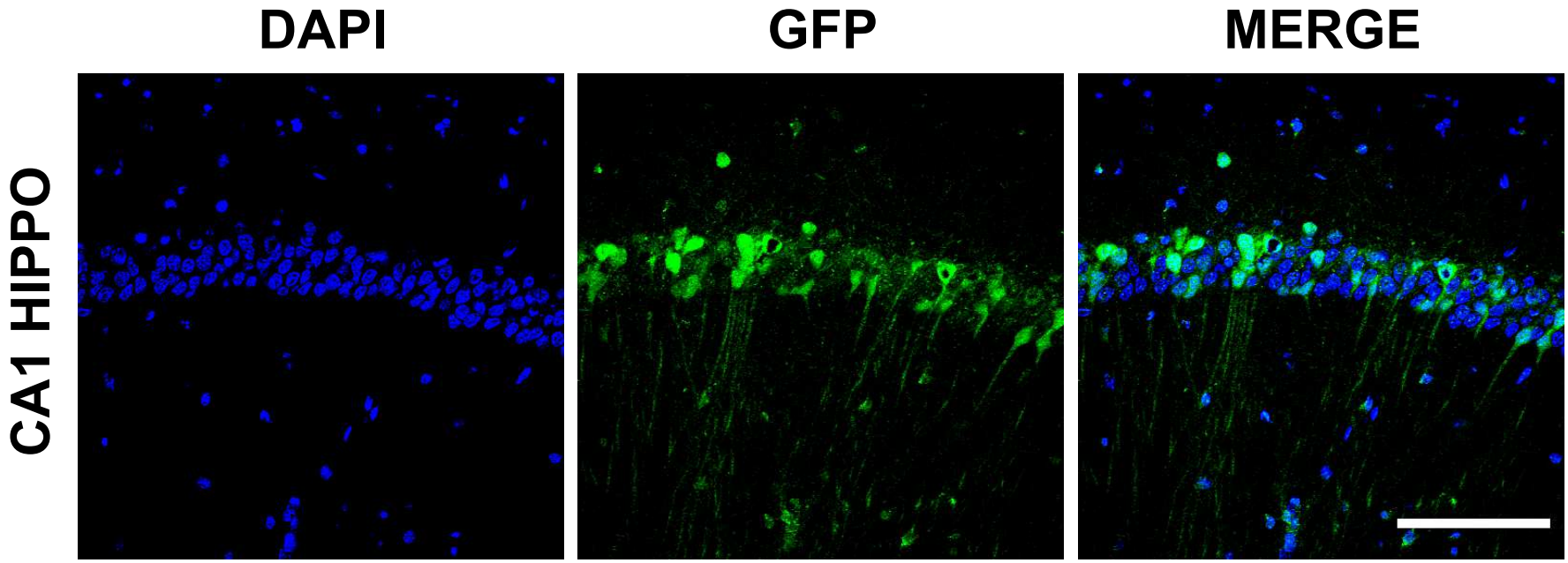


Fig 4

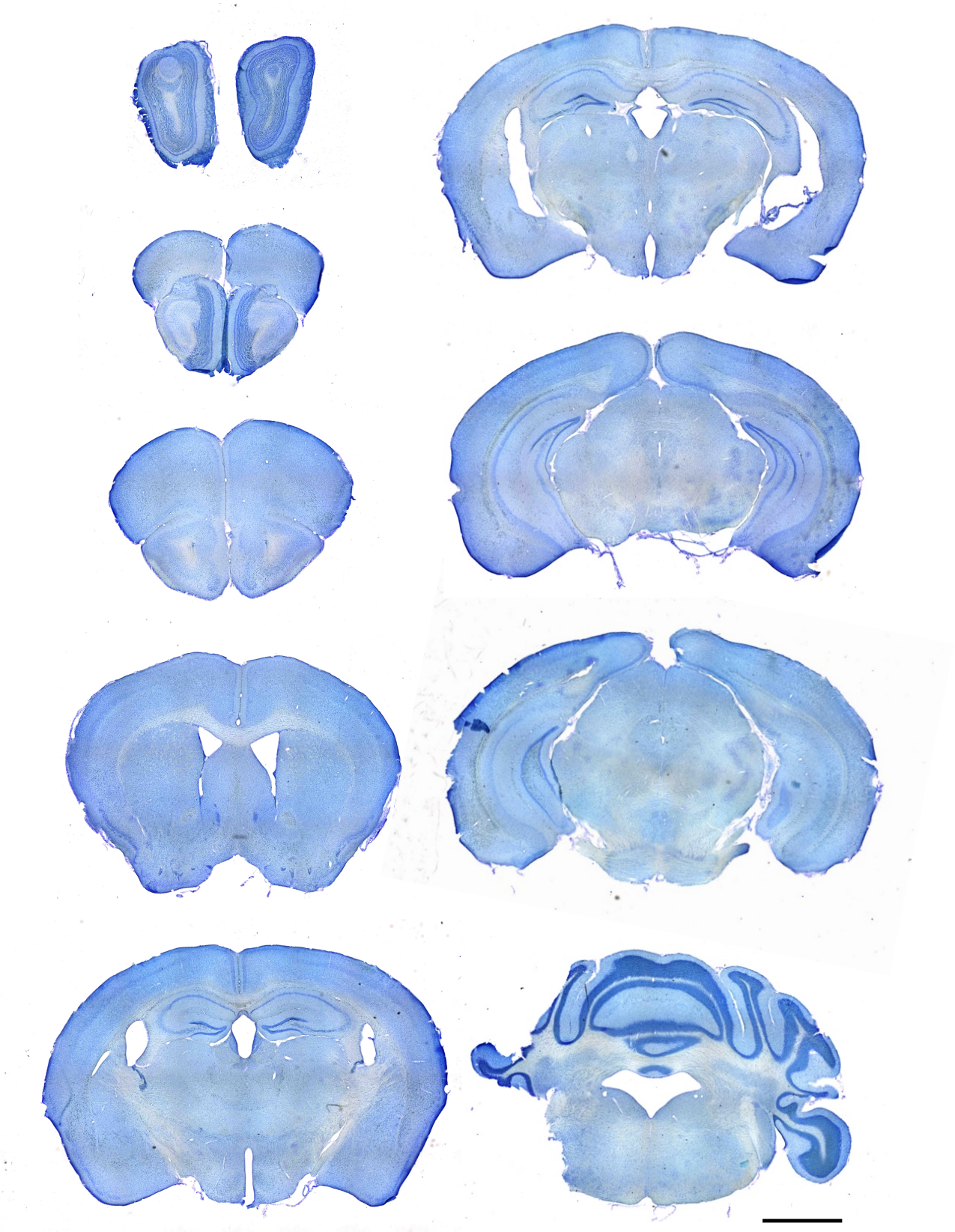
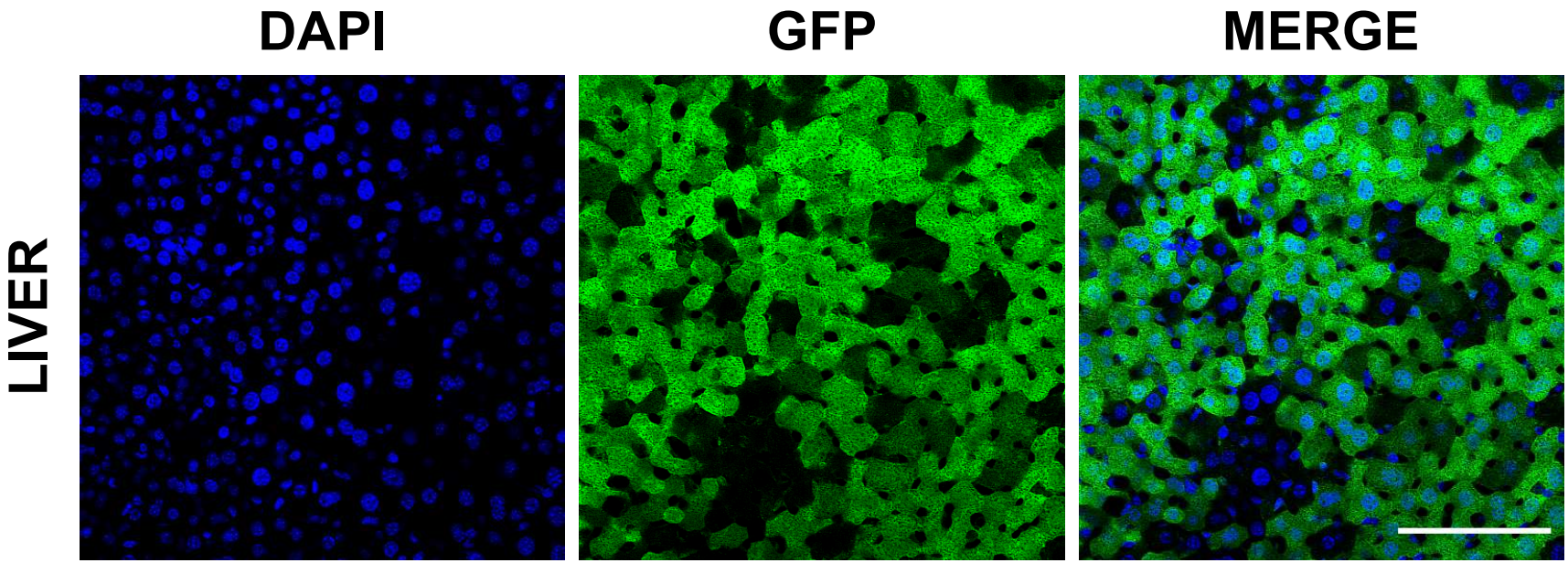


Fig 5



Name of Material/Equipment	Company	Catalog Number	Comments/Description
12-well plates	Corning	3513	
6-well plates	Corning	3516	
Clear nail polish	User preference	N/A	
DAPI	Sigma-Aldrich	D9542	
Embedding molds	Thermo Scientific	1841	
Ethylene glycol	User preference	N/A	
Formalin solution	Fisher Scientific	SF98-4	
Horse serum, heat inactivated	Gibco	26050088	
Microscope slide boxes	Electron Microscopy Services	71370	
PBS	User preference	N/A	
Primary antibody	User preference	N/A	
Rectangular Coverslips	VWR	48393-081	24 x 50 mm
Rectangular staining dish	Electron Microscopy Services	70312	
Round artist paintbrush #2	Princeton Select Series	3750R	Brand not important
Secondary antibody	User preference	N/A	
Specimen matrix for embedding	OCT Tissue-Tek, Sakura	4583	
Stain tray – slide staining system	Electron Microscopy Services	71396-B	Use dark lid
Sucrose	User preference	N/A	
Superfrost Plus Micro Slides	VWR	48311-703	
TBS	User preference	N/A	
Triton X-100	Sigma-Aldrich	X100	
Vectashield antifade mounting medium	Vector Laboratories	H-1000	Non-hardening
Well inserts for 12-well plates	Corning Netwells	3477	
Well inserts for 6-well plates	Corning Netwells	3479	
Whatman filter paper	Millapore-Sigma	WHA1440042	

THE UNIVERSITY OF RHODE ISLAND
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Kingston, July 1, 2020

Alisha DSouza, Ph.D.
Senior Review Editor
Journal of Visualized Experiments
1 Alewife Center, Suite 200
Cambridge, MA 02140

Response to Reviewers and Revisions

Dear Dr. DSouza,

We thank you for providing us with expert referees and we appreciate their thoughtful and constructive comments, which has helped us to improve the manuscript. We have made every effort to address the reviewers' concerns. textual revisions in the manuscript file are indicated in red color. Below are our detailed responses to the referees' and editorial comments.

Sincerely,

Jaime M. Ross, Ph.D.
Assistant Professor
George & Anne Ryan Institute for Neuroscience
College of Pharmacy, Department of Biomedical and Pharmaceutical Sciences

Reviewer 1

– We thank the referee for the careful and insightful review of our manuscript. We have addressed the reviewer's concerns and modified our manuscript accordingly.

1) "Manuscript Summary: In this method manuscript, the authors try to emphasize the advantages of using free-floating over slide-mounted sections when performing immunofluorescence (IF) protocols. In my opinion though, the information here provided is not enough to appreciate to which extent this asset is better than any other alternative already in use by the scientific community since long time, as also explained in the I introduction by the authors. In multiple occasions through the manuscript, the authors list positive and negative aspects of both their new technique and other alternatives, but I had the impression those comments are sometimes a little bit stretched, and not exactly representing

reality. Furthermore, within the protocol part, some details are missing, making the protocol itself vague and potentially non-reproducible. And finally, being this protocol proposed as an alternative to others, I would have expected the authors to clearly shown those comparisons in the figures, whereas they opted for only showing the results obtained via their protocol. Overall, I think that the manuscript is not considerable for publication as it is presented here, but it can nevertheless be improved with some modification and addition."

– We understand the concerns of the referee and changed the text in order to present the free-floating technique not as a better way to perform IHC but as an alternative to the slide-mounted technique that presents advantages when the sections are thick and many slices are to be processed simultaneously.

2) "Concerns: ABSTRACT: 1. In the abstract, the authors state that the free-floating approach utilizes fewer resources (than the slice-mounted counterpart) and further discuss this point in the introduction (p.4) and in the discussion (p10), stating how lower concentrated antibodies can be used in free-floating based IF compared to mounted slices. I do not really agree, since even if floating sections ease the penetration of the antibodies, this does not really justify a drastically reduction of antibody concentration. In addition, the volumes needed for processing slices is far bigger in floating than in mounted sections based IF."

– We have changed the text throughout the manuscript to better explain our point. Since we usually process up to 40 slices in a microcentrifuge tube with 1mL of antibody using the free-floating method, this is a much smaller volume than the amount that would be required to completely cover the same number of sections if mounted on a slide. Moreover, after incubation the antibodies can be easily recovered, as compared to incubation done on mounted sections, and can be preserved with 0.02 % (w/v) sodium azide and reused multiple times. An additional savings comes from the fact that during the mounting the slices can be attached and detached until the desire location on the slide is achieved, allowing the researcher to mount up to 12-16 sections per slides, thus decreasing the number of slides and coverslips that are utilized.

3) INTRODUCTION: 2. The authors proceed in listing the disadvantages of using slide-mounted sections. Although some of them are correct, some others are overstated. The limited antibody penetration for example can be solved by decreasing the slice thickness; washes can be efficiently performed by immerging slides in PBS-Tween or PBS-TritonX in slides' jars and let them oscillate for 5 minutes at RT and repeat up to 5 times (but 3 is normally enough) and finally, unprocessed slides can be stored vertically, even paired back-to-back, in plastic slides' boxes, hence not occupying so much storage space as the authors suggest. Concerning the fact that staining has to be performed relatively quickly, I do not really see the issue. Adherent-sections-based methods have been and still are successfully used by many in the field, suggesting they retain great reliability, and in some cases cannot be substituted by thick-floating-sections methods (i.e. colocalization experiments, complementary staining on adjacent sections etc.). I suggest the authors should focus more on the advantages their new method will bring in specific contexts, more than devaluating alternative methods. Overall, it is important for the authors to highlight the advantages of their method, but this does not necessarily imply that any other pre-existing method is ineffective and hence needs to be substituted.

– We have changed the text accordingly and presented the advantages and disadvantages of both methodologies. One advantage that we have now discussed in the manuscript is that when free-floating slices are to be collected the time required to section the entire brain is much less. In addition, the way of collecting slices allows researchers to have almost all brain regions present within one aliquot thus making it quick to stain an entire brain.

4) "PROTOCOL 3. Step 1.1, the authors should state the developmental stage and if different ages are used (i.e. perinatal, pups, p21 and adults), indicate the relative adaptations (if any)"
– We indicated the developmental stage of the animal in the protocol.

5) "4 Step 2.2, is it compatible with thinner sections? One of the advantages of cryostat vs vibratome cutting is the possibility to slice very thin sections. Although 20um is already thinner than the minimum obtainable by vibratome sectioning, is still pretty thick, I wonder if the method is compatible with thinner sections, this would broaden even more the spectrum of applications."
– In our experience 20 μm is the minimum thickness for sections to be handled. Thinner sections would be very difficult to be handled. This has been added to the manuscript.

6) "5. Steps 3.2, 4.5 and 5.4 the authors say they place the sections in 2 ml Eppendorf tubes. The number of sections per each tube is missing."
– We specified in the text that the number of sections per tube depends on the way the sections are collected. Typically, when we prepare a library for each brain, we collect series of 12 which translates to about 18 to 24 sections per tube. In our experience, a tube can easily accommodate up to 40 slices.

7) "6. Step 4.1, time span indication is missing."
– We have specified the time in the protocol to acclimate the sections (about 10-20 min).

8) "7. Step 7.1, time span indication is missing."
– We specified the time in the text (about 10-15 min or until sections looks opaque).

9) "REPRESENTATIVE RESULTS 8. Processed instead of processing and I believe the liver figure is the number 5 and not 4."
– We corrected the errors. Thank you.

10) "FIGURE AND TABLE LEGENDS 9. "Freeze on dry ice and store at -80°C""
– Corrected. Thank you.

11) "10. FIGURE1: differently from the main text, in the figure the secondary antibody step is depicted as performed in plates instead of tubes. Make sure the information is correct and consisting throughout the manuscript."
– We changed Figure 1 accordingly to depict the secondary antibody incubation being performed in tubes.

12) "11. FIGURE 2-5: In the f. legends, thickness of the sections is not stated. Furthermore, although the images quality is high, it would be much more informative to see these results compared to the same staining performed on slice-mounted sections. And finally, an overview image of the entire section would be useful to better evaluate the overall quality of the staining, such as homogeneity of the signal and noise levels."
– The thickness of all the sections shown in the relevant figures is 40 μm, which is mentioned in the figure legends. We added new images to Figure 2 (upper panel), which show immunofluorescent IHC using free-floating at a lower magnification in order to better convey the overall quality of the staining.

13) "DISCUSSION 12. After ref.39, remove "Replace with"."
– We corrected the error.

Reviewer 2
– We thank the referee for the careful and insightful review of our manuscript. We have addressed the reviewer's concerns and modified our manuscript accordingly.

1) "Manuscript Summary: In principle, this is a nicely written protocol describing the use of immunofluorescence in free-floating sections. However, there is nothing novel about it. Therefore, it should be stated somewhere in the manuscript that it describes a very well-established technique already used by numerous researchers worldwide. Also, before the manuscript is acceptable major errors/misconceptions with regard to IHC and NHS need to be corrected."
– We have added additional text to communicate that the free-floating technique is not novel as well as to illustrate when it should be used over the adhered slides and vice versa.

2) "Major Concerns: The authors claim at several locations that they use IHC, but then at the same location they go on describing how they use a secondary antibody coupled to a fluorophore. This is a protocol for free-floating immunofluorescence NOT for free-floating immunohistochemistry. The description of the protocol must be made clearer so that the reader is not confused. In the same vein, it is stated in the last sentence of the 1st paragraph of the Introduction "...is immunohistochemistry (IHC) is utilized, which is the immunostaining method that we will focus on throughout this paper", and in the first paragraph of the Discussion they also refer to IHC, although their protocol itself describes immunofluorescence. P.S.: At the core of IHC is the "histochemical" enzymatic detection, where a chromogen is altered by an enzyme coupled to an antibody through an enzymatic reaction, hence "immune+histochemistry". The authors even included this point in their current Discussion."
– We agree with the reviewer that a little confusion has being made on the use of IHC terminology. According to definition, "Immunohistochemistry is a laboratory method that uses antibodies to check for certain antigens (markers) in a sample of tissue. The antibodies are usually linked to an enzyme or a fluorescent dye." (https://www.cancer.gov/publications/dictionaries/cancer-terms/def/immunohistochemistry). The definition does not indicate if the secondary antibody is conjugated to an enzyme or fluorochrome. IHC and ICC by themselves do not specify the labeling method, thus it can help to combine them with the detection technique to clearly delineate the sample type and labeling method, i.e. ICC/IF, fluorescent IHC. With time IHC has been used incorrectly to refer to chromogenic immunohistochemistry. We have reviewed the text in the manuscript accordingly in order to be consistent with the terminology used.

3) "Since the major goal of this manuscript is to provide a general protocol for the use of free-floating sections, the authors should leave their current description of immunofluorescence. In addition, they should refer in the protocol itself to alternative detection methods based on IHC in free-floating sections based on enzymatic detection, e.g., with alkaline phosphatase or peroxidase in conjunction with appropriate substrates and chromogens and with non-aqueous mounting media."
– We have adjusted the text accordingly to communicate that the protocol presented is for fluorescent IHC and that it can be adapted for different IHC stainings.

4) "One cannot not use always normal horse serum, because researchers may also use a primary antibody generated in horse. Instead, the authors should state that normal serum should be use and mention NHS as an example."

– We modified the text accordingly.

5) "In our experience handling of 20µm thick free-floating sections is extremely difficult and often damages of the sections. When bulk staining is aimed for, but many sections become damaged, this defeats the purpose. For this reason, it is not advisable to use brain sections thinner than 40µm for bulk staining. Or if individual 20µm thick sections are stained, then more time and effort needs to be invested in careful handling of sections. Also, a 20µm thick section is rarely thick enough to follow dendrites and axons. These points have to be added to the Discussion."
– We included these points to the Discussion.

6) "Minor Concerns: In sentence below (in Discussion) delete "Replace with" "……organization and morphology39. Replace with Our lab has also successfully applied this protocol to many different tissues, including brain, liver, heart, kidney,….."; Correct sentence below (in Discussion) to: "…., such as a jewelers lamp, during the mounting process can be helpful for piecing together sections…."""
– We have corrected all these concerns. Thank you.

Editorial Comments
– Thank you for the suggestions and comments on our manuscript. We have addressed the concerns and modified the text accordingly.

*1) "**Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol."*
"1) Please include an ethics statement before your numbered protocol steps indicating that the protocol follows the animal care guidelines of your institution."
– The focus of this manuscript is on how to perform histological-based staining using free-floating sections and not on how rodent perfusion is performed. An ethical permit is not needed to perform free-floating immunohistochemistry. Nevertheless, we have added a short statement to Step 1.1.
"2) 1.1: mention strain, sex, age. How is the perfusion performed?"
– Since the focus of this manuscript is on the performing histological-based staining using free-floating sections, we will not be showing how rodent perfusion is performed. We have adjusted Step 1 in the protocol accordingly to reflect this change, added age, sex, species information, and refer to a suitable reference for how to perform prefusion in adult rodents (Gage, G. J., Kipke, D. R., Shain, W. Whole Animal Perfusion Fixation for Rodents. *J. Vis. Exp.* (65), e3564, doi:10.3791/3564 (2012)).
"3) 7.4: mention magnification and other settings."
– The choice of microscope and magnification varies greatly on the tissue used (e.g. brain versus liver) and the structures or proteins that are being imaged (e.g. mitochondria versus cytoplasmic expression). Examples of the type of microscope and magnifications often used are included but are researcher dependent.

*2) "**Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol."*

– We have taken care to ensure that the Discussion text includes all these topics.

*3) "**Commercial Language:**JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are cryomold, netwell 1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names."*

– We have removed commercial sounding language (i.e. cryomold, netwell, OCT) and adjusted the text accordingly. Thank you.

4) "If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation].""

– All the figures are original and have not been previously published.