Journal of Visualized Experiments

Detecting Amyloid-β accumulation via Immunofluorescent Staining in a Mouse Model of Alzheimer's Disease

--Manuscript Draft--

Article Type:	Invited Methods Collection - Author Produced Video
Manuscript Number:	JoVE62254R1
Full Title:	Detecting Amyloid-β accumulation via Immunofluorescent Staining in a Mouse Model of Alzheimer's Disease
Corresponding Author:	Chao Liu Xuzhou Medical University Xuzhou, Jiangsu CHINA
Corresponding Author's Institution:	Xuzhou Medical University
Corresponding Author E-Mail:	flowingsands@hotmail.com
Order of Authors:	Zijian Song
	Miao Zheng
	Jiahui Ding
	Yidi Xu
	Miao-Jin Ji
	Chao Liu
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$1200)
Please specify the section of the submitted manuscript.	Neuroscience
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please provide any comments to the journal here.	Author Produced Video
Please indicate whether this article will be Standard Access or Open Access.	Standard Access ($1400)

TITLE:

Detecting Amyloid-β accumulation via Immunofluorescent Staining in a Mouse Model of Alzheimer's Disease

AUTHORS AND AFFILIATIONS:

Zijian Song[1*], Miao Zheng[1*], Jiahui Ding[1], Yidi Xu[1], Miao-Jin Ji[1], Chao Liu[1]

[1]Jiangsu Province Key Laboratory of Anesthesiology and Jiangsu Province Key Laboratory of Anesthesia and Analgesia Application Technology, School of Anesthesiology, Xuzhou Medical University, Xuzhou, China

*These authors contribute equally.

Correspondence to: Chaoliu@xzhmu.edu.cn

KEYWORDS:

Neuroscience, Alzheimer's disease (AD), Amyloid-β (Aβ) plaque, 5×FAD transgenic mouse, immunofluorescent staining, 6E10 anti-Aβ antibody

SUMMARY:

In the neuropathology of Alzheimer's disease, one of the most crucial characteristics is the deposition of amyloid-β. In this protocol, we describe the method of immunofluorescent staining in 5×FAD transgenic mouse to detect amyloid-β accumulation in plaques. The process of perfusion, cryosectioning, staining and quantification will be described in detail.

ABSTRACT:

Alzheimer's disease (AD) is a neurodegenerative disease that contributes to 60-70% dementia around the world. One of the hallmarks of AD undoubtedly lies on accumulation of amyloid-β (Aβ) in the brain. Aβ is produced from the proteolytic cleavage of the beta-amyloid precursor protein (APP) by β-secretase and γ-secretase. In pathological circumstances, the increased β-cleavage of APP leads to overproduction of Aβ, which aggregates into Aβ plaques. Since Aβ plaques are a characteristic of AD pathology, detecting the amount of Aβ is very important in AD research. In this protocol, we introduce the immunofluorescent staining method to visualize Aβ deposition. The mouse model used in our experiments is 5×FAD, which carries five mutations found in human familial AD. The neuropathological and behavioral deficits of 5xFAD mice are well-documented, which makes it a good animal model to study Aβ pathology. We will introduce the procedure including transcardial perfusion, cryosectioning, immunofluorescent staining and quantification to detect Aβ accumulation in 5×FAD mice. With this protocol, researchers can investigate Aβ pathology in an AD mouse model.

INTRODUCTION:

Alzheimer's disease (AD) is a neurodegenerative disease that causes 60%-70% dementia around the world and costs much social resources[1]. It is well-known that accumulation of amyloid-β (Aβ) is a pathological hallmark in Alzheimer's disease. Amyloid precursor protein (APP) is an

integral membrane protein that exists in many tissues. Aβ peptide, consisting of 36-42 amino acids[2], is produced by the subsequent cleavage of β- and γ-secretase in APP[3,4]. Changes in APP cleavage and mutations in *APP* gene lead to overproduction of Aβ. Aβ molecules can aggregate to form oligomers or fibrils, which are believed to be neurotoxic[5,6]. In previous studies, the accumulation of Aβ was demonstrated to be correlated with neuronal death in AD[7-9].

The 5×FAD (C57BL/6J) transgenic mice contain 3 mutations in *APP*, and 2 mutations in *PSEN1*. The accumulation of intracellular Aβ starts as early as 1.5 month of age. Extracellular accumulation of Aβ was found around 2 months, in the cortex and hippocampus. The accumulation increased rapidly with age[10]. The well-documented Aβ pathology makes it a good animal model for our protocol.

The goal of the described staining method is to visualize and quantify Aβ deposition in the brain of AD mice model. The procedure including transcardial paraformaldehyde perfusion, cryosectioning, immunofluorescent staining and quantification to detect Aβ accumulation in 5×FAD mice will be introduced. This protocol is a reliable and easy method to investigate Aβ pathology in AD mouse model.

PROTOCOL:

All experimental procedures were performed with the approval of the Institutional Animal Care and Use Committee of Xuzhou Medical University and in accordance with the guidelines of the Chinese governmental regulations for the care and use of laboratory animals.

1. Perfusion of Mice

NOTE: More details of the perfusion procedure can refer to the video from Wiliam Shain's lab[11].

1.1 Anesthetize 5×FAD (C57BL/6J) transgenic mice with 1% pentobarbital sodium (50 mg/kg body weight) by intraperitoneal injection[12,13]. Loss of response to hind paw-pinching indicates proper anesthetization. Do not start perfusion before the mouse is properly anesthetized.

1.2 Use four pins to fix the limbs on the polyfoam plank. Set the abdomen of the anesthetized mouse upwards, with its limbs adequately stretched. Use a pair of iris scissors to make an incision at the xiphoid process. Then the diaphragm will be exposed.

1.3 Use surgical scissors to make an incision on the diaphragm, and carefully continue the diaphragm incision to the upper bound of the rib cage. Cut the ribs and thoracic muscles, dissect the attached tissues to expose the heart.

1.4 Find the right atrium of the mouse. Cut open the right atrium using iris scissors.

1.5 Inject 20 mL of 37 °C PBS (0.01 M, pH 7.2-7.4) from the left ventricle of the heart to flush out blood. Then slowly inject 20 mL of 4% PFA of room temperature from the left ventricle to

fix the tissues. The injection rate of PBS and 4% PFA is around 5 mL/min. Fixation tremors should be observed within seconds.

CAUTION: 4% PFA can irritate eyes and airways, and may provoke allergic reactions. This step should be done in ventilated places, and the operator should wear safety goggles and face masks.

1.6 Use surgical scissors to cut off the head, and carefully remove the cranium use dissecting forceps and extract the brain. Then submerge the brain into 4 mL of 4% PFA in a 5 mL plastic tube for 12-24 h at 4 °C.

2. Embedding and Cryosectioning

2.1 After fixation in 4% PFA, transfer the brain into 4 mL of 15% sucrose in a 5 mL plastic tube at 4 °C. After 12-24 h, the brain should sink to the bottom.

2.2 Transfer the brain into 4 mL of 30% sucrose in a 5 mL plastic tube at 4 °C for 12-24 h. Then the brain is ready for embedding. The purpose of 15% and 30% sucrose stepwise soaking is to dehydrate the brain, which avoids the formation of ice crystals inside the cells during embedding and cryosectioning. The dehydrated brain can be stored at 4°C for one week.

2.3 Leave roughly 1 mL of sucrose with the brain in the tube. Then add the same volume optimal cutting temperature (OCT) compound into the tube and mix properly. This helps OCT compound to wrap the brain more sufficiently.

2.4 Mount a thick layer of OCT compound on the knob surface and freeze at -21 °C before cryosectioning. Cut the brain sagittally from the middle; use either half for sectioning in a cryostat.

2.5 After the OCT compound solidifies, mount the knob on the specimen head and trim it into a platform surface. Lay one half of the brain on the trimmed surface with the middle side downwards.

2.5.1 Put a tinfoil ring around the brain to avoid leakage of OCT compound, and then fill the ring with OCT compound until the brain is submerged. When OCT compound embedding solidifies, it is ready for sectioning.

2.6 Set the thickness of the section to 20 μm. Set the chamber temperature to -21 °C, and the specimen head temperature to -19 °C. Section the brain from the rostral end to the caudal end.

2.7 Brush some PBS on the glass slides. Pre-coat the glass slides in poly-lysine to prevent the sections from peeling off. Attach the section onto the slide. If the section is folded, use a soft brush to unfold the sections with PBS.

2.8 Dry the sections overnight at room temperature and store at -20 °C. Sections can be kept at -20 °C for up to one month before Aβ staining.

3. Aβ Staining Procedure

3.1 Warm up the sections in room temperature, dry the slide surface, and use a hydrophobic pen to draw a circle around the section for antibody incubation.

3.2 Soak the slide in PBS in a 30 mL plastic staining box to wash off OCT compound. Use at least 20 mL of PBS.

3.3 Add 100 µL of 1x antigen retrieval solution for frozen sections onto the slide. Incubate for 5 min at room temperature.

3.4 Wash the retrieval solution with at least 20 mL of PBS of room temperature for 3 times (5 minutes for each).

3.5 Add diluted primary antibody (6E10) on the sections and incubate at 4 °C for 16-24 h in a wet, dark box (primary antibody dilution solution: 1% BSA, 0.3% Triton X-100, 0.01% sodium azide in PBS, 1:500 dilution). BSA functions as blocking agent.

3.6 Wash the sections with PBS for 3 times, 5 min each time.

NOTE: Perform steps 3.7, 3.8 and 3.9 in a dark place.

3.7 Incubate sections in secondary antibody solution (Goat anti-Mouse IgG (H+L) Alexa Flour 594, diluted with PBS 1:200) for 1 h at room temperature in a wet, dark box.

3.8 Wash the secondary antibody off the sections with at least 20 mL of PBS for 3 times, 5 min each time.

NOTE: Do not pour PBS onto sections during washing to prevent sections peeling off. Keep the sections wet.

3.9 Absorb the liquid around sections and add a drip of mounting medium on each section. Seal the sections with cover glass, avoiding bubbles.

3.10 Observer the stained sections with a fluorescence microscope. Keep the imaging parameters consistent to avoid derivation. Keep the slides at 4°C in a dark place. Analyze the sections within one week, since the staining will fade with time.

4. Imaging and Quantification of Aβ accumulation by ImageJ

4.1 Capture images by a fluorescence microscope connected with a digital camera and imaging software. We used Image Pro Plus software and the imaging parameter was set as follows: **Exp Pvw:** 450 ms, **Exp Acq:** 450 ms; **Pvw:** 1 × 1, **Acq:** 1 × 1; **Pvw Resolution:** Width 1 × Height 1, **Acq Resolution:** Width 1 × Height 1; **Capture Depth:** 8-bit mono; **Gain: Pvw:** 13, **Acq:** 13, **Gamma: Pvw:** 1, **Acq:** 1, **Offse: Pvw:** -700, **Acq:** -700.

4.2 In ImageJ Fiji 2.0.0 (https://imagej.net/Fiji), select **Plugins** | **Stitching** | **MosaicJ** in the menu. Then select **Files** | **Open Image Sequence** to open the images to be stitched (**Figure 1A**).

4.3 All the selected images will be displayed on the bottom. After stitching the images manually, select **File** | **Create Mosaic** (**Figure 1B, C, D**).

4.4 Select **Image** | **Adjust** | **Brightness/Contrast …**, and adjust the brightness and contrast of the image (**Figure 3A**). Then the image is able to be saved (**Figure 1E**).

4.5 To quantify the accumulation of Aβ, open the image through **File** | **Open…** in the menu (**Figure 2A**).

4.6 Select **Image** | **Type** | **8-bit** to adjust the image to 8-bit. Then choose the area intended to be counted using **Polygon** selection (**Figure 2B**).

4.7 Select **Image** | **Adjust** | **Threshold** to select the proper threshold of signals. Threshold can be adjusted via dragging the scroll bars or change the numbers directly in the textbox (maximum: 255, minimum: 0). When all the Aβ signals in the section has become red, the threshold is appropriate for the measurement (**Figure 2C**). Make sure that **Dark Background** box is checked.

4.8 Selecting **Analyze** | **Set Measurement…** in the menu to choose the parameters that will show in the results. Confirm that **Integrated density** and **Limit to Threshold** is selected. **Integrated density** (total intensity) is the target of the measurement (**Figure 2D**).

4.9 Select **Analyze** | **Measure** to get the results. The results will be displayed and ready for statistical analysis (**Figure 2E**).

REPRESENTATIVE RESULTS:

We used the above-described immunofluorescent staining procedures to investigate the deposition of Aβ accumulation in 5×FAD mice of different age. **Figure 3** represents typical results and suboptimal results using our protocol. Brain slices of 5-month and 8-month heterozygous 5×FAD transgenic mice and 4 to 6-month wild-type control were stained with 6E10 antibody and detected under a fluorescence microscope. **Figure 3A** shows Aβ accumulation in plaques in the sagittal section of the mouse brain. They are mainly deposited in cortex and hippocampus, with the highest density in subiculum of hippocampus, which is consistent with previous study[10]. As the image shows, Aβ accumulation was clearly detected by the antibody in 5×FAD mice. The integrated density (integrated density = Area × Mean.

Integrated density: the sum of fluorescence intensity of this area; Mean: the average fluorescence intensity in this area; Area: the proportion of fluorescence in the image) of Aβ accumulation in 8m 5×FAD mice was significantly higher than that in 5m 5×FAD mice (unpaired students' t-test, $p = 0.0071$), which shows that the accumulation of Aβ will gradually increase with the age. In addition, no Aβ signal was observed in the wild-type control, which shows the specificity of 6E10 antibody. Some suboptimal results (an 8-month heterozygous 5×FAD transgenic mouse and an 8-month wild-type control) are also shown in **Figure 3C**. The deficits include slice breakage and curled edge. How to prevent this situation will be discussed. In general, immunofluorescent staining using 6E10 anti-Aβ antibody is a specific method that can easily quantify Aβ accumulation in 5×FAD mice.

FIGURE AND TABLE LEGENDS:

Figure 1. Stitching brain slice images by ImageJ. (A) In the menu of ImageJ/Fiji, Select **Plugins** | **Stitching** | **MosaicJ** to begin stitching. **(B)** Select **File** | **Open Image Sequence** and select the images to be stitched. Stitch the images manually according to the anatomical structures of the section. **(C)** After stitching, select **File** | **Create Mosaic** to create the whole image of the section. **(D)** An example of a stitched image. **(E)** Select **Image** | **Adjust** | **Brightness/Contrast…** to adjust brightness and contrast of the whole image. The middle panel shows the adjustment menu. Select **File** | **Save as…** to save the image into Tiff or other format.

Figure 2. Quantification method of Aβ fluorescence. (A) Set the image type as 8-bit through selecting **Image** | **Type** | **8-bit. (B)** Use the **Polygon** selection in the menu of ImageJ and depict the contour of the whole brain. **(C)** Select **Image** | **Adjust** | **Threshold…** to exclude the background noise. Make sure that **Dark background** box is checked. Drag the red lines or scroll bars until the Aβ fluorescence is properly highlighted. **(D)** Use **Analyze** | **Set Measurements** to choose what will be presented in the result table and set counting method. The menu of Set Measurements is shown. Confirm that **Integrated density** and **Limit to threshold** are selected. **(E)** Select **Analyze** | **Measure** to get the results. **Integrated density** (shown as IntDen) represents the total intensity of Aβ fluorescence (Integrated Density = Area × Mean).

Figure 3. Representative results of immunofluorescent staining of Aβ deposition in 5×FAD mice. (A) The immunostaining of Aβ deposition in the sagittal section of 5-month and 8-month 5×FAD transgenic mice and 4 to 6-month wild-type mice brains using anti-Aβ antibody 6E10. Images were captured by an Olympus IX-81 fluorescence microscope under a 4x or 20x objective. Upper panel, stitched whole brain images in lower magnification. Lower panel, cortex images in higher magnification. As the white arrows indicate, the florescence signals of Aβ deposition were observed clearly in 5×FAD groups, but no Aβ signal was observed in wild-type group. **(B)** The bar chart shows quantitative analysis of total intensity (Integrated Density = Area × Mean) of Aβ fluorescence in the brains of 5-month and 8-month 5×FAD transgenic mice. Unpaired students' *t*-test: **, $p < 0.01$ ($n = 3$ for each group). Error bars represent SD. **(C)** Cases of suboptimal slices in immunofluorescent staining, representing an 8-month 5×FAD transgenic mouse and an 8-month wild-type control. Left, section breakage; Right, curled edge of the section. Scale bar, 200 μm.

DISCUSSION:

Immunofluorescent staining using 6E10 antibody can specifically detect Aβ accumulation in the brain, which is easy to be quantified by Image J. What is worth noticing is that some crucial steps in this protocol may affect the results.

To prevent slices from peeling off or breakage shown in **Figure 3C**, a few key points should be noticed. Perfusion should proceed rapidly after making the incision on the diaphragm because of the irreversible pathophysiological effects caused by hypoxia. These effects may profoundly influence the results[11]. The brain tissue is required to be properly fixed and dehydrated before cryosectioning. Temperature should be properly set for the cryostat (chamber -21 °C, specimen head -19 °C), and the situation of the cutting blade and anti-roll plate should be checked. No dust or frost should be on the cutting blade or anti-roll plate, and they should be positioned correctly. The sections should also be flattened properly before attaching. In addition, slides should be pre-coated to prevent slices from peeling off during staining. Popular methods include poly-lysine or gelatin-coating. Poly-lysine coated microscope adhesion slides can be bought from companies.

Some steps in the staining process will also affect the results. Before incubation, antigen retrieval solution should be applied to the sections to enable full exposure of the antigen. In order to prevent evaporation of the primary antibody during incubation, the sections should be incubated in a wet box. In addition, the sections should be kept in dark after the secondary antibody incubation to avoid fluorescence quenching. Several staining methods have been developed for Aβ detection, such as Thioflavin-S staining, and Congo Red staining and Gallyas silver staining, but immunofluorescent staining using 6E10 antibody has unique advantages. Thioflavin-S and Congo Red can bind with all the β-sheets containing proteins, therefore, these chemical dyes are less specific for Aβ detection[14,15]. Compared with chemical staining, immunofluorescent staining is more specific. The 6E10 anti-Aβ monoclonal antibody is specifically reactive to amino acid residues 4-10 of Aβ, according to a previous high-resolution mapping[16]. Previous studies also indicated that immunostaining using 6E10 antibody can detect greater plaque deposition than Gallyas silver staining and Thioflavin-S staining, which indicates that Aβ detection using antibody might be more sensitive than chemical staining methods[17]. However, the critical limitation of 6E10 staining is that this antibody can still recognize full-length APP and other cleaved peptides containing 6E10 epitope, but the fluorescence signal outside Aβ plaques is very weak in 5xFAD mice. Previous studies showed that 6E10 antibody bound with Aβ peptides in both conformation- and sequence-dependent ways[16]. Despite the limitations, this protocol is still a practical and specific method that can help researchers investigate the Aβ pathology of AD.

ACKNOWLEDGMENTS:

This research was supported by a general project of Natural Science Foundation of China (grant number: 81974157) from the Natural Science Foundation of China, a Jiangsu Special Appointed Professorship (to C.L.) from Jiangsu Education Department, a starting grant of excellent talent (D2019025) and Innovation and Entrepreneurship Training Program (201910313038Z to Z.J.S., Y.D.X., M.Z. and J.H.D) from Xuzhou Medical University. This research was also supported by

National Demonstration Center for Experimental Basic Medical Science Education (Xuzhou Medical University).

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1 Burns, A., Iliffe, S. Alzheimer's disease. *BMJ.* **338** b158 (2009).

2 Leong, Y. Q., Ng, K. Y., Chye, S. M., Ling, A. P. K., Koh, R. Y. Mechanisms of action of amyloid-beta and its precursor protein in neuronal cell death. *Metabolic Brain Disease.* **35** (1), 11-30 (2020).

3 Tiwari, S., Atluri, V., Kaushik, A., Yndart, A., Nair, M. Alzheimer's disease: pathogenesis, diagnostics, and therapeutics. *International Journal of Nanomedicine.* **14**, 5541-5554 (2019).

4 Murphy, M. P., LeVine, H., 3rd. Alzheimer's disease and the amyloid-beta peptide. *Journal of Alzheimer's Disease.* **19** (1), 311-323 (2010).

5 Hardy, J. A., Higgins, G. A. Alzheimer's disease: the amyloid cascade hypothesis. *Science.* **256** (5054), 184-185 (1992).

6 Reitz, C. Alzheimer's disease and the amyloid cascade hypothesis: a critical review. *International Journal of Alzheimer's Disease.* **2012**, 369808 (2012).

7 Bozyczko-Coyne, D. *et al.* CEP-1347/KT-7515, an inhibitor of SAPK/JNK pathway activation, promotes survival and blocks multiple events associated with Abeta-induced cortical neuron apoptosis. *Journal of Neurochemistry.* **77** (3), 849-863 (2001).

8 Canevari, L., Abramov, A. Y., Duchen, M. R. Toxicity of amyloid beta peptide: tales of calcium, mitochondria, and oxidative stress. *Neurochemical Research.* **29** (3), 637-650 (2004).

9 Zhu, X. *et al.* Oxidative stress signalling in Alzheimer's disease. *Brain Research.* **1000** (1-2), 32-39 (2004).

10 Oakley, H. *et al.* Intraneuronal beta-amyloid aggregates, neurodegeneration, and neuron loss in transgenic mice with five familial Alzheimer's disease mutations: potential factors in amyloid plaque formation. *Journal of Neuroscience.* **26** (40), 10129-10140 (2006).

11 Gage, G. J., Kipke, D. R., Shain, W. Whole animal perfusion fixation for rodents. *Journal of Visualized Experiments.* 10.3791/3564 (65) (2012).

12 An, S. *et al.* Medial septum glutamatergic neurons control wakefulness through a septo-hypothalamic circuit. *Current Biology.* 10.1016/j.cub.2021.01.019 (2021).

13 Cao, J. L. *et al.* Activation of peripheral ephrinBs/EphBs signaling induces hyperalgesia through a MAPKs-mediated mechanism in mice. *Pain.* **139** (3), 617-631 (2008).

14 Ly, P. T., Cai, F., Song, W. Detection of neuritic plaques in Alzheimer's disease mouse model. *Journal of Visualized Experiments.* 10.3791/2831 (53) (2011).

15 Klunk, W. E., Jacob, R. F., Mason, R. P. Quantifying amyloid beta-peptide (Abeta) aggregation using the Congo red-Abeta (CR-abeta) spectrophotometric assay. *Analytical Biochemistry.* **266** (1), 66-76 (1999).

16 Hatami, A., Albay, R., 3rd, Monjazeb, S., Milton, S., Glabe, C. Monoclonal antibodies against Abeta42 fibrils distinguish multiple aggregation state polymorphisms in vitro and in Alzheimer disease brain. *Journal of Biological Chemistry.* **289** (46), 32131-32143 (2014).

17 Shi, X. Z., Wei, X., Sha, L. Z., Xu, Q. Comparison of beta-Amyloid Plaque Labeling

353 Methods: Antibody Staining, Gallyas Silver Staining, and Thioflavin-S Staining. *Chinese Medical*
354 *Sciences Journal.* **33** (3), 167-173 (2018).
355

Figure 1

Click here to access/download;Figure;Fig. 1.tif

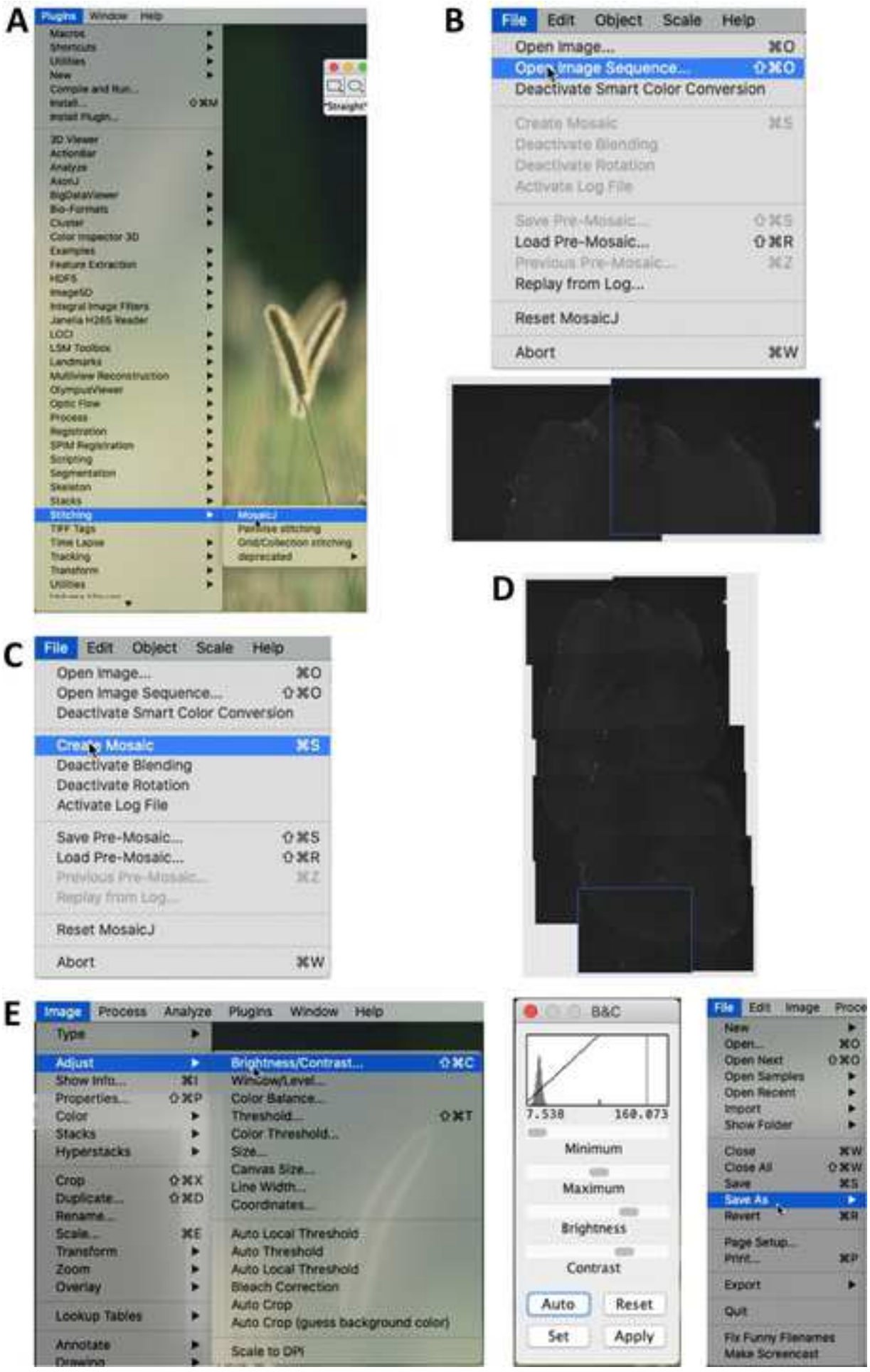


Figure 2

Click here to access/download;Figure;Fig. 2.tif

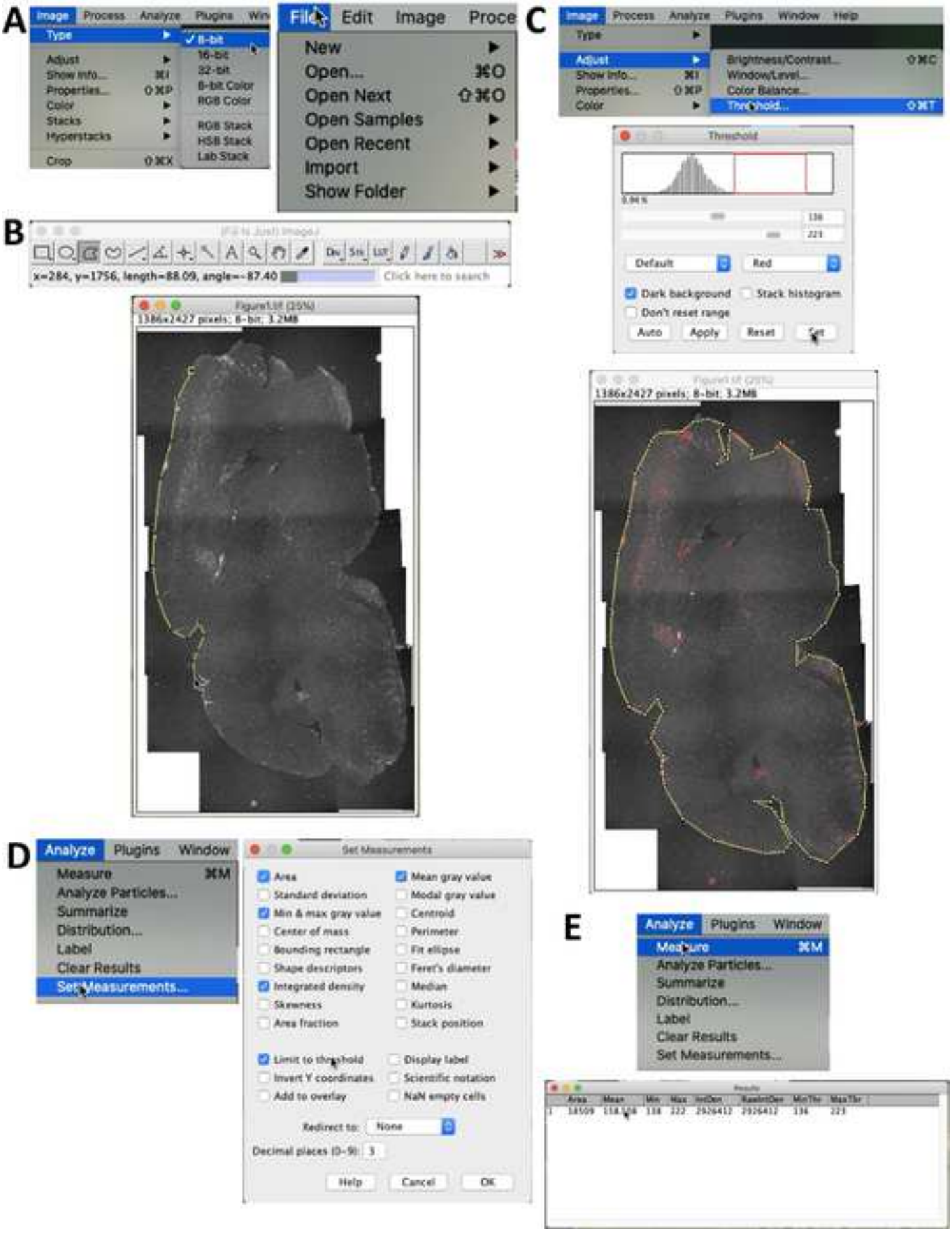
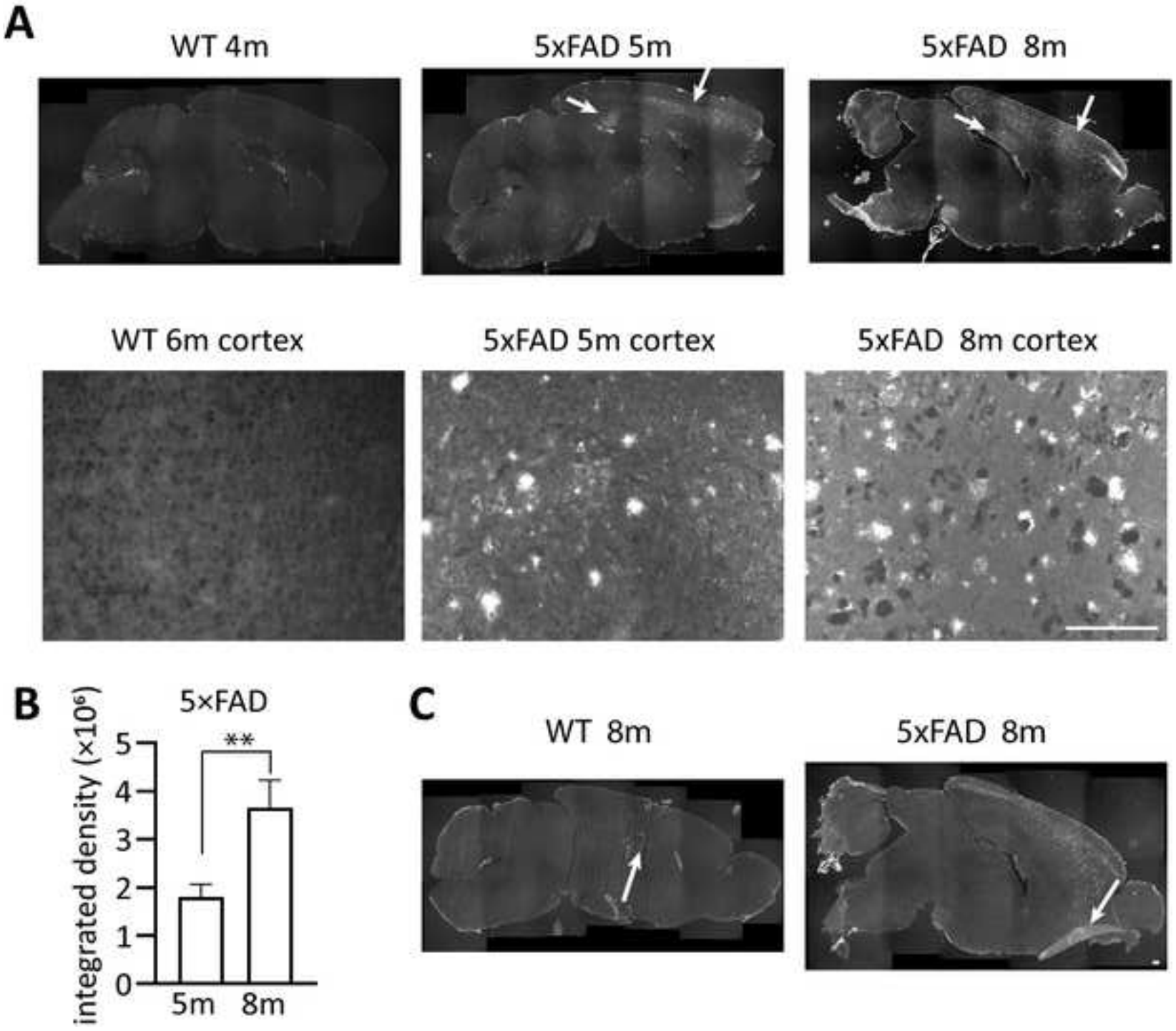


Figure 3



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Anesthetia			
Injection syringe	KLMEDICAL		1 mL
Pentobarbitual sodium	Sigma-Aldrich	P3761	1% in saline for i.p. injection
Thoracotomy:			
IRIS-Fine Straight iris scissors (~11.5 cm)	RWD	S12010-11	
Sharp curved surgical scissors(11.5 cm)	RWD	S14016-11	
Straight dissecting forceps (~10.5 cm)	RWD	F12010-10	
Perfusion			
Centrifugal tube (5 mL)	Biosharp		
Injection syringe	KLMEDICAL		20 mL
Paraformaldehyde	Vicmed	VIH100	
PBS 0.01M (PH7.2-7.4) powder	Vicmed	VC2001P	Add deionized water to make solution
Fixation, Dehydration and Cryosectioning			
Adhesion microscope slides	CITOTEST	188105	76.2 mm × 25.4 mm
Microtome Cryostat	LEICA	CM1950	
Optimal cutting temperature compound (OCT)	Sakura Finetek	4583	
Sucrose	VETEC	V900116	
Immunofluorescence staining			
Fluorescence microscope	OLYMPUS	IX-81	
Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 594	Thermo Fisher Scientific	A-11005	200X
Image-Pro Plus 7.0C	Media Cybernetics		Scientific graphing and data analysis software
Immunohistochemical Wet Box (black)	Sinylab	Customized	300 mm × 100 mm × 38 mm, groove depth 27 mm, can contain 10 standard slides
Pipet	Thermo Scientific		
Pipette tips	Well-offer		
Plastic staining box	Sinylab	Customized	30 mL, can contain 5 standard slides

Primary Antibody Dilution Buffer	made in our lab		1% BSA 1g, 0.3% Triton X-100 300ul, 0.01% sodium azide 10mg in 100ml PBS
Purified anti beta amyloid,1-16 antibody (6E10)	Biolegend	SIG-39320	500X
Quick Antigen Retrieval Solution for Frozen Sections	KeyGEN BioTECH	KGIHC005	5X
Quantification			
Graphpad Prism 8.0.1	Graphpad		Medical mapping software
Image J Fiji 2.0.0	National Institute of Health		Scientific graphing and data analysis software

Dear Editors and Reviewers,

Thank you for your comments on our manuscript entitled “Detecting Amyloid-β plaques via Immunofluorescent Staining in a Mouse Model of Alzheimer’s Disease” (ID: JoVE62254). We have read the comments carefully and made corrections accordingly. Revised portion are marked in red in the manuscript.

Response to the reviewer’s comments:

Changes to be made in the written manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: According to the reviewer's instruction, we have revised the whole manuscript carefully and tried to avoid any grammar or syntax error. In addition, we have invited several colleagues to help polish the manuscript. We hope that the language is acceptable now.

2. Is pentobarbital rather than phenobarbital used?

Response: We are sorry for the mistake. “pentobarbital”, rather than “phenobarbital”, was used to anesthetize the mice. We’ve corrected it in Protocol step 1.1. Thank you for the correction.

3. Please include an ethics statement before all of the numbered protocol steps indicating that the protocol follows the animal care guidelines of your institution.

Response: The ethics statement has been included before the numbered protocol:
All experimental procedures were performed with the approval of the Institutional Animal Care and Use Committee of Xuzhou Medical University and in accordance with the guidelines of the Chinese governmental regulations for the care and use of laboratory animals.

4. Please mention how proper anesthetization is confirmed.

Response: The way to confirm proper anesthetization has been included in Protocol step 1.1: Loss of response to hind paw-pinching indicates proper anesthetization.

5. Please specify the euthanasia method.

Response: Transcardial perfusion needs the circulatory system to deliver the perfusing solution. Therefore, the mouse was not euthanatized before perfusion. According to the ethics protocol, we provided the method of proper anesthetization of the mouse in Protocol step 1.1.

6. Please specify the surgical tools used throughout.

Response: The surgical tools, including iris scissors, surgical scissors and dissecting forceps, have been specified in Protocol step 1.2, 1.3, 1.6.

7. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?

Response: We've added more details to our protocol steps. For example, in Protocol 1.3, we supplemented some details in removing the rib cage: "Use surgical scissors to make an incision on the diaphragm, and carefully continue the diaphragm incision to the upper bound of the rib cage. Cut the ribs and thoracic muscles; dissect the tissues attached to make the heart exposed."

8. 1.4: How much 4% PFA is injected and at what rate?
Response: 20mL 4% PFA of room temperature was injected transcardially at the rate of 5mL/min. This has been supplemented in Protocol step 1.5.

9. 2.1: Fix the brain in 15% sucrose until the brain floats?
Response: The brains were soaked in 15% and 30% sucrose stepwise for dehydration. The brain will float in the sucrose at first because it still contains much water. After dehydration of 12-24 h, the brain will sink to the bottom. We've supplemented this in Protocol step 2.1.

10. 2.2: How long is the brain fixed in 30% sucrose?
Response: The brain should be dehydrated in 30% sucrose for 12-24 h, as we've supplemented in Protocol step 2.2.

11. How much PBS is used for the wash?
Response: The amount of PBS used in washing is 20 ml, as amended in Protocol step 3.2.

12. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
Response: We've already eliminated all the commercial language from the manuscript. Thank you for your correction.

Changes to be made in the written manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
Response: According to the reviewer's instruction, we have revised the manuscript carefully and tried to avoid any grammar or syntax error. In addition, we have invited several colleagues to help polish it. We hope that the manuscript is acceptable now.

2. On-screen Text:
• Main title card: Detecting Amyloid-β plaques via immunofluorescent staining in a Mouse Model of Alzheimer's Disease
Capitalize the "s" in "staining" (Staining)
• For the introduction and conclusion, consider adding some name cards for the

speaker
Response: We've corrected the title and added name cards for the speakers in the video.

3. Overall, the pacing is fast. Optionally, you may want to edit this video so that some of the pacing is spread out a little bit. Sometimes, the video feels like it is moving too quickly.
Response: We've remade the video to make the pace slower. We hope that the pace is acceptable now.

4. The text states that phenobarbital at 40 mg/kg of body weight is used for anesthesia. Did the authors mean pentobarbital? Pentobarbital is not ideal and is not used for general anesthesia in the US. Could the authors provide a reference for the use of this drug and the dose? The authors should also state that reflexes are checked prior to the thoracotomy.
Response: We are sorry for the mistake. We used pentobarbital sodium in our protocol, and we've corrected this in both manuscript (Protocol step 1.1) and the video. Due to the restriction of the public safety department of the government, we have the only choice of chloral hydrate or pentobarbital sodium as anesthetics in our university. The pentobarbital sodium is not ideal because of respiratory depression/poor analgesia, but it is acceptable for the non-survival procedures. Please refer to the following references for the use of this drug and the dose:

An, S. et al. Medial septum glutamatergic neurons control wakefulness through a sep to-hypothalamic circuit. Curr Biol. 10.1016/j.cub.2021.01.019, (2021).

Cao, J. L. et al. Activation of peripheral ephrinBs/EphBs signaling induces hyperalgesia through a MAPKs-mediated mechanism in mice. Pain. 139 (3), 617-631, (2008).

For deeper anesthesia, we have changed the dose to 50 mg/kg body weight. We've also stated that loss of response to hind paw-pinching should be checked prior to the thoracotomy in Protocol step 1.1 and in the video.

Responds to the reviewer's comments:
Reviewer #1:
Major Concerns:
1. The authors propose this antibody-based staining for the quantification of Aβ deposition or of Aβ plaques. However, in the discussion and in the video they report that, according to previous studies, the 6E10 antibody can still recognize full-length APP and other cleaved peptides containing 6E10, and that it combines with amyloid accumulation in both conformation- and sequence-dependent ways. The intensity threshold in the quantification uniforms the intensity from different aggregate species, but it is not clear whether it would include the signal from monomers, oligomers and smaller aggregates. The type of species included may also depend on

the threshold and resolution. Therefore, it would be useful to specify the size of the aggregates detected in 5xFAD mice, and possibly the minimum detectable size. Moreover, the authors could specify that the protocol is aimed at the quantification of Aβ accumulation in plaques instead of the quantification of Aβ plaques, which is misleading as it might also refer to the number of plaques.

Response: We thank reviewer #1 for the comment. We have used "the quantification of Aβ accumulation" instead of "the quantification of Aβ plaques", and changed the title accordingly. Indeed, the antibody can't discriminate different conformations of Aβ peptides, but the staining outside Aβ plaques is very weak in our protocol. Using 6E10 antibody to detect Aβ accumulation is common in 5xFAD mice. Please see the below references for further information. Thank you sincerely for your comment.

Lee, C. Y. D. *et al.* Elevated TREM2 Gene Dosage Reprograms Microglia Responsivity and Ameliorates Pathological Phenotypes in Alzheimer's Disease Models. *Neuron.* **97** (5), 1032-1048 e1035, (2018).

Spangenberg, E. et al. Sustained microglial depletion with CSF1R inhibitor impairs par enchymal plaque development in an Alzheimer's disease model. Nat Commun. 10 (1) , 3758, (2019).

von Saucken, V. E., Jay, T. R. & Landreth, G. E. The effect of amyloid on microglia-neur on interactions before plaque onset occurs independently of TREM2 in a mouse mod el of Alzheimer's disease. Neurobiol Dis. 145 105072, (2020).

2. For the perfusion of mice, 1% phenobarbital sodium is cited as the anaesthetic agent. Phenobarbital is commonly used for epilepsy treatment; however, the most common anaesthetics for veterinary surgery is pentobarbital sodium. As the names are very similar and often confused, the authors could double-check which one they used.

Response: Yes, the anesthetic agent used in our protocol is pentobarbital sodium instead of phenobarbital. This has been corrected in Protocol 1.1. We feel sorry for the mistake.

3. The perfusion of mice might not be detailed enough to be replicated properly. Step-by-step drawings or a reference to a similar illustrated perfusion protocol would be extremely helpful, since preliminary dissection is not shown in the video. For a reference, please see Gage, G. J., Kipke, D. R., Shain, W. Whole Animal Perfusion Fixation for Rodents. J. Vis. Exp (65), e3564, doi:10.3791/3564 (2012). Moreover, more information on the tools, an estimation of the total amount of 4% PFA for a mouse and of the total expected perfusion time could be added to the text.

Response: We've enriched the details in perfusion and cited the reference suggested in Protocol 1.1. The amount of 4% PFA used for perfusion was 20ml, and total expected perfusion time is 4min. Thank you sincerely for your suggestion.

4. In the Aβ staining procedure, the secondary goat anti-mouse antibody might recognize other mouse epitopes as well as the target Aβ epitope marked with the mouse primary antibody, especially as the sample is not incubated in a blocking

solution before hybridization. In principle, this might cause some background staining. From the image of the wild-type brain, the background seems very low, as stated also in the video; to confirm this, the authors could include the intensity quantification for the wild-type and for the 5xFAD mice without thresholding, and mention in the discussion how the intensity threshold would cut off the background.
Response: Actually, we have included 1% BSA as blocking agent in primary dilution solution. In our staining, the background is very low. There is almost no signal in wild-type mice because the 6E10 antibody is against human species. Actually we didn't cut off the background in ImageJ quantification. While adjusting the threshold, **Dark Background** should be selected. This has also been introduced in Protocol step 4.7. Thanks for your comment.

5. Recommended imaging parameters could be added in the discussion or the quantification section in the protocol.
Response: The imaging parameters should keep consistent to avoid derivation. In our protocol, the imaging parameter was set as: **Exp Pvw:** 450 ms, **Exp Acq:** 450 ms; **Pvw:** 1 × 1, **Acq:** 1 × 1; **Pvw Resolution:** Width 1 × Height 1, **Acq Resolution:** Width 1 × Height 1; **Capture Depth:** 8-bit mono; **Gain: Pvw:** 13, **Acq:** 13, **Gamma: Pvw:** 1, **Acq:** 1, **Offse: Pvw:** -700, **Acq:** -700.
We have added the setting in Protocol step 4.1. Thank you for your suggestion.

6. In the quantification of Aβ plaques by ImageJ, the plugin MosaicJ is not in the basic ImageJ version. The source should be included in the text.
Response: We've added the source of ImageJ Fiji, which includes the plugin MosaicJ, in Protocol step 4.2.

7. Image quality needs to be substantially improved.
Response: Sorry for the poor image quality displayed in PDF file. We've enhanced the image quality, and uploaded the tif image separately.

Minor Concerns:
1. The protocol could be made more acknowledgeable by correcting some theoretical flaws in the abstract and introduction:
a. neurofibrillary (not neurofibril) tangles are made of highly phosphorylated Tau, not Aβ;
b. the production of Aβ is due to proteolytic (not photolytic) cleavage of APP by the β- and γ- secretases;
c. APP should be the only (not "main") source of Aβ.
Response: All of these mistakes have been corrected in INTRODUCTION part. We sincerely thank you for the correction.

2. In the embedding and cryosectioning, the chamber temperature is set to -20°C, whereas in the discussion it is -21°C. As the authors stress that temperature is a crucial factor for final quality, they should be uniformed.

Response: We've uniformed the chamber temperature to -21°C. Thank you for your suggestion.

3. It is worth mentioning that the slides must be coated in the embedding and cryosectioning section of the protocol.
Response: As suggested, we've added that the slides must be coated before mounting sections onto them in Protocol step 2.7.

4. In the antibody staining procedure, it may be useful to report the composition of the primary antibody solution, or to refer to the reagent table.
Response: We have included the composition of primary antibody solution buffer (1% BSA, 0.3% Triton X-100, 0.01% sodium azide in PBS) in Protocol step 3.5.

5. More details on plasticware (tube volume) and on storage time (for instance, how long can the sections be stored at -20°C before Aβ staining without deteriorating) could be added to the protocol.
Response: We have added more details on plasticware in step 1.6, 2.1, 3.2; and storage time in Protocol 2.2 and 2.8.

6. Sentences are generally short and clear, but the grammar could be improved by checking phrasal verbs, irregular verbs and vocabulary, with special regard to technical terms.
Response: According to the reviewer's instruction, we have revised the whole manuscript carefully and tried to avoid any syntax error. In addition, we have invited several colleagues to help polish the manuscript. We hope that the grammar is acceptable now.

Reviewer #2:
Major Concerns:
1. More details like time spend and temperature could be added in the below step.
"1.4 Inject 20mL PBS from the left ventricle of the heart to flush out blood. Then slowly inject 4% PFA from the left ventricle to fix the tissues. During the injection of 4% PFA, the limbs and tails of the mice will twitch."
Response: The injection rate at the rate of 5mL/min and PBS temperature were added into Protocol step 1.5.

Minor Concerns:
2. All the images included in the paper are in poor resolution in the PDF view and some of the images included in the video for example WT 8m image at the at 05:30 is in poor condition and need to be further improved.
Response: We have improved the image quality, and uploaded the tif images. The WT 8m image is shown for demonstration of suboptional staining.

And in fact, Using Thioflavin staining, which is more simple and affordable, we can

already get good qualified Immunofluorescence photograph of Aβ plaques, why the 6E10 is employed if we only want to stain the plaques?

Response: Compared with chemical staining, immunofluorescent staining is more specific because the antibody only recognizes the 6E10 epitope; while Thioflavin S can stain all the beta-sheet rich proteins, such as neurofibrillary tangles, neuropil threads and vascular amyloid (Kawa R et al., ACS Chem Neuroscie, 2018, doi:10.1021/acschemneuro.7b00389). Neurofibrillary tangles can be discriminated by their shape, but this brings troubles for quantification. Besides, Thiofalvin S can't stain Aβ oligomer, which is believed the most neurotoxic forms of misfolded Aβ. We have realized that it's not proper to use "the quantification of Aβ plaques", so we have rephrased the words to "the quantification of Aβ accumulation", and changed the title accordingly. Thank you for the comment.

3. Captions should be added in the video.

Response: Thank you for your suggestion. We have consulted the editors, and they didn't recommend adding captions into the video, as other videos on *Journal of Visualized Experiments* are usually without captions. However, we've added name cards in the Introduction part of the video, and corrected the mistakes in the video. In addition, we have made the pace of the video slower and added the key points into the video. We hope that the video is satisfying now.

Reviewer #3:

Minor Concerns:

1. Abstract - Neurofibrillary tangles (NFTs) instead of "neurofibral tangles"

Response: We've corrected the mistake in Abstract.

2. Introduction – "Beta-amyloid precursosr protein (APP)…"- please rephrase the sentence.

Response: We've rephrased the sentence in Line39-41 as "Amyloid precursor protein (APP) is an integral membrane protein that exists in many tissues. Aβ peptide, consisting of 36-42 amino acids4, is produced by the subsequent cleavage of β- and γ-secretase in APP."

3. Introduction – "The 5×FAD (C57BL/6J) transgenic mice contain 3 mutations…" - please rewrite the entire paragraph avoiding repetition of the word „begin" (begins, begins, beginning)

Response: We've rewritten the entire paragraph in Paragraph 2 of Introduction as suggested: "The 5×FAD (C57BL/6J) transgenic mice contain 3 mutations in *APP*, and 2 mutations in *PSEN1*. The accumulation of intracellular Aβ starts as early as 1.5 month of age. Extracellular accumulation of Aβ was found around 2 months, in the cortex and hippocampus. The accumulation increased rapidly with age. The well-documented Aβ pathology makes it a good animal model for our protocol."

4. Protocol step 1.4 - it should be stated the PBS and PFA temperature. Room

temperature or 4°C?
Response: The temperature of PBS should be 37°C and the temperature of PFA should be of room temperature. We have stated it in Protocol step 1.5.

5. Protocol step 1.4 - it should be stated the used PBS pH.
Response: The PBS pH was 7.2-7.4. We have stated it in Protocol step 1.5.

6. Protocol step 2.4 - why only the left half of the brain? Why not the whole brain?
Response: Both halves of the brain can be used for sagittally sectioning. We always use the left half for consistency.

7. Protocol step 2.5 - the tissue orientation on the OCT for cutting should be described.
Response: We've described the orientation of the tissue in Protocol step 2.5 and 2.6.

8. Protocol step 2.6 - the glass slides are of what kind? Superfrost, precoated or common? The authors mention this in the discussion section, but it should also be included in the protocol step properly.
Response: The glass slides should be coated by poly-lysine. We've included this in Protocol step 2.7.

9. Protocol step 3.4 - PBS temperature should be stated.
Response: We have stated "PBS of room temperature".

10. Protocol step 3.7, 3.8 and 3.9 - Sections should be protected from light? The authors describe this in the discussion section, but it should be included in the protocol step for better comprehension.
1.1 **Response:** It's true that the sections should be protected from light after being applied with antibodies. Fluorescence quenching might happen if the protection is improper. We've stated that "Protocol step 3.7, 3.8 and 3.9 should be proceeded in a dark place."

11. Protocol step 3.10 - Storage conditions for the slides should be stated.
Response: The storage conditions for the stained slides should be at 4°C in a dark place. However, the sections should still be analyzed as soon as possible to avoid fading of the fluorescence. We have stated that in Protocol step 3.10.

12. Figure 3 - A) in the upper panel a 4-month wild-type brain is presented and in the lower panel is a 6-month? C) Here an 8-month wild-type brain is presented? Please revise the entire figure and rewrite the legend properly.
Response: Many thanks for the comment. We have revised the figure and rewrited the Figure 3 legend.

We are looking forward for your response.

Best regards,

Chao Liu, PhD
Professor
Jiangsu Province Key Laboratory of Anesthesiology
Xuzhou Medical University
209 Tongshan Road, Xuzhou, China, 221004
Fax: +86 0516 83262697
Phone: +86 0516 83262697