FACT For Fast And Three-Dimensional Imaging of Intact Tissues

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Methodology

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Abstract

Free of Acrylamide Sodium Fast Free-of-Acrylamide Clearing Tissue (FACT) is a developed technique using no acrylamide for clearing tissues. As the lipid removal normally is a harmful process and it causes loss of biological molecules such as proteins and on the other hand is crucial for transparency and efficient antibody staining throughout the whole tissue especially for microscopy and imaging, the FACT technique is suitable since it makes chemical bonding of membrane and intracellular proteins with the extracellular matrix creating a massive three-dimensional (3D) matrix and structural support to fortify the tissue during processing. Compared to other acrylamide-based techniques, FACT requires less labor, toxic, and harmful chemicals. Here we describe protocols encompassing every angle and dimension of the FACT protocol for antibody staining and imaging of whole-cleared tissues while preserving the structure and increasing the image quality. The entire protocol includes tissue perfusion, fixation, clearing, antibody staining, Refractive Index Matching (RIM), microscopy, and imaging; this timing varies due to the size, weight, different kind of tissues and the type of immunostaining. This technique has been favorably performed on different types of tissues for molecular interrogation analysis of large tissues.

Introduction

Biological systems are capable of forming a complex neural network of forwarding feeding, feedback, and horizontal circuits [1]; the most sensitive biological systems are the nerve base of spatial codes [2]. For decades, research on the neurobiology of neural tissue imaging focused mainly on anatomical structures of the nerves by mechanical slicing techniques, which laid the foundations for understanding the neural maps of two dimensional (2D) spatial displays [2]. However, texture transparency and light scattering greatly limit the depth of the tissue that can be drawn up optically [2].

The three dimensional (3D) image has enabled studying cellular and extracellular structures, such as the structure of vasculature, or neuronal networks in the brain [3, 4]. Various protocols have been developed to clean the entire tissue and 3D imaging [5]. Benzyl alcohol and benzyl benzoate (BABB) were among the first materials that made 2 cm thick fixed tissue transparent for deep microscopic imaging and were used in conjunction with conventional immunohistochemical techniques compared to tissues of less than 50 μm [5, 6]. Besides, numerous advances have been made for high resolution and large-scale imaging of cleared tissue, including Scale [7], dibenzyl ether (DBE) [8], 3D imaging of solvent cleared organs (3DISCO) [9], See Deep Brain (seeDB) [10], Clear T [11], Clear Unobstructed Brain/Body Imaging Cocktails (CUBIC) [12], System-Wide control of Interaction Time and kinetics of Chemical (SWITCH) [13] and ultimate DISCO (uDISCO) [14]. The aforementioned techniques require a very clear texture and transparent tissue to detect [5].

Considering the limitations of the above-mentioned techniques, including the isolation of fluorescence from samples, incomplete cleansing samples, and the lack of feasibility for antibody labeling, several other techniques have been developed [5]. However, these protocols were still limited by the isolation of fluorescence from samples, the inadequate purification of samples, and the inability to allow antibody

labeling [2]. Efforts to address these issues and to modify tissue processing conditions have provided the primary inputs for optical clearing techniques [2]. The cell membranes as the main source of light scattering in tissues and lipid removal, is a potential way to increase tissue transparency [5].

Several techniques have been developed to eliminate the transparency of lipid for 3D imaging, including the use of acrylamide protocols such as CLARITY [15], passive CLARITY [4], passive clarity technique (PACT) [16], and Perfusion-Assisted Agent Release in Situ (PARS) [16]. These methods use embedding hydrogel such as CLARITY and PACT [5]. These hydrogel-based techniques also require toxic chemicals, labor work, and specific lab equipment. Not only they are expensive, but also they change the volume of the texture even after using the refractive index matching solutions (RIMs) [5]. The cutting edge technique CLARITY developed by Chung and Deisseroth [17] has created a new tissue processing platform to illuminate a 3D cell conjugation and make-up in general [2]. This clearing technique, the most commonly used method for the study of healthy tissue image and can be applied to explore structural molecular and intact tissue infrastructure, is largely constrained due to the use of tissue-specific reagents or special application [2]. Three transparent roles have been created through these techniques: the stabilization of tissue structures using embedding hydrogel [17], the use of large-scale compatible clearing reagents [12], and enhanced tissue imaging [2, 18]. Whole-tissue clearing takes days to weeks to disrupt the fluorescent signaling of the labeled chemical, and ultimately cannot prevent the separation of fluorescent protein signals for a long time. These limitations are more problematic in developing countries laboratories with limited equipment, so, a simple method is needed for laboratories in developing countries [5].

The other techniques have been developed without applying acrylamide methods including Free of Acrylamide Sodium Fast Free-of-Acrylamide Clearing Tissue (FACT) [19] and Dodecyl Sulphate (SDS)-based Tissue Clearing (FASTClear) [20]. The FACT requires less labor, and toxic and harmful chemicals to the environment than the acrylamide-based ones [5]. Another limitation in developing countries is the lack of advanced microscopes, i.e. confocal, two-photon excitation, and light-sheet microscopes [5]. To date, all protocols for 3D imaging of tissues have been used by advanced microscopes. The use of the FACT approach with a conventional epifluorescence microscope is another goal [5]. Therefore, there are two procedures described in this paper that the goal of the first procedure is to speed up the structural analysis of whole tissue clearing and to apply to the fluorescent imaging of mouse brain tissue. The aim of the second procedure is to evaluate the FACT protocol for clearing the entire mouse tissue and 3D imaging of brain cortical vasculature using the FACT method by a simple epifluorescence microscope in imaging laboratories [5, 21, 22]. FACT has also been used for monkey brain imaging using a Zeiss LSM 880 scanning confocal microscope [23].

Clarifying large tissue volumes

The FACT was firstly obtained by perfusion with phosphate-buffered saline (PBS) and then from 4% paraformaldehyde (PFA) (w/v) in 1 M PBS as a fixative solution. After collecting a 1 mm coronal section of mouse brain sample, brain fragments were supposed to be fixed in a fixative solution at 4°C for 3 days.

SDS, which is a highly effective surfactant, was used to remove fatty spots (Figure 1A) [19]. The samples were observed daily until obtaining visual confirmation of full transparency of the tissue by viewing black grid lines on a white sheet of paper through the tissue itself (Figures 1B-D) [19]. Other tissues of mice and rats have been shown in Figures 2 and 3, respectively.

After clearing the fragments of the brain with the FACT protocol, for staining the cleared tissue with the antibodies, SDS was removed by washing in PBS with Triton X 100 (PBST) for 12 h. The PBST solution was replaced every 6 h. Glycine, Triton X 100, donkey serum, and dimethyl sulfoxide (DMSO) dissolved in PBS was injected into the tissues and blocked the permeability at 37°C. The tissues were washed twice in PBST for 1 hour at 37°C. Brain slices were incubated with primary antibodies, Tween-20 (as a nonionic surfactant), DMSO, donkey serum and sodium azide in PBS for at least 2 days at 37°C. The specimens were washed three times in PBST for 1 hour and incubated at 37°C. Then, sections with secondary antibodies were incubated in Tween-20, DMSO, serum, and sodium azide in PBS for at least 2 days [19]. All steps were performed by shaking at 37°C. The specimens were washed three times in PBST for 1 hour and then incubated at 37°C or transferred to a refrigerator (4°C), and thereafter for 7 days in aluminum foil-coated tubes contained PBST and sodium azide [19].

Imaging large clarified tissue volumes

The next major challenge is the development of high-resolution optical techniques and high-resolution in deep textures [4]. First, frozen coronal brain sections with 30 μm of thickness were prepared on a cryostat [19, 24]. And for the first time, the clearing of the rat tissues with the FACT protocol was shown for effective tissue clearing and 3D imaging of the brain cortex vasculatures [5] .

The FACT method has undergone some changes, including imaging changes to adapt this method to non-equipping laboratories [5]. The images were taken with a Nikon A1R + upright confocal microscope [19]. Though the main part of the whole-tissue clearing is optical sectioning for 3D imaging that can be achieved optimally with the expensive confocal microscope, the accessibility of this type of microscope is a great challenge for matching the whole tissue imaging in a typical laboratory with limited resources. Therefore, in the FACT method, we used an epifluorescence microscope with a motorized stage for auto-fluorescence vessel imaging in the z plane [5]. However, this approach has limitations, including the lower depth of the fluorescent light image in an epifluorescence microscope compared to a laser in a confocal microscope. This can be solved by cutting 1 to 2 mm of cleared tissue for imaging. In addition, the lower epifluorescence power for collecting high signals compared with the laser-enhanced fluorophores in the confocal imaging of the tissue for a maximum depth of 200 to 300 μm [5].

Overview of the procedure

We here describe the FACT protocol (Figure 4). Before starting the process, reagents are prepared, and tissues are collected (Step 1 in method#1 and Steps 1-2 in method#2). The tissue clarification process

includes fixation of the perfused brain (Steps 2-3 in method#1 and Steps 3-4 in method#2), clearing of the tissue (Steps 4-5 in method#1 and Steps 5-8 in method#2), and RIM (Step 12 in method#1 and Steps 9 in method#2). If required, tissues are going under antibody staining procedure (Steps 6-11 in method#1 and Steps 10-17 in method#2) (Figure 5) in advance of RIM. The cleared tissues undergo confocal microscopy and imaging (Steps 13-16 in method#1 and Steps 18-22 in method#2) (Figures 1E, F), followed by 3D reconstruction and image analysis (Steps 17-23) (Figures 1G, H); we describe the application of FACT protocol, which is suitable for lipid removal in different kinds of cleared tissues.

The lipid removal is crucial for transparency and efficient antibody staining throughout the whole brains. The lipid removal normally is a harmful process and causes loss of biological molecules such as proteins, but now with the FACT technique, chemical bonds of membrane and intracellular cytoplasmic proteins with the cytoskeleton and the extracellular matrix assist the creation of a massive 3D matrix and structural support to fortify the tissue during processing (Figure 6) [19, 25].

The hydrogel-based techniques as mentioned earlier require toxic chemicals, labor work, and specific lab equipment. Not only are they expensive, but also they change the volume of the texture even after using the RIMs [5]. The CLARITY developed is the most commonly used method for studying whole tissue image and can be applied for exploring structural molecular and intact tissue infrastructure [2, 17]. Another limitation is the timing in which the whole tissue clearing takes days to weeks to disrupt the fluorescent signaling of the labeled chemical and ultimately cannot prevent the separation of fluorescent protein signals for a long time [5]. On the other hand, The FACT requires less labor, and toxic and harmful chemicals to the environment than the acrylamide-based ones [5]. Additional to that limitation, in developing countries, there is a lack of advanced microscopes, i.e. confocal, two-photon excitation, and light-sheet microscopes [5]. To date, all protocols for 3D imaging of tissues have been used by advanced microscopes. The use of the FACT approach with conventional epifluorescence microscopy is recommended instead of methods using advanced microscopes [5].

As a final comment for future work, we think that the removal of polyacrylamide hydrogels increases the rate, amount, and depth of antibody penetration and provides a clear image compared to endogenous fluorescent labeling. Maintaining the primary or secondary antibodies in the hydrogel networks or crosslinking of these antibodies with the hydrogel creates unexpected non-specific staining and increases the background during imaging, which significantly reduces the image quality.

There are two protocols for FACT. One with confocal microscopy (Protocol #1) and another one with epifluorescence microscopy (Protocol #2) that can be used depending on the laboratory equipment.

Methods

Protocol #1

REAGENTS

Tissue preparation

- C57BL/6 N mice and B6.129P2(Cg)-Cx3cr1tm2.1(cre/ER,-EYFP)Litt/ WganJ mice (The Jackson Laboratory)

! CAUTION Experiments on animals must conform to all relevant governmental and institutional regulations.

- Mice are anesthetized with intraperitoneal injections of pentobarbital (0.35 mg/kg). The mice are perfused transcardially with PFA solution.

! CAUTION PFA is toxic. Perform all procedures in a fume hood.

Fixative solution

- Ice-cold PBS solution (1M, pH 7.6)
- 4% (wt/vol) PFA in 1M PBS

Clearing solution

- 8% (wt/vol) SDS in 0.1M PBS (pH7.5) for FACT

! CAUTION SDS is a toxic, irritant, and poses a risk of severe damage to the eyes. It may be harmful by inhalation, ingestion, or skin absorption. (SDS protocol) Perform all procedures in a fume hood.

Washing and Imaging

- PBS with 0.1% Triton X-100 (PBST)

Permeabilizing and blocking tissues

- 0.6 M glycin
- 0.2% Triton X-100
- 6% donkey serum
- 20% DMSO

All the above dissolved in PBS

Antibody staining reagents

- Primary antibody (Iba1, Abcam) diluted 1:50 in 0.2% Tween-20, 5% DMSO, 3% donkey serum, and 0.01% sodium azide in PBS.
- Secondary antibodies (Alexa594) diluted 1:200 in 0.2% Tween-20, 5% DMSO, 3% donkey serum, and 0.01% sodium azide in PBS.

Incubation

- In aluminum foil-covered tubes containing PBST and 0.01% sodium azide

EQUIPMENT

Confocal microscopy

- Horseshoe-like piece of putty (1-mm thickness)
- Wellco dish (Pelco (Ted Pella), cat. No. 14032E120)
- Nikon A1R$^+$ upright confocal microscope
- Water immersion 25X objective lens (1.1-NA, 2mm-WD, Nikon, USA)

3D reconstruction and image analysis

- Dell server board T7910
- Two Intel E5-2687WV4 CPUs
- Four 32GB DDR4 ECC RAM
- 4 TB hard disk (Dell SAS 7.2K)
- Nvidia Quadro 5000 graphics card

Statistical analysis

- Bitplane Imaris software (version 7.4.2)
- GraphPad Prism (version 6.00 for Windows, GraphPad Software, La Jolla, CA, USA)
- IBM SPSS Statistics for Windows (Version 22.0, 2013; IBM Corp., Armonk, NY)

REAGENT SETUP

- Prepare the PBS solution (1 M, pH 7.6) and keep at 4°C for perfusion.
- Prepare 4% (wt/vol) PFA solution at 4°C for perfusion.
- Prepare FACT clearing solution (pH 7.5) by mixing 0.1 M PBS with 8% (wt/vol) SDS.
- Prepare RIM by mixing 87.5% (wt/vol) sorbitol with 0.02X PBST which titrated to pH 7.5 (sRIM).

PROCEDURE

Transcardial perfusion and tissues collection

1| Anesthetize mice with intraperitoneal injections of pentobarbital (0.35 mg/kg). Perfuse the mice transcardially with PFA solution. Remove the mice's brains.

! CAUTION Process the samples in tubes covered with aluminum foil to prevent fluorescent photobleaching after brain sampling and during the clearing procedure

! CAUTION Perform all procedures in a dimly lit room.

Fixation

2| Perfuse mice transcardially with 40 mL ice-cold PBS solution (1 M, pH 7.6) followed by 20 mL of 4% (wt/vol) PFA in 1 M PBS.

3| After collecting 1 mm coronal sections with a mouse brain mold, fix the brain slices in the same fixative solution.

 PAUSE POINT Tissues should be kept at 4°C for 3 days.

Clearing

4| Clear the slices with 8% (wt/vol) SDS in 0.1 M PBS (pH 7.5) at 37°C with gentle rotational shaking at 80 r/min speed.

5| Refresh the solutions daily until visual confirmation of complete tissue transparency by viewing black grid lines on a white sheet of paper through the tissue itself.

• POINT The cleared tissues can be directly passed into the immunostaining step, or it can be preserved in PBS with 0.02% sodium azide at 4°C for several weeks.

Antibody staining of FACT-cleared tissue

6| Remove the SDS by washing in PBS with 0.1% Triton X-100 (PBST) for 12 h.

• POINT PBST solution should be replaced every 6 h.

7| Permeabilize and block the tissues overnight at 37°C with 0.6 M glycine, 0.2% Triton X-100, 6% donkey serum, and 20% DMSO dissolved in PBS.

8| Wash the tissues two times in PBST for 1 h at 37°C.

9| Incubate the washed brain slices with primary antibody (Iba1, Abcam) diluted 1:50 in 0.2% Tween-20, 5% DMSO, 3% donkey serum, and 0.01% sodium azide in PBS for a minimum of 2 days at 37°C. Wash the samples again three times in PBST for 1 h and incubate overnight at 37°C. Table 1 lists examples of antibodies for staining a 1-mm-thick tissue block in 1ml PBS or PBST.

Table 1
Antibodies for staining a 1-mm-thick tissue in 1ml PBS or PBST.

Volume (µl)	Antibody	supplier
5–20	Iba1 antibody	Abcam, no. ab5076
5–20	Alexa594 Secondary antibody	Abcam, no. ab150160
5–20	Anti-CD31 primary antibody	Abcam, no. ab24590
5–20	FITC-IgG secondary antibody	Abcam, no. ab6785
5–20	Hoechst 33342	Sigma-Aldrich, no.14533

10| Incubate sections with secondary antibodies (Alexa594) diluted 1:200 in 0.2% Tween-20, 5% DMSO, 3% donkey serum, and 0.01% sodium azide in PBS for a minimum of 2 days at 37°C.

• POINT Conduct all procedures with shaking at 37°C.

• POINT The incubation time depends on the thickness of the tissue.

11| Wash the samples again three times in PBST for 1h each, and then incubate the tissues overnight at 37°C or transfer to a refrigerator (4°C) where they remain for 7 days in aluminum foil-covered tubes containing PBST and 0.01% sodium azide.

RIM

12| After clearing, either incubate the brain slices at 4°C (for a maximum of 3 weeks for transgene-labeled slices or 1 week for antibody-labeled slices) until imaging or transfer the cleared tissues directly into FocusClear (CelExplorer Labs) for 1 h before imaging.

Confocal microscopy

13| Embed the brain slices in a chamber formed by a 1-mm thick and flattened horseshoe-like piece of putty acting as a wall on a glass slide. Fill the chamber with FocusClear, and gently seal the upper part of the chamber using a Wellco dish (Pelco (Ted Pella), cat. no. 14032E120) with the glass surface facing down and thus preventing the formation of small bubbles on the surface of the brain slice.

14| Focus the laser onto the specimen with a water immersion 25× objective lens (1.1-NA, 2 mm-WD, Nikon, USA).

15| After defining the edges of the tissue slice and capturing a large-scale image, place the objective lens on the cortex area and scan three fields (XY = 1024 × 1024 µm2) with whole tissue depth (Z = maximum visible signals down to 1000 µm) (speed = 0.5, step distance = 1 µm). Define the laser power, light gain, and offset of the upper and lower visible surfaces of the slice before Z-scanning, for the highest acquisition of excitation and emission of microglial YFP signals using the *intensity correction* option of the Nikon NIS software.

16| Transfer the TIFF image sequences to Bitplane Imaris software (version 7.4.2) for 3D reconstruction and image analysis, after obtaining the images of the brain cortex.

3D reconstruction and image analysis

17| Perform the 3D reconstruction and tracing of microglial morphology using Imaris software and its algorithms, including Surface and Filament, and automatic or semiautomatic counting.

• POINT Because of the large amount of data, a workstation server should be used for the data analysis.

18| Perform the 3D reconstruction of the cell surface based on the signal thresholds of microglia cells in two steps in Imaris Surface.

19| In the first step, define and measure an index for all of the Z-depth images to show the similarity of the number of microglia cells in different parts of the cortex and to remove the effect of tissue expansion when counting the numbers of cells per unit volume. For this purpose, count the numbers of cells in three virtual squares using the *region of interest* option of the Imaris Surface software (XYZ = 200 × 200 × 100 μm3 from the surface layer).

20| In the next step, using the *entire image* option of Imaris Surface, reconstruct the cells in each of the Z-depth images (surface area detail = 0.2 μm and manual adjustment of threshold to cover the whole visible microglia volume).

21| Correct the total cell numbers by multiplying the calculated number index of the first step in each sample to measure the total cell number in each imaged volume. Furthermore, extract the data for the cell surface area and the mean signal intensity in the detected cells from the Imaris Surface analysis of the entire images and evaluate as indices of cell signal quality with the different clearing protocols.

22| Categorize and image the cells in Imaris Vantage according to the cell surface area. Use Imaris Filament to determine the accuracy of the methods in detecting the fine branches of microglial cells. For this purpose, select three complete cells that were imaged at the 100 μm depth of the hole using the 3D crop option of the Imaris Filament. Use the semiautomatic settings to reconstruct the cell centers and filaments of the selected cells, and analyze the filament levels and lengths as indices of the visibility of the branches and sub-branches of the individual cells in each method.

23| Classify the 3D reconstructed images according to the filament levels using Imaris Vantage.

•Timing

Steps 1–5, Transcardial perfusion, tissues collection, fixation, and tissue clearing: 3 days

Steps 6–10, Antibody staining of FACT-cleared tissue: approximately 5 days

Steps 11, Washing: 7 days

Steps 12, Complete transparency and RIM: 3–12 h

Steps 13–16, Confocal microscopy: 3–5 h

Steps 17–23, 3D reconstruction, and image analysis: 3–5 h

REAGENTS

Tissue preparation

- This protocol describes the imaging of brain samples prepared from 3 adult female wild-type (WT) BALB/c mice and 3 female Wistar rats.

! CAUTION Experiments on animals must conform to all relevant governmental and institutional regulations.

- Laboratory Animal Center of the Center of Infertility, Shahid Sadoughi University of Medical Sciences, Yazd, Iran. (The experiment was performed according to Shahid Sadoughi University of Medical Sciences Guidelines for Animal Handling and the Ethics Committee of Research and Clinical Center for infertility No. 91/8/2/2168).
- The rats and mice were euthanized by ether inhalation and then cervical dislocation.

Fixative solution

- 4% PFA (Merck KCaA, Germany)
- PBS (Gibco, UK) solution (0.01 M)

Clearing solution

- 8% (wt/vol) SDS in 0.01 M PBS with 0.02% sodium azide

Washing and Imaging

- PBS 1X/sodium azide

RIM and imaging solution

- Glycerol (80%, vol/vol)

3D epifluorescence microscopy

- 80% glycerol

Comparing antibody stained and auto-fluorescent vessels

- PBS with 0.1% Triton X-100 (PBST)
- Anti-CD31 primary antibody (1:10, mouse species, Abcam, USA)
- FITC-IgG secondary antibody (Goat anti-mouse, 1:100, Abcam, US)

- Hoechst 33342 (1:100, Bis Benzimaide H 33342, Sigma, USA)
- Glycerol

EQUIPMENT

FACT Protocol

- Shaker incubator (Jaltajhiz, Iran).
- DP71 camera (Olympus, Japan)
- Loop microscope (SZX16, Olympus, Japan)

3D epifluorescence microscopy and comparing antibody stained and auto-fluorescent vessels

- Horseshoe-like chamber (1-mm thickness wall)
- Epifluorescence microscope (BX51 with a DP72 camera, Olympus, Japan)
- Air/dry objective lens 10× (UPlanSApo, Olympus Co. Ltd.; numerical aperture: 0.4 and working distance: 3.1 mm)

REAGENT SETUP

Fixative solution

- Dilute 4% PFA in PBS solution (0.01 M) (pH = 7.5, room temperature).

Clearing solution

- Dilute 8% (wt/vol) SDS in 0.01 M PBS (pH = 7.5) with 0.02% sodium azide at 3°C with mild rotational horizontal shaking (100 r/min) in a shaker incubator.

Imaging

- Wash the brain slices once in PBS with 0.02% sodium azide, and then shake gently 12 h in the same solution at 37°C in horizontally fixed falcon tubes. For complete transparency and RIM, samples should be placed in 80% glycerol in double-distilled water for 3 to 12 h at room temperature before imaging.

Comparing antibody stained and auto-fluorescent vessels

- After clearing, wash the residual SDS from the brains by slow shaking in PBS with 0.1% Triton X-100 (PBST) for 24 h at 37°C. Incubate the samples for 24 h with anti-CD31 primary antibody diluted in PBST with shaking at 37°C. Subsequently, wash the samples in PBST buffer for 24 h with shaking at 37°C. Then, incubate them with the FITC-IgG secondary antibody diluted in PBST for 24 h with shaking at 37°C in a tube covered with an aluminum sheet. To label cell nuclei, add Hoechst 33342 to the secondary antibody mixture for the final 12 h of incubation with shaking at 37°C. Before

mounting and imaging, wash samples in PBST for 24 h with shaking at 37°C. Submerge samples in glycerol for 24 h at room temperature.

EQUIPMENT SETUP

Acquire the images in TIFF format from the microscope and import them to Imaris software (version 7.4.2, ImarisX64, Bitplane AG). To renovate the name of the channels and the colors, use the “Display Adjustment” tab. For adjusting the tissue thickness according to the z-stack imaging information of an epifluorescence microscope, employ the “Edit” button and “image Properties” panel. Erase the unwanted sections apply the “Edit” button and then the “Crop 3D” option for finalizing the images. Modifying the size of the image is available by dragging the borders. Based on the sensed signals, rebuild brain cortex 3D vessels through the filament algorithm, in the “Surpass” panel by composing new “Filament” in the “filament” button. Outline the thinnest and the thickest imaged vessels in the “Slice” panel. The space between the selected two points at the maximum width of the thinnest and thickest vessels is displayed automatically on the “Measure” panel. Then, log the data in the” Surpass” panel and adjust the threshold of starting and seed points. By applying the "Select" tab in the "Camera" panel, track vessels’ length and dismiss the undesired seeds by pressing the shift button on the keyboard and left-clicking on the point. To ensure that the grain points are correctly preserved, rotate the 3D image by selecting "Navigate" and moving the mouse cursor. Subsequently, choose the highest threshold for the local contrast. Finally, complete the regeneration of blood vessels without selecting the "Detect Spines". Remove extra parts of the vessels that do not match the signal on the "Edit" panel. Use the “Color” tab to revise the color of regenerated cylinders.

PROCEDURE

Fixation

1| Euthanize the rats and mice by ether inhalation and then dislocate the cervical vertebrae.

! CAUTION Follow appropriate institutional guidelines for handling animals.

2| Dissect out the brain.

3| Separately transfer them into 4% PFA diluted in PBS solution (0.01 M) as a fixative solution.

! CAUTION PFA is toxic. Perform all procedures in a fume hood.

 PAUSE POINT Fix tissues in the fixative solution at 4°C for 3 days.

4| Cut the brain into 1 mm slice, coronally.

Clearing

5| Clear the tissues with clearing solution at 37°C with mild rotational horizontal shaking (100 r/min) in a shaker incubator.

 PAUSE POINT Refresh the clearing solution daily for 3 days and then replace weekly until confirming the visual of 80% tissue transparency by observation through the tissue of clear black grid lines printed on a white paper Figure

6| Image transparency of the tissue over the clearing process using a DP71 camera on a stage of a loop microscope for background illumination. Record the start and end date of clearing for all tissues.

Imaging

7| Wash the brain slices once in PBS with 0.02% sodium azide.

8| Shake them gently for 12 h in the same solution at 37°C in horizontally fixed falcon tubes.

Complete transparency and RIM

9| Place samples in 80% glycerol in double-distilled water for 3 to 12 h at room temperature before imaging.

Steps 10–17, Comparing antibody stained and auto-fluorescent vessels: 1 week

Steps 17–22, 3D Epifluorescence microscopy: 1–3 h

Comparing antibody stained and auto-fluorescent vessels

 PAUSE POINT 10| Wash the residual SDS from the brains by shaking slowly in PBS with 0.1% Triton X-100 (PBST) for 24 h at 37°C.

 PAUSE POINT 11| Incubate the samples for 24 h with anti- CD31 primary antibody (1:10) diluted in PBST with shaking at 37°C. Table 1 lists examples of antibodies for staining a 1-mm-thick tissue block in 1ml PBS or PBST.

 PAUSE POINT 12| Wash the samples in PBST buffer for 24 h with shaking at 37°C.

 PAUSE POINT 13| Incubate them with the FITC-IgG secondary antibody (1:100) diluted in PBST for 24 h with shaking at 37°C in a tube covered with an aluminum sheet.

 PAUSE POINT 14| To label cell nuclei, add Hoechst 33342 (1:100) to the secondary antibody mixture for the final 12 h of incubation with shaking at 37°C.

 PAUSE POINT 15| Before mounting and imaging, wash samples in PBST for 24 h with shaking at 37°C.

 PAUSE POINT 16| Submerge samples in glycerol for 24 h at room temperature.

17| Image the antibody signals and auto-fluorescent vessels by an epifluorescence microscope and use CellSens imaging software.

3D epifluorescence microscopy

18| Mount the brain slices individually between two glass slides, which are surrounded by the same thickness non-colorful putty that formed a horseshoe-like chamber (1-mm thickness wall) to protect the tissues' thickness from pressing between the slides and provide a chamber for the RI matching solution.

• POINT This chamber between two slides should be filled with a fresh 80% glycerol.

19| Take the image of the auto-fluorescent vessels by an epifluorescence microscope and use CellSens imaging software.

20| After fixing the apparatus on the microscope stage, image the specimen by an air/dry objective lens 10×, which is water immersed to increase working distance.

21| Apply the EPI illumination mode and red excitation (650 nm) and deep red emission (690 nm) for imaging.

22| Image selected area of the tissue surface for 150 μm depth on a z-stack manner (each 10-μm step), automatically.

•Timing

Steps 1–4, Fixation solution: 3days

Steps 5–6, Tissue clearing: approximately 1 week

Steps 7–8, Imaging: 12 h

Steps 9, Complete transparency and RIM: 3–12 h

Steps 10–17, Comparing antibody stained and auto-fluorescent vessels: 1 week

Steps 17–22, 3D Epifluorescence microscopy: 1–3 h

Results And Discussion

This work presented rapid creation of transparent brain slices with the passive clearing using the FACT protocol. In the first method we prove that the FACT protocol provides an optimum signal from transgene labeled microglia. The protocol is remarkably effective for immunohistochemistry in both antibody-based and transgene-based imaging in brain slices with antibody staining and YFP-labeled microglia in transgenic mice. However, in comparing the Iba1 immunostaining and transgene-YFP-microglia, more microglia signal is detected within the Iba1 immunostaining image. Additionally, it is elucidated that SDS concentration is crucial in fat removal of the tissue and PBS plays an important role in the preservation of endogenous signals. Besides, the optimum temperature was deduced to be 37°C. Another goal of the current study was to set up a method to shorten the clearing time step without damaging the structure of microglial branches. Due to the size change in tissue for transgene-labeled microglia during clearance,

the reduction of sample thickness in the immunostaining protocol and the removal of hydrogel from the fixation step, resulted in less cell deformation and preserved cellular structure. Since microglia are smaller than neurons in the CNS, the FACT protocol also conserves imaging of their fine processes and their structural interactions. For immunostaining of FACT-cleared brain slices, antibody trapping in the hydrogel networks or crosslinking of antibodies with the hydrogel may cause unexpected lower image quality because of the increased background staining. The quality of the optical microscope is limited below the micron resolution due to the operation of the optical lens spacing in both confocal and light-sheet microscopes. Hence, using such microscopes are feasible for microglia with a depth range of 300 µm to 700 µm.

On the other hand, in the second method, the FACT clears the 1-mm thick brain slices in both mice and rats within 3 days. A microvasculature containing red blood cells was examined by an epifluorescence microscope. Moreover, the Imaris Filament algorithm was used for segmenting the blood vessels. In order to demonstrate the possibility of imaging vessels using a non-antibody-based approach on the basis of auto-fluorescent characteristics of the RBC and to approve their detectability in this protocol, vessels must be imaged after applying the FACT technique. For this purpose, the brain slices corresponding to non-perfused and perfused mice were stained in the same staining condition and the same tube containing CD31. The non-perfused vessels of the brain cortex were checked by blue (WB) and green (WG) filter boxes to detect the auto-fluorescent RBC signals. Vessels, which are not visible in the WG filter, are not also observed in the image of the WB filter. This phenomenon shows that the auto-fluorescent RBC signals and CD31-labeled vascular endothelium are completely overlapped. In both non-perfused and perfused mice, vascular endothelium nuclei line-shaped structures can be observed with Hoechst 33342 staining.

This approach has some limitations including (i) a lower depth of imaging of fluorescent light in an epifluorescence microscope in comparison to a laser in a confocal microscope that this problem can be resolved by cutting a 1 to 2 mm piece of cleared tissue for imaging, (ii) a lower power of the epifluorescence to collect the raised signals compared to the laser-enhanced fluorophores in confocal resulted in restriction in imaging tissues for the depth up to 200 to 300 µm.

One advantage of this approach is that the whole tissue clearing such as our results, can demonstrate the 3D architecture for the blood vessels and can be applied to seek vasculature relations in neurons [26].

Abbreviations

2D, two dimensional

3D, three dimensional

3DISCO, three dimensional imaging of solvent cleared organs

BABB, Benzyl alcohol and benzyl benzoate

CUBIC, Clear Unobstructed Brain/Body Imaging Cocktails

DMSO, dimethyl sulfoxide

FACT, Free of Acrylamide Sodium Fast Free-of-Acrylamide Clearing Tissue

FASTClear, Dodecyl Sulphate (SDS)-based Tissue Clearing

PACT, passive clarity technique

PARS, Perfusion-Assisted Agent Release in Situ

PBS, phosphate-buffered saline

PBST, phosphate-buffered saline with Triton X 100

PFA, paraformaldehyde

RIM, Refractive Index Matching

SWITCH, System-Wide control of Interaction Time and kinetics of Chemical

uDISCO, ultimate DISCO

Declarations

Ethics approval and consent to participate

The experiment was performed according to Shahid Sadoughi University of Medical Sciences Guidelines for Animal Handling and the Ethics Committee of Research and Clinical Center for infertility No. 91/8/2/2168.

Consent for publication

Not applicable.

Availability of data and material

Not applicable.

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Competing interests

There is no conflict of interest.

Authors' contributions

Z.F., A.K., and A.T. conceived and designed the format of the manuscript. Z.F., A.K., N.B., F.N., and A.T. drafted and edited the manuscript. I.N., K.T., Y.H., and A.T. reviewed the manuscript. All authors contributed to the critical reading and discussion of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Figures

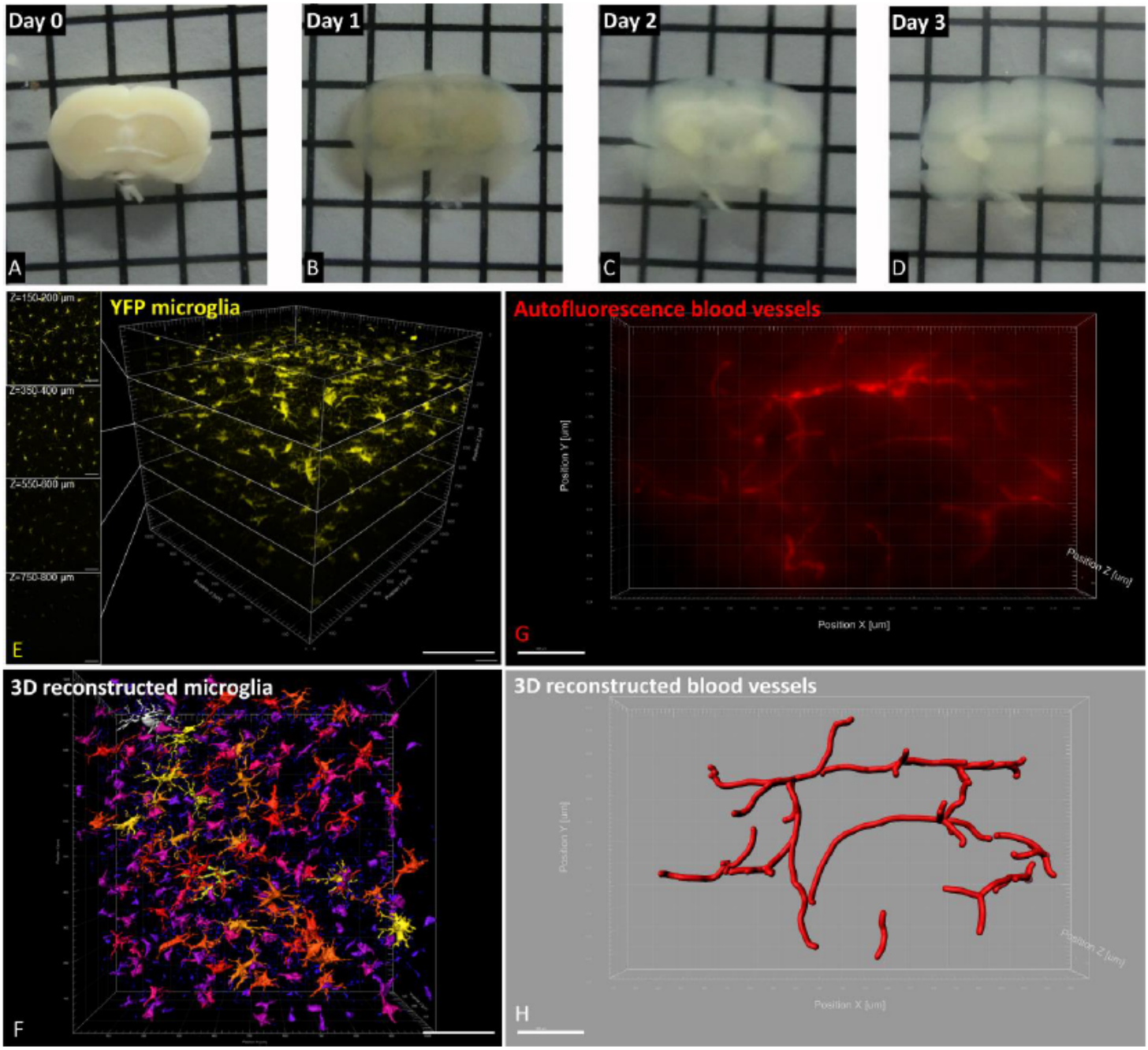

Figure 1

Mouse brain tissue clearing using the FACT. (A-D) The process of tissue clarification over time. Optical transparency is achieved within 3 days without almost no change in tissue size. The tissue is 1-mm adult mouse sagittal blocks and a maximum of 80% transparency is achieved. Squares are 3×3 mm2. (E) High-resolution imaging using confocal microscopy. Microglia expressing in cerebral cortex within 1024 × 1024 µm2 and at different depths of mice followed by the clarification with FACT protocol using confocal microscopy at 25x magnification. The data was obtained in almost 4 h; the left column of images displays the maximum-intensity projections over a 50-µm-thick volume at different depths and it was detected to a depth of 948 µm (each section in Z is 200 µm). (F) Three-dimensional microglia renovation in 400-µm-thick volumes of the cerebral cortex. The different surface regions of constructed microglia are

shown with the spectrum of colors which is composed by the Imaris Surface algorithm and ordered from white (highest area) to purple (lowest area) via Imaris Vantage. (G) Three-dimensional (3D) imaging of blood vessels in the mouse brain cortex using epifluorescence microscopy. 2000×1200×150 μm3 of brain cortex obtained from clarified mouse brain. The data was acquired in approximately 2 h. Blood vessels that have not been perfused, scattered the fluorescent signal from auto-fluorescent heme in red blood cells. (H) 3D segmentation of reconstructed vessels of the cortex is presented at the depth of 150 μm by Imaris software. The scale bar is 300 μm.

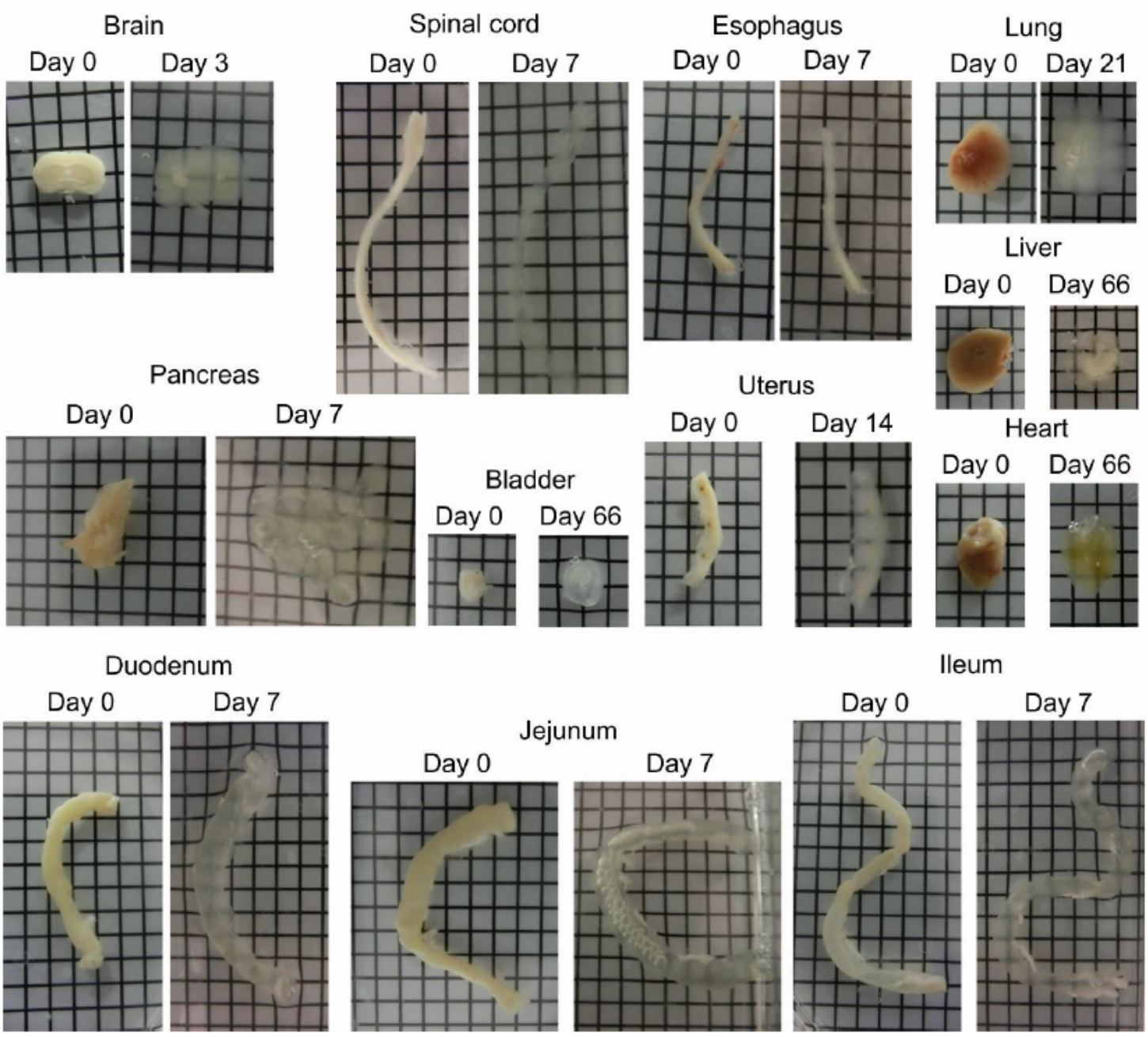

Figure 2

Cleared mouse tissues using the FACT. Mouse tissues including 1-mm slice of brain, spinal cord, esophagus, lung, pancreas, bladder, uterus, heart, duodenum, jejunum, ileum using FACT technique with

maximum transparency of 80%. Squares are 3×3 mm2.

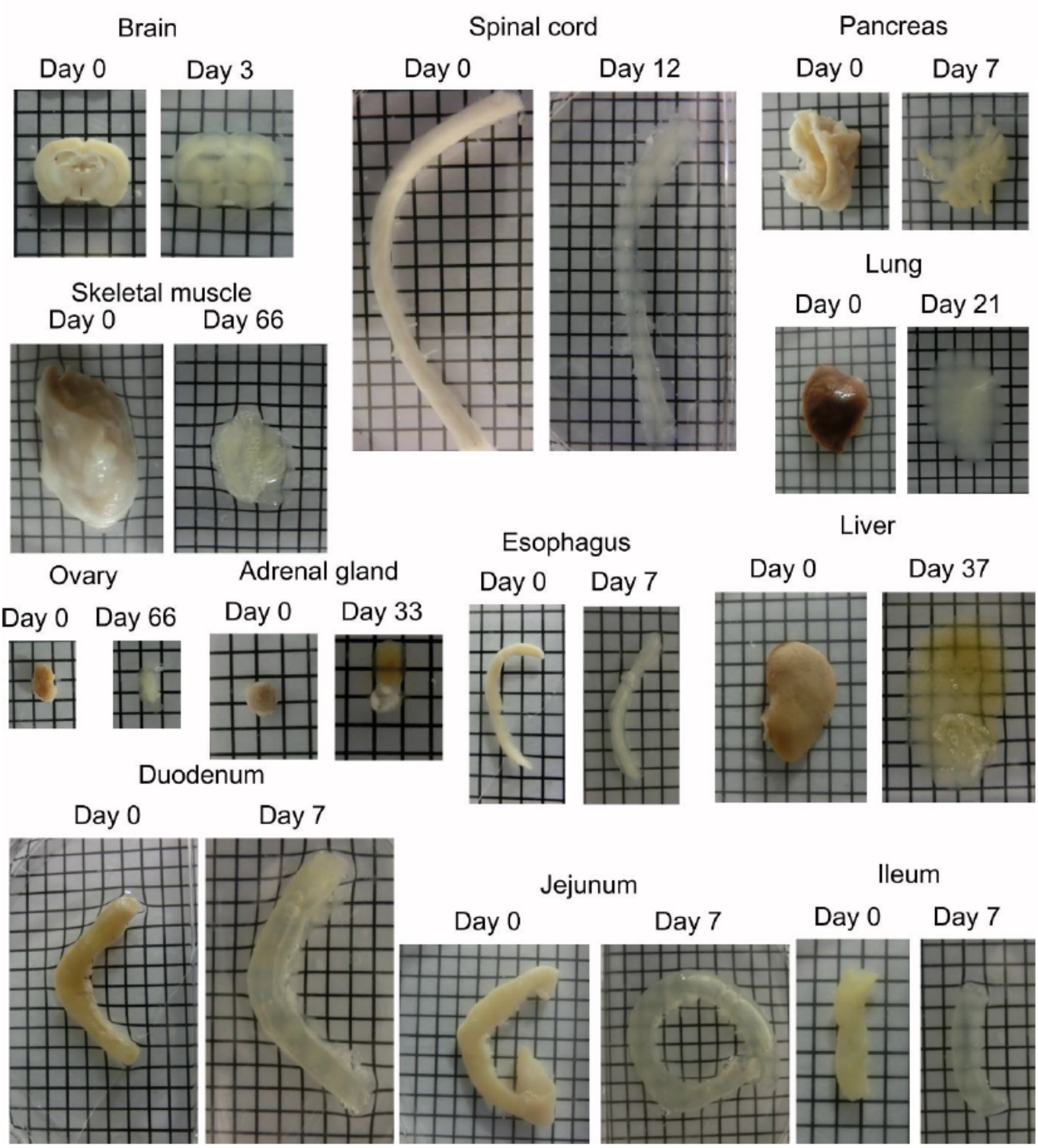

Figure 3

Cleared rat tissues using the FACT. Rat tissues including 1 mm brain slice, spinal cord, pancreas, skeletal muscle, lung, ovary, adrenal gland, esophagus, liver, duodenum, jejunum, and ileum using FACT technique with maximum transparency of 80%. Squares are 3×3 mm2.

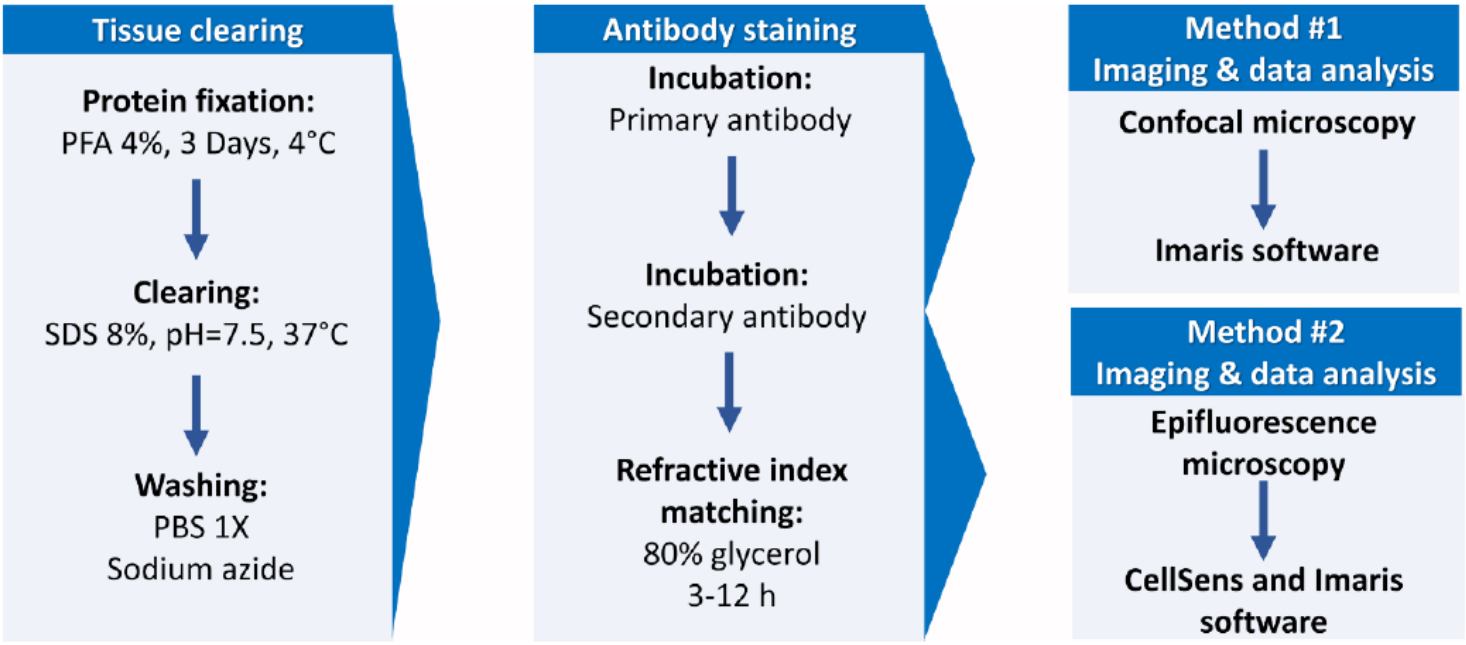

Figure 4

FACT overview. The tissue sample is fixed with PFA and then is cleared by SDS. After washing with the solution containing PBS and sodium azide, the next step begins by incubating the tissue with antibodies at 37°C. The resulting tissue can undergo multiple rounds of molecular and structural interrogation using immunohistochemistry and then it goes through testing the Refractive index matching (RIM) with glycerol. For imaging and data analysis, a confocal microscope is employed with Imaris software and an epifluorescence microscope is employed with CellSens and Imaris software. All animal experiments were performed with Institutional committee approval.

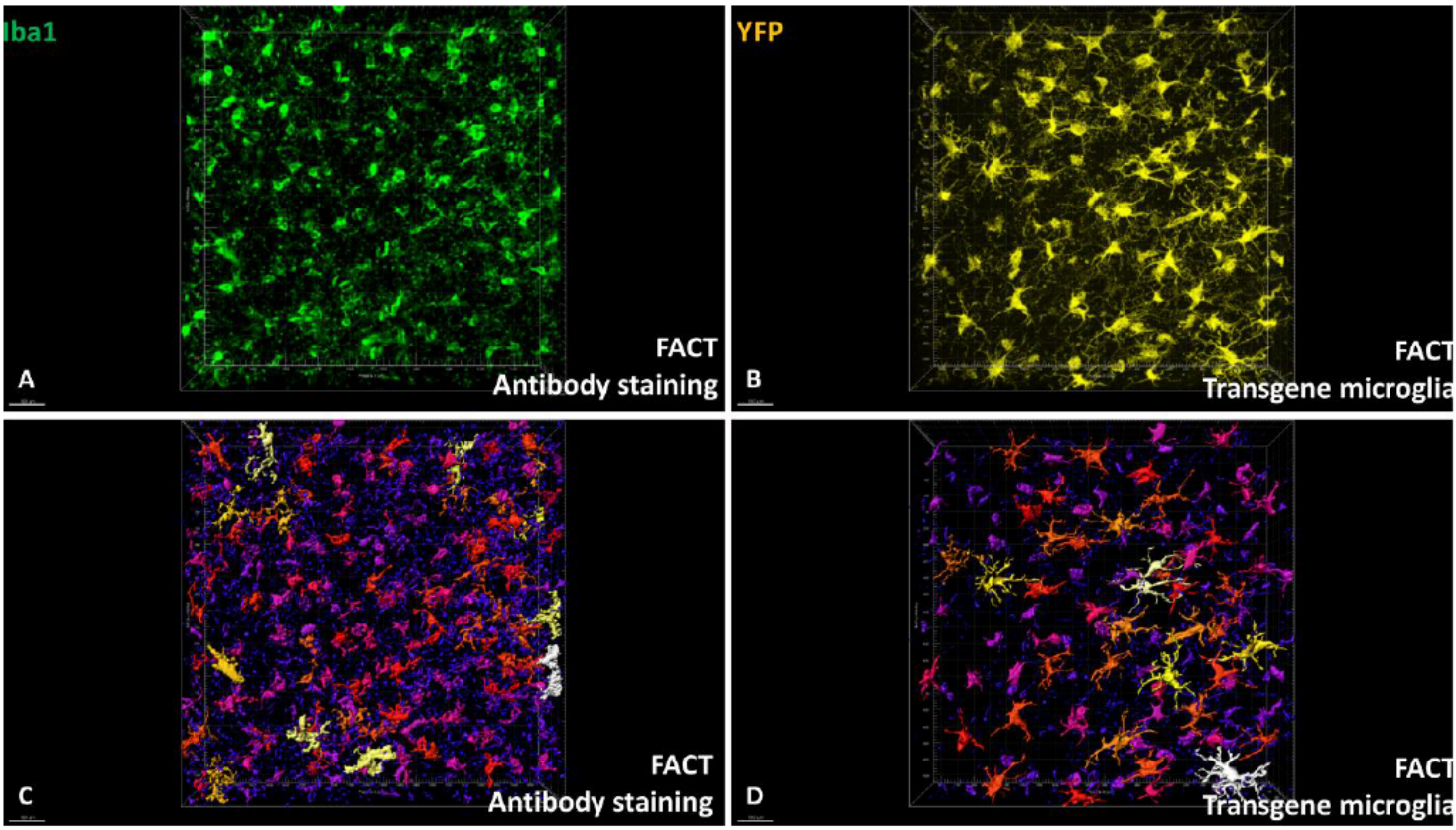

Figure 5

Antibody-based and transgene-based imaging of brain by the FACT protocol. The FACT protocol was remarkably effective for immunohistochemistry in both antibody-based and transgene-based imaging. 200-μm-thickness image with (A) antibody staining and (B) YFP-labeled microglia in transgenic mice. However, in comparing the (C) Iba1 immunostaining and (D) transgene-YFP-microglia, more microglia signal was detected within the Iba1 immunostaining image. Scale bars are 100 μm.

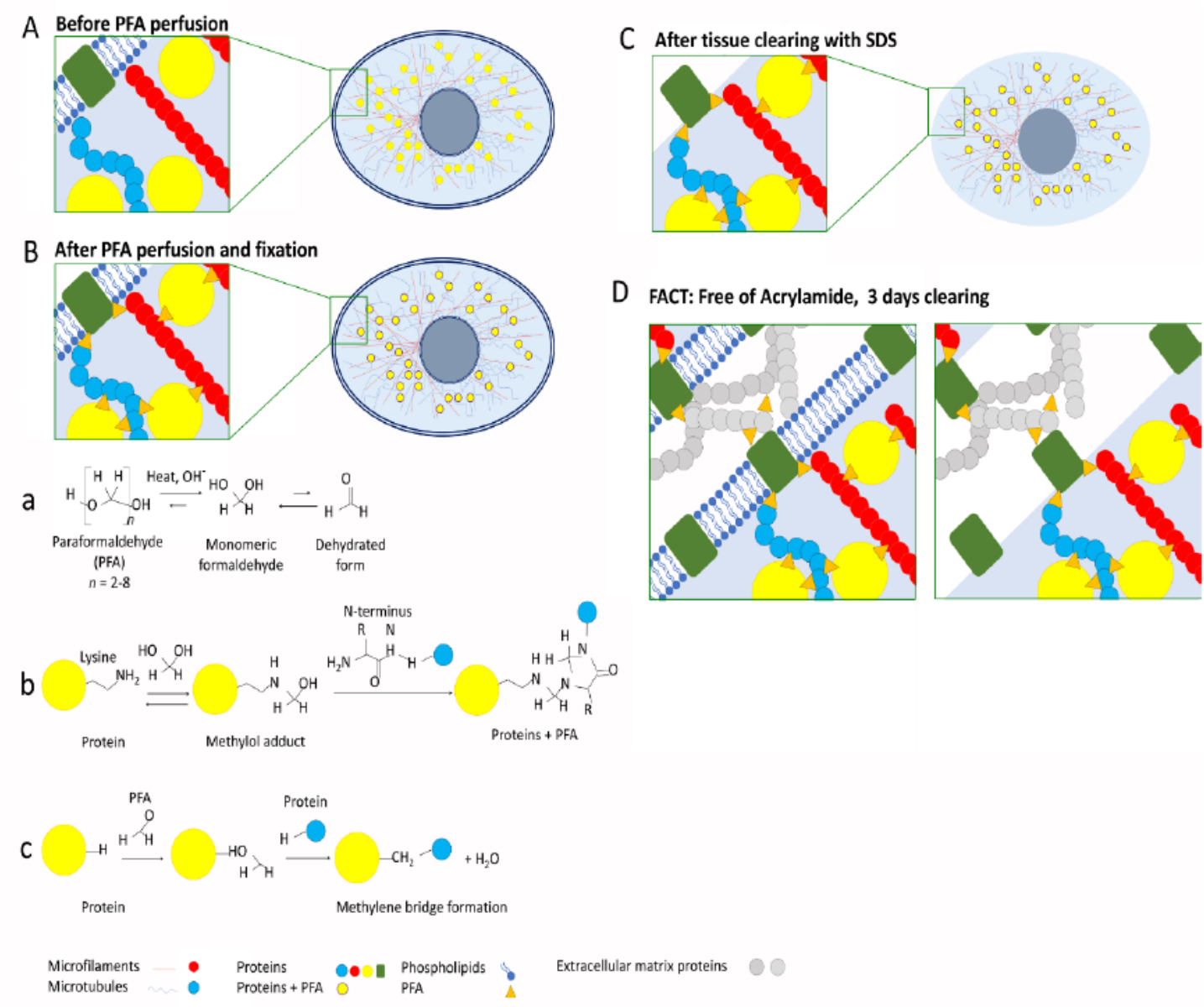

Figure 6

Basic mechanisms of the FACT. (A, B) During the fixation with paraformaldehyde (PFA), membranes and intracellular cytoplasmic proteins (including transgenic fluorescent proteins) bond chemically with cytoskeletons, including microfibers and microtubules, or with extracellular matrices such as proteoglycans. These bonds help build a massive three-dimensional matrix that provides structural support to the tissue. (C) After removing cell walls with 8% SDS at pH 7.5 (optimal pH for maintaining natural protein structures), PFA forms a chemical bond with tissue scaffolds. (D) Incubation of tissue with SDS speeds up and helps lipid clearance in this technique. (a-c) The assumed chemistry of this process is shown.