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Study on the transparent dFC-Tesos microscopic imaging method for large tissues in human brain

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Abstract

Objective: Human brain has features with high fat, high density, difficulty in deep and even labeling, and strong background fluorescence, which made it uneasy to get high-resolution imaging. To solve the above problems, this study successfully explored a new method for human brain transparency called dFC-Tesos by steps including human brain tissue electrophoresis degrease, antibody labeling, decolorization, transparency, three-dimensional high-resolution imaging, to clearly observe the spatial relationships and pathological changes between microglia and Aβ plaques in Alzheimer's disease.

Methods: The 3 mm thick slices of the superior temporal gyrus of the human brain were sectioned by a vibration slicer, then degreased in 4% SDS solution with 3 V, 0.01 A electric field until the brain slices were lightly permeable and the interface between gray matter and white matter was fuzzy. Antibodies of microglia were incubated and Aβ plaques were stained by thioflavin S. Additionally, to exclude endogenous fluorescence interference of the samples, the brain slices were decolorized by 8% ammonia, hydrogen peroxide containing 0.08% KOH and 25% *N*,*N*,*N*,*N*-tetra(2-hydroxypropyl) ethylenediamine (w/v), respectively, which successfully reduced the interference of vascular auto-fluorescence and improved the imaging quality. Human brain tissue transparency was accomplished by tert-butanol gradient degreasing, tert-butanol, and tetraethylenediamine dehydration, benzyl benzoate, bisphenol A ethoxylate diacrylate and tetraethylenediamine for refractive index matching. They were then imaged by LS18 lightsheet micro-imaging instrument with a resolution of 1.65 μm × 1.65 μm × 3.5 μm.

Results dFC-Tesos method was sequentially performed by electrophoretic degreasing of human brain samples, electrophoretic antibody labeling, decolorization, transparent human brain sample preparation, light-sheet microscopy, and splicing of big data to successfully obtain ultrastructural information and spatial location relationships of microglia and Aβ plaques in Alzheimer's disease (AD) brain, which provided methodological support for the study of new mechanisms of the pathology of AD. The dFC-Tesos method also provided an important tool for the study of macroscopic and ultrastructural structures of the pathological brain, the mechanism of major brain diseases, as well as the

study of drug targets, and so on.

Keywords: electrophoretic defatting, large tissue transparency of human brain, decolorization, electrophoretic antibody incubation, 3D imaging

Introduction

The traditional histological tools include gross dissection and tissue sectioning: gross dissection could observe organ location, morphology, and structure, but the internal structure was not observed; tissue sectioning could obtain cellular or subcellular structural information with the help of microscopy (Feng et al., 2000), however, sectioning destroyed the integrity of the tissue and no macroscopic continuous information could be observed. For larger and thicker *ex vivo* samples, the traditional labeling method would cut the tissue into thin slices and incubate slices in antibodies (Ullmann et al., 2013). While this method could only reflect the information of the local monolayer of the sample and could not obtain the overall sample information, as well as the spatial location relationship of various cellular proteins within the sample (Tainaka et al., 2018), also, the slicing process would cause damage to the sample and loss of information (Daeschler et al., 2023). The traditional microscopic imaging observation depth was only a few hundred microns, which was difficult to meet the needs of thicker tissues or intact organs imaging (Ke et al., 2016).

Technological advances had given birth to the transparency technique and such a method could observe the internal microstructure of tissues without destroying the organ (Zhan et al., 2023). In recent years, transparency had been used for large tissues before imaging (Pende et al., 2020). The principle of the transparency technique was twofold: firstly removed substances with large differences in refractive coefficients in tissues such as water and fat, and secondly, adopted a solution with a similar refractive coefficient to the tissue. For example, AliErturk et al. (2016), at the University of Munich, Germany, improved the previous uDISCO technique to develop the vDISCO method. It firstly soaked the whole body of mice in organic solvents to degrease and decolorize them, followed by pumping nano-antibodies into the circulatory system of dead mice to label specific cells with fluorescent antibodies, which ensured the structural integrity of the tissues and allowed fluoroscopic observation of mouse cells, the tissue and organ states, as well as their connections. However, the PEGASOS method could be applied to almost all types of tissues, including bones and teeth. Jing et al. (2018) applied the PEGASOS method to image the vertebrae after transparency treatment, which not only revealed the distribution pattern of nerves within the medullary cavity of long bones, but also helped to elucidate the relationship between the peripheral nervous system and the central nervous system.

However, the above studies were applicable to low-fat, non-dense organ tissues. Human brain has features with high fat, high density, difficulty in deep and even labeling, and strong background fluorescence, which made it uneasy for big sample transparency so as to get high-resolution imaging (Hama et al., 2011). For the human tissue brain, which had been grown for 70–80 years, there were few relevant reports in the literature because of its high-fat content, high denseness, and not easy to obtain. Our research group had the honor to explore the dFC-Tesos method in collaboration with the National Human Brain Bank for Development and Function for human brain tissues, which successfully immunolabeled and stained Aβ plaques and microglia in the supratemporal gyrus area of 3-mm-thick human brain tissue and achieved transparent microscopic imaging. This study successfully explored a new method for human brain transparency called dFC-Tesos by steps including human brain tissue electrophoresis degrease, antibody labeling, decolorization, transparency, three-dimensional high-resolution imaging, so as to clearly observe the spatial relationships and pathological changes between microglia and Aβ plaques in Alzheimer's disease.

Finally, it provided a useful tool for studying the mechanism of disease development from microstructure.

Material and method

1 Material

1.1 Reagents

Reagent	Company name	State	Country
SDS	Beijing Biorigin Biotechnology Co., Ltd	Daxing	China
TritonX-100	Sigma	Germany	Germany
Bovine serum albumin	Sigma	Germany	Germany
Anti-TMEM119	Abcam	Britain	Britain
Goat Anti-Rabbit IgG H&L (Alexa Fluor® 568)	Abcam	Britain	Britain
Thioflavin S	FEIYUBIO	Nantong	China
KOH	RHAWN	Shanghai	China
Ammonia solution	Beijing tongguang fine chemicals company	Chaoyang	China
H_2O_2	Luoyang Shangchun Biotechnology Co., Ltd	Luoyang	China
N,*N*,*N*,*N*-Tetrakis (2-hydroxypropyl) ethylenediamine	Shanghai Aladdin Bio-Chem Technology Co., LTD	Shanghai	China
Tert-butanol	Shanghai Aladdin Bio-Chem Technology Co., LTD	Shanghai	China
N,*N*,*N*,*N*-Tetramethylethylenediamine	Shanghai Aladdin Bio-Chem Technology Co., LTD	Shanghai	China
Benzyl benzoate	Shanghai yuanye Bio-Technology Co., Ltd	Shanghai	China
Bisphenol A ethoxylate diacrylate	Shanghai Macklin Biochemical Technology Co., Ltd	Shanghai	China
KH_2PO_4	Beijing Biorigin Biotechnology Co., Ltd	Daxing	China
KCl	Beijing Biorigin Biotechnology Co., Ltd	Daxing	China
DMSO	Beijing Biorigin Biotechnology Co., Ltd	Daxing	China
Ethanol	Beijing tongguang fine chemicals company	Chaoyang	China

NaCl	Beijing Biorigin Biotechnology Co., Ltd	Daxing	China
Na_2HPO_4	Shanghai yuanye Bio-Technology Co., Ltd	Shanghai	China

1.2 Human brain tissues

Tissues provided by: National Human Brain Bank for Development and Function, Chinese Academy of Medical Sciences, and Peking Union Medical College, Beijing, China. This study was supported by the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, Neuroscience Center, and the China Human Brain Banking Consortium. The experiment focused on the temporal lobe and was divided into two groups: Negative group (donor without Alzheimer's Disease) and Positive group (donor with Alzheimer's Disease). Negative group's temporal lobe was from brain tissue donor PTB484, male, 40 years old, PMD = 8 h. Positive group's temporal lobe was from donor PTB504, female, 94 years old, PMD = 8 h.

2 Method

2.1 Brain sample preparation

The formalin-fixed tissue of the superior temporal gyrus of the human brain was placed in a Petri dish, then the residual liquid was blotted with filter paper and the sample was glued to a vibrating microtome sample holder pre-coated with a small amount of 502 in the orientation required for the experimental design. The carrier table with the glued brain block was placed in the Cutting Solution buffer tray and the vibrating microtome (Leica VT1200S, Leica, Germany) was operated to cut the tissue surface flat and then sliced at 3 mm thickness. (Cutting Solution (in mM): 228 sucrose; 11 glucose; 26 $NaHCO_3$; 1 NaH_2PO_4; 2.5 KCl; 7 $MgSO_4$; 0.5 $CaCl_2$).

2.2 Defatting stage of human brain slices in the superior temporal gyrus

Since proteins were the target molecules for most biomedical research, it was necessary to use the defatting method to achieve tissue transparency. The more thorough the defatting was, the better the tissue transparency would be, especially in human brain tissue which contained high quality of lipid. Delipidation also removed obstacles to subsequent antibody incubation, allowing full antibody binding deeper into the tissues and achieving full antibody labeling in all parts of the tissue and organs.

When defatting brain slices, the defatting solution used was Sodium dodecyl sulfate (SDS) solution with a 4% concentration. SDS was an anionic surfactant with strong defatting ability, which moved directionally under the action of the electric field force generated by the electrode sheet inside the electrophoresis tank with the constant voltage or current provided by the linear DC power supply. In this regard, it could be defatting deep inside the sample tissue, and with the motor intelligent controller controlling the electric field direction reversal, the sample avoided deformation by unidirectional force, and the force was uniform in all parts, which could effectively preserve the original form of the sample. After the brain slices were put into the electrophoresis tank with 4% SDS solution, the linear DC power supply was turned on. Then, the required voltage, current, and electrode turning interval length were set to start the defatting. When the color of the sample tended to be "translucent, light penetrating the sample" from milky white, the state of no obvious internal structure texture was considered the end of defatting. The defatting conditions of the brain slices were 2.7 V, 0.01 A, 13 days, 13 nights, and 10 h, with the electric field reversing direction every 20 s. The samples before and after defatting were shown in Figure 1.

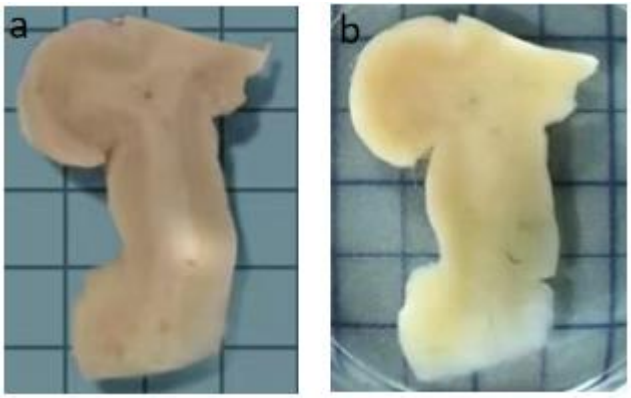

Figure. 1. Sample status before and after defatting.

Figure 1a shows the original sample, the border between grey and white matter can be seen clearly. Figure 1b shows the state after defatting, the border between grey and white matter was blurred.

2.3 Microglia immunolabeling and Aβ plaque staining in human brain slices from the superior temporal gyrus

After the defatting of brain slices, the immunolabeling stage was entered, which consisted of four parts: sample pretreatment, primary antibody incubation, secondary antibody incubation, and Aβ plaque staining.

Firstly, the brain slices were pretreated after defatting. All tissues of organisms contain a certain amount of endogenous enzymes and endogenous biotin and in immunohistochemical staining using affinity reagents, endogenous biotin tends to bind affinity and form affinity-biotin complexes, leading to false positives. Therefore, the sample was rinsed with 0.025% tris-buffered saline solution to close the sample endogenous peroxidase, conditions: 20 min/time, rinsed for 3 times, 4 °C ice water bath, 40 r/min on a shaker. After rinsing, the sample needs to be sealed with the closure solution before incubation, the reason was that antibody would be combined in other locations due to electrostatic adsorption and other non-specific binding. Bovine serum albumin contained in the closure solution was prepared in the principle that a protein was firstly added to bind as much as possible to the non-specific binding sites. Closure conditions: 4 °C ice water bath overnight, 40 r/min on a shaker. 4 °C was the most suitable temperature for antibody incubation and shaking the sample could be fully exposed to the closure solution. After the closure of the sample, tris-buffered saline solution was used again to rinse for 3 times, 20 mins each time in a 4 °C ice water bath with 40 r/min on a shaker. The aim of this procedure was to wash away the unbound solid BSA, barely leaking the antigen.

In the primary antibody incubation stage, the pretreated brain slices were placed into a semi-permeable bag and the bag was suspended in the antibody dilution solution, then, the bag orientation was adjusted so that the maximum cross-sectional area of the brain slices in the semi-permeable bag was placed parallel to the electrode sheet (perpendicular to the direction of the electric field), which enabled uniform and efficient use of the electric field and facilitated the full penetration of the antibody into the interior. Finally, when the linear DC power supply was turned on, the antibodies would move directionally under the electric field force, reversing the electrode direction back and forth several times, which facilitated the full incubation of the antibodies in the sample. The voltage in antibody incubation shall not be over 24 V, otherwise, it would cause protein denaturation and aggregation, which would precipitate out of the solution. The voltage could be reduced and the incubation time could be extended appropriately. Semi-permeable membrane bags with an MWCO of 3000 were selected and the antibody protein heavy chain molecular weight was 50,000~75,000 with the light chain molecular weight being about 25,000, so the antibody was trapped in the semi-permeable bag, which could save a lot of antibody compared with adding antibody directly to the electrophoresis tank. The antibody used for

immunolabeling microglia was primary antibody Ab185333 (0.1 μg/ml), electrophoresis conditions: 3 V, 0.01 A, 3 days and 3 nights, 5 ml/pc, 4 °C ice water bath, 40 r/min on a shaker.

In the secondary antibody incubation phase, the brain slices were rinsed 3 times with 0.025% tris-buffered saline solution for 20 min each time, 4 °C ice water bath, 40 r/min on a shaker. The secondary antibody incubation method was the same as the primary antibody with the incubation condition being: ab175471(2 μg/ml), 3 V, 0.01 A, 26 h, 5 ml/pc, 4 °C ice water bath, 40 r/min on a shaker.

For final Aβ plaque staining, brain slices incubated by microglia antibody were placed into 0.5% (g/100 ml) thioflavin-S dissolved in 50% ethanol and incubated for 12 h (e.g. 500 mg thioflavin-S + 20 ml DMSO + 80 ml 50% ethanol) under the following conditions: 3 V, 0 A, 12 h, 4 °C ice water bath, 40 r/min on a shaker. Then floating colors were washed with 80% ethanol for 10 s and transferred into 5 times the concentration of phosphate buffer saline solution at 4 °C for 1 h. Stored at 4 °C in the refrigerator, it can be used for transparent sample preparation. Brain slices after Aβ plaque staining are shown in Figure 2.

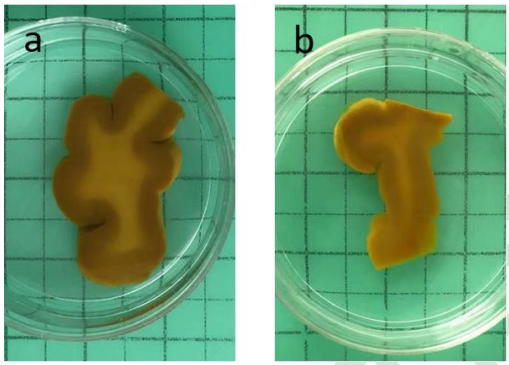

Figure 2. Brain slices after Aβ plaque staining, temporal lobe samples turned dark yellow in color.

Figure 2a shows temporal lobe tissue from the Negative group (donor without Alzheimer's Disease). Figure 2b shows temporal lobe tissue from the Positive group (donor with Alzheimer's Disease). Both of the samples had taken 17.5 days from sample defatting to Aβ plaque staining.

2.4 Transparent sample preparation of human brain slices in the superior temporal gyrus

Transparent sample preparation includes decolorization, gradient defatting, dehydration, and refractive index matching.

Decolorization

The brain slices need to be decolorized first due to endogenous fluorescence interference. The method was as follows: the brain slices were immersed in 20 ml of 8% ammonia, 37 °C constant temperature shaker, 100 r/min overnight, after which they were replaced with hydrogen peroxide solution containing 0.08% KOH, 37 °C constant temperature shaker, 100 r/min, 40 min. They were once again immersed in 20 ml of 8% ammonia overnight. Finally, the sample was decolorized with 25% *N*,*N*,*N*,*N*-tetra (2-hydroxypropyl) ethylenediamine (w/v) in aqueous solution for 48 h. After the above steps, the interference of vascular auto-fluorescence was successfully reduced and the imaging quality was

improved (Figure 3).

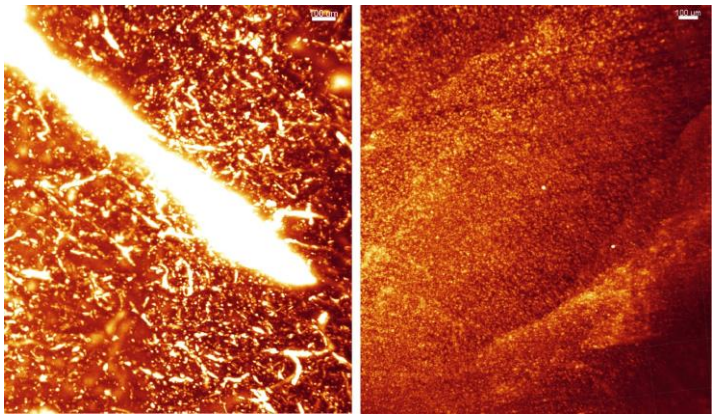

Figure 3. The first figure shows a sample without decolorization, the white zones were endogenous fluorescence of blood vessels, which affected the recognition for microglia marked by TMEM119. The second figure shows the sample after decolorization. The details could be seen more clearly without the endogenous fluorescence of blood vessels.

Gradient defatting

The 30%/50%/70% defatting solution consisted of 30%/50%/70% (v/v) tert-butanol (Aladdin, L2128284) and 5% (w/v) tetra (2-hydroxypropyl) ethylenediamine in aqueous solution respectively. After decolorization, the brain slices were sequentially immersed in a gradient of 30%/50%/70% defatting solution. The duration of this process was 11 days and 11 nights, which could be assisted by adding collagenase II (0.2 mg/ml) to the 70% tert-butanol solution because the white matter region of the brain slices was not easily transparent. The whole process was performed at 37 ℃ on a constant temperature shaker at 100 r/min.

Dehydration

Dehydration solution consisted of 70% (v/v) tert-butanol and 30% (w/v) tetraethylenediamine, dehydrated for 2 days, 20 ml/pc, 37 ℃ constant temperature shaker, 100 r/min, refresh the solution per day.

Refractive index matching

Finally, the sample was placed in the refractive index matching solution which consisted of 47% (v/v) benzyl benzoate (Sigma W213802), 48% (v/v) bisphenol A ethoxylate diacrylate (BED468) (Sigma 413550), 5% (v/v) tetraethylenediamine (Aladdin, G2131334), followed by the addition of 2% (w/v) 2-hydroxy-4-(2-hydroxyethoxy)-2-methylpropiophenone (Sigma, 410896) as UV initiator. The matching conditions were 3 days, 20 ml/pc, constant temperature, shaker at 37 °C, 100 r/min, refresh the solution per day, until the brain slices were transparent and homogeneous for imaging. The state of the brain slices after transparency was shown in Figure 4. The sample treating processes were shown in Figure 5.

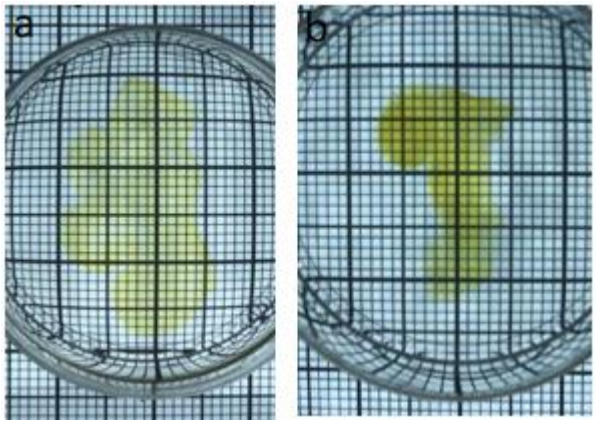

Figure 4. After brain slice transparency, the sample became transparent.

Figure 4a shows temporal lobe tissue from the Negative group. Figure 4b shows temporal lobe tissue from the Positive group. Both of the tissues had taken 38.5 days from sample defatting to be transparent.

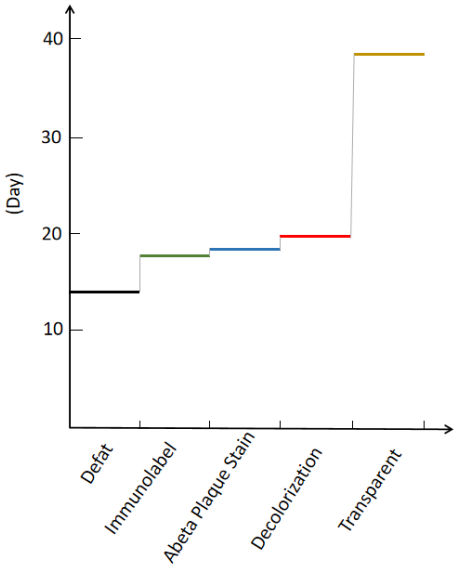

Figure 5. Shows the relationship between sample processing and time.

2.5 LS-18 micro-optical imaging demonstration

The brain slices of human were prepared into a transparent state and imaged by LS-18 3D microscope, with imaging resolution 1.65 × 1.65 × 3.5 μm. After spliced with the self-developed software TD Combiner 1.0 for large data splicing and 3D reconstruction, the ultrastructure of microglia and Aβ plaques in the superior temporal gyrus part of Alzheimer's brain were shown in Figure 6. We used antibody Iba1/AIF-1 together with antibody TMEM119 to observe their co-location to verify the specificity of antibody TMEM119 (Figure 7).

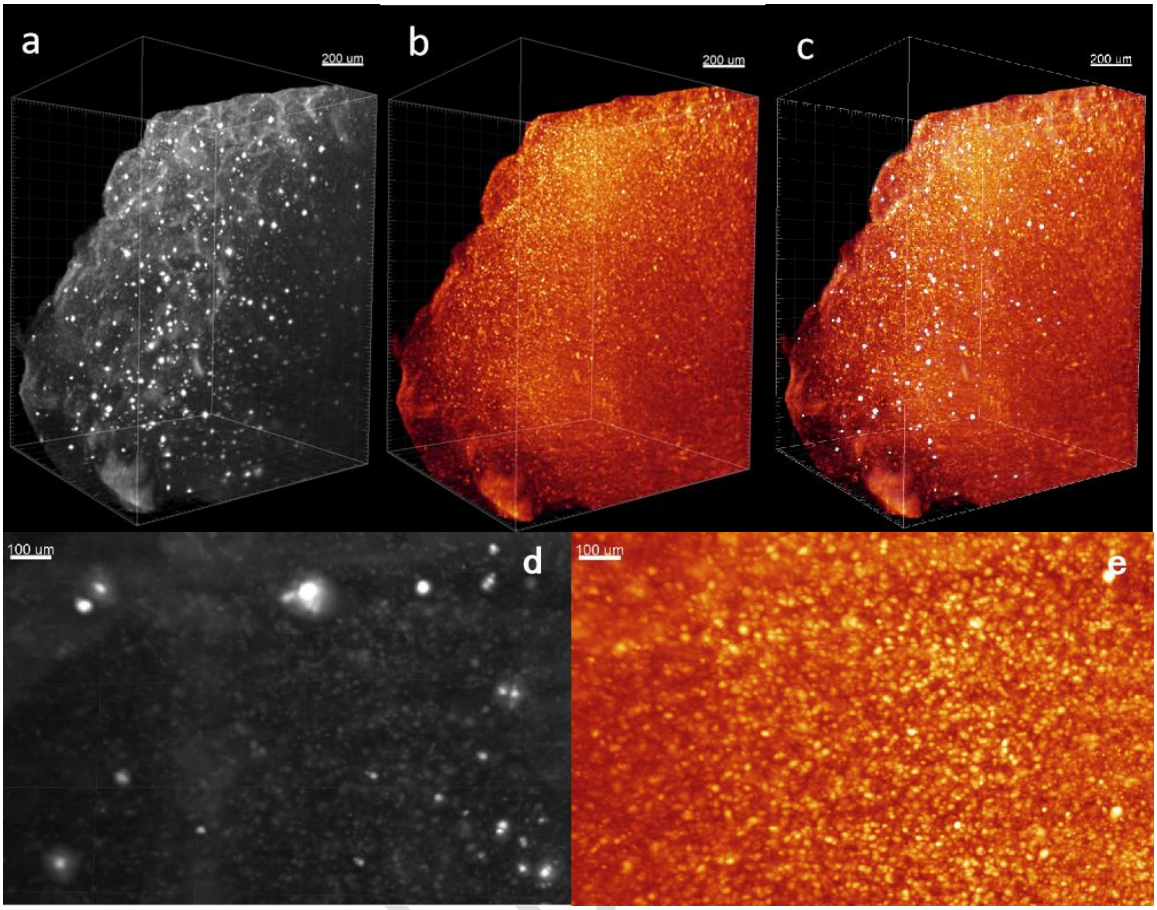

Figure 6. Microscopic morphology of Aβ plaques and microglia in Alzheimer's brain.

Figure 6a shows Aβ plaque (scale bar 200 μm). Figure 6b shows microglia (scale bar 200 μm). Figure 6c shows Aβ plaque with microglia (scale bar 200 μm). Figure 6d shows Aβ plaque (scale bar 100 μm). Figure 6e shows microglia (scale bar 100 μm).

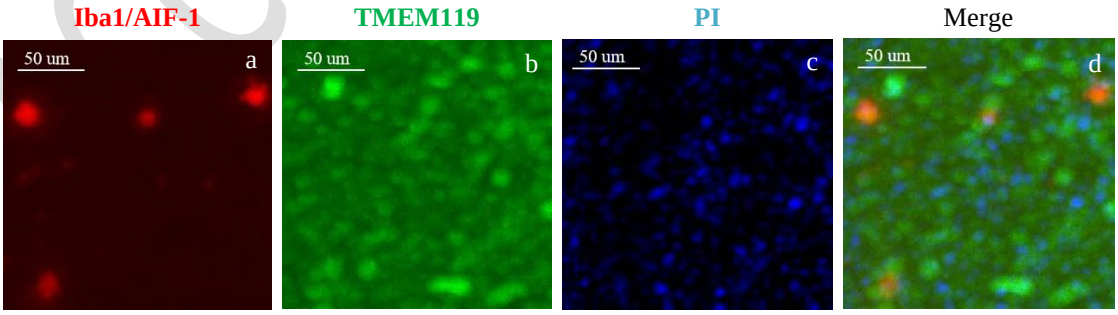

Figure 7. Microscopic channel images after Iba1/AIF-1 together with antibody TMEM119 staining and the image after merging. Figure 7a shows Iba1/AIF-1 antibody channel (scale bar 50 μm). Figure 7b shows the TMEM119 antibody channel (scale bar 50 μm). Figure 7c shows the PI channel (scale bar 50 μm). Figure 7d shows the image after three channels were merged (scale bar 50 μm).

Results

This method successfully completed the microglia antibody incubation and Aβ plaque staining of 3 mm thick human brain superior temporal gyrus brain slices, and achieved quantitative statistics of microglia and Aβ diameters, volumes, and spatial location relationships by identifying the images. dFC-Tesos method, by immersing in a 4% SDS solution with strong lipid removing ability and by applying the electric field, allowed lipids removed deep inside the human brain sample, realized micro-punching of biological cell membrane phospholipid bilayer, and opened a channel for antibody labeling deep in the tissue sample. The relationship between sample processing and time was shown in Figure 5. The traditional antibody incubation could only achieve thin sample labeling, we achieved antibody incubation for the thick human brain sample by applying an electric field. In addition, to exclude the endogenous fluorescence interference of the samples, the brain slices were decolorized by 8% ammonia, hydrogen peroxide containing 0.08% KOH, and 25% *N*,*N*,*N*,*N*-tetra(2-hydroxypropyl)ethylenediamine (w/v) respectively, which successfully reduced the interference of vascular endogenous fluorescence and improved the imaging quality.

The dFC-Tesos method overcame the problems of strong endogenous fluorescence of human brain blood vessels, high-fat content, difficulties in transparency, dense human brain tissue, uneven incubation of antibodies in thick samples, and the inability of antibodies to penetrate into tissue deeply. It greatly broadened the depth and size of antibody labeling in human brain tissue and this experiment also provided important imaging data for the field of Alzheimer's disease research. Moreover, the method would also provide an important means for study on heavy brain disease mechanism and drug target.

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