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Optimizing Tissue Clearing and Imaging Method for Human Brain Tissue: Preparing Methods, Clearing Efficiency, Staining Methods, and Imaging Strategy

사람 뇌 샘플을 위한 조직 투명화 및 시각화 기법 최적화

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Abstract

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Currently, the tissue clearing method is actively used in neuroscience research. Recent advances in tissue clearing method allow visualization of neural networks inside the unsectioned whole brain tissues. However, a protocol applicable for human brain tissue has not yet been established. Our goal is to optimize human brain clearing and imaging methods using fresh human samples.

Two types of human brain samples were used: Fresh samples and cadaver samples. Fresh samples were obtained at autopsy

and fixed for about one week. Cadaver samples were obtained for medical education and research and fixed about two years post mortem. Active electrophoretic clearing was used for human brain tissue. The degree of transparency, artifacts (causing auto fluorescence and scatter phenomenon) and quantity of DAPI staining were quantified using Image J. DAPI (Nucleus), Lectin (Blood vessel) and GFAP (Astrocyte) were stained for 4 days (4DP), 11 days (11DP) in PBS. Commercial staining kit (DeepLabel staining kit, 4DP-C) and staining method which is using the electrophoresis device (perfectSTAIN, 1DA) were used to human brain tissue. The staining efficiency of various staining strategy was compared (staining time, antibody penetration depth, complexity of the staining method, degree of tissue contamination and cost). Confocal microscope was used to investigate staining depth.

Fresh samples showed higher clearing efficiency compared to cadaver samples (time required for 80% transparent; 4 hr vs. 40 hr). Fresh samples showed fewer artifacts compared to cadaver samples. Regarding the antibody staining efficiency, fresh and cadaver samples showed similar penetration depth (about 65um). In fresh samples, DAPI was stained over 500μm in depth after 16hrs of incubation. The DAPI fluorescent brightness of the surface was increased in accordance to the incubating duration. Samples showed deeper GFAP staining depth when they were incubated longer in PBS buffered antibody solution. In the results of antibody penetration depth, 11DP showed a trend of deeper staining compared with 4DP (4DP: 65um vs 11DP: 125um). Furthermore, 4DP-C showed significantly deeper staining compared with 4DP (P=0.006) (4DP: 65um vs. 11DP: 125um vs. 4DP-C: 160um). Although statistically not significant (n=1), the using electrophoresis device showed highest efficiency.

In conclusion, we have successfully visualized human brain 3D

microstructure with optimized clearing and staining methods. The present study provided and compared various cleared specific and protocols for human brain clearing and staining. The most optimized human brain clearing method of the present study will open new paradigm for disease diagnosis and human brain research.
Key words: Human, Brain, Tissue Clearing, 3D imaging
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Contents

List of Tables

List of Figures

Introduction

Brain is the most functionally and structurally complex organ in the human body. Different anatomical brain regions are complexly connected with different types of neurons, and each region influences one another and creates conscious or unconscious behavior. Therefore, understanding the 3D structure and connectivity is prerequisite for brain research. Furthermore, brain is the pathogenetic cause of various neurodegenerative diseases. In order to overcome various devastating diseases, studies of the human brain must be performed. The attempt to understand the function/structure of human brain has been pursued for a long time. but However, there has been huge constraints due to several technical limitations. The main reason is that it is problematic to obtain sufficient human brain samples due to ethical problems, legal problems and accessibility. Therefore, the practical methods for human brain study has not been sufficiently developed compared with other species (such as rodents) or other organs.

Recently, several imaging techniques including confocal microscopy and tissue clearing technology have been developed [1]. It enables researchers to observe various tissues including the brain in a 3-dimensional image [2-6]. The new era has opened in which the tissues can be investigated without sectioning [7]. These tissue imaging techniques are now being used extensively, possibleallowing researchers to observe the structure and connectivity of large tissue samples in 3D, which was not feasible with sectioned tissue. Nowadays, tissue clearing

technology has been essential in numerous studies published in leading journals [2].

Various tissue clearing techniques have been developed to allow visualization of tissues' 3D image including confocal microscope [2,8-10]. In particular, CLARITY(Clear, Lipid-exchanged, Acrylamide-hybridized Rigid, Imaging/immunostaining compatible, Tissue hYdrogel) is the novel technique that is highly utilized in biological research [1,11]. In CLARITY, the formaldeghyde-based fixative tissues are infiltrated with hydrogel to form biomolecule crosslinking. Then polymerizing the tissue to make stable the tissue structure, furthermore this process allows deeper diffusion of antibodies and other labeling probes. Subsequently, reducing the lipid molecules using ETC (electrophoretic tissue clearing) chamber that accelerates the extraction process. Once the lipid has been removed, the tissue becomes transparent, which makes it suitable for 3D observation with a confocal microscope or a light sheet microscope. So far, a lot of published reports has been described the successful use CLARITY particularly in fixed rodent brain tissue, because there is a way to observe without staining such as fluorescent transgenic mouse or fluorescent virus injected mouse [9].

However, there are few articles which applies tissue clearing technique to human brain. Lately, several studies have reported initial preliminary results using tissue clearing technique in human brain and investigated pathogenesis of Alzheimer's [8,12], Lewy body [13] and neurodevelopmental [11,14] diseases in 3D. Most studies have been conducted on cadaver samples. Although fresh samples and cadaver samples were used in several papers [2,13], the studies did not quantitatively compare the clearing

time, artifacts (including auto fluorescence and scattered fluorescence) level and staining efficiency [2]. In addition, there are only few studies, directly comparing antibody staining, which is thought to be the bottle neck in human brain tissue clearing research [15]. Due to these limitations, till these days, there is no established recommended method for applying clearing technique in human samples.

The aim of the present study was to propose optimal sample selection and staining strategies for human brain tissue clearing study. To do this, our study compared the clearing efficiency and artifact level of the donated cadaver samples with the samples obtained at autopsy. More importantly. staining efficiency using antibodies and chemical dyes was compared between both samples. Furthermore, various staining methods were compared and evaluated. Several staining methods were applied to the human brain and scored according to several comparison aspects (staining time, staining depth, complexity of method, tissue contamination, cost). The results will provide optimized protocols for human brain 3D histological investigation using tissue clearing.

Methods

Acquisition and storage of human brain tissue

Collection and analysis of autopsied human specimens were approved by the Institutional Review Board for Human Subjects Research and carried out by a certified staff in the Department

of Forensic Medicine of the Seoul National University (Seoul, Korea) following the standard operating procedure for conducting autopsies. The collection of 8 autopsied cortex samples was performed within 80h post mortem, as follows. After the autopsy procedures, the following information was collected from the Department of Forensic Medicine of the Seoul National University (Seoul, Korea): gender, age at death, height, weight, cause of death, estimated postmortem interval. A small portion of cortex from each individual was fixed 4%PFA for 1 week before being stored in PBS solution with 0.02% sodium azide.

The cadaver brain tissue samples were given by the Department of Anatomy at Seoul National University College of Medicine. Brain cortex tissue blocks of cadavers were stored in 4% PFA for more than 1 year. Tissue blocks were immersed in PBS solution with 0.02% sodium azide before proceeding (Table 1,2).

Tissue Clearing

The tissue blocks were manually sectioned (~1mm in thickness) using razor blades and were incubated in hydrogel for 1 day. Polymerization process was then operated for 3hrs using X-CLARITYTM Polymerization System (-90kPa). The tissue blocks were then washed with PBS for 3 hours and were cleared using X-CLARITYTM. The fresh brain samples were cleared for about 30 hours and the cadaver samples were cleared for approximately 50 hours.

Immunofluorescence and Chemical Dye Staining

Four different types of staining methods were applied. The first

method was “4 days passive staining” (4DP). Pre-processed samples were incubated in primary antibody solution (1:500 GFAP, 0.02% sodium azide in PBS) for 4 days, washed in PBS for 2 days, incubated in secondary antibody solution (1:250 Rabbit 488nm, 1:1000 DAPI, 1:500 Lectin) for 4 days, and was washed in PBS for 2 days. The second method was “11 days passive staining” (11DP). Every step was same as in 4DP method except for the primary antibody solution incubation time (11days) and the secondary antibody solution incubation time (11days). The third method was “DeepLabel kit” (4DP-C; 4 Days Passive staining with Commercial staining kit). The brain tissue samples were immersed in permeabilization buffer for 2 days. The samples were then incubated in Primary antibody solution (1:500 GFAP in DeepLabel kit staining buffer) for 4 days, washed in PBS for 2 days, incubated in Secondary antibody solution (1:250 Rabbit 488nm, 1:1000 DAPI, 1:500 Lectin in DeepLabel kit staining buffer), and washed in PBS additionally for 2 days. The final method was perfectSTAIN (1DA; 1 Day Active staining with a commercial device). 1DP method used a electrophoresis device, which pushpushes antibody into the tissue in bidirectional manner. The samples were incubated in saponin for 10 minutes, washed in PBS for 10 minutes, then washed in 10% Bovine Serum solution for 30 minutes. Next, the samples were minimally trimmed and were put inside the Perfect Stain™ with perfectSTAIN chemical dye solution (1:1000 DAPI, 1:500 Lectin in perfectSTAIN staining buffer). The samples were stained by the Perfect Stain™ for 1 hour and were incubated in perfectSTAIN chemical dye solution overnight (Table 3).

Image acquisition and Data Analysis

The imaging of stained tissue was performed using a Carl Zeiss LSM 800 Confocal Microscope (Carl Zeiss, Oberkochen, Germany) at the Cancer Research Institute in Seoul National University and Leica TCS SP8 Confocal Microscope (Leica microsystems, Wetzlar, Germany) at the Center for Medical Innovation in Seoul National University. After being stained, the samples were submerged in the mounting solution for 4 hours for the homogenization of the refractive index. The images were then acquired by the confocal microscope using the laser excitation wavelengths of 405nm, 488nm, 594nm, 647nm. A x10 objective (working distance 200-500um, imaging interval 0.9-2.0um), and a x20 objective (working distance 200um, imaging interval 2.0um) was used.

Image visualization was performed using Zen Blue (Carl Zeiss, Germany) software and LAS X (Leica microsystems, Germany); 3D renderings were performed using Zen Blue, LAS X and Imaris (Bitplane, Belfast, UK) software.

Calculating Tissue Opacity

The opacity of cleared samples was evaluated using Image J program. The images of sample before clearing and after clearing were trimmed as 1cm x 0.8cm sized rectangles. The raw opacity of each rectangle was estimated as the closed area of xy-plot shown in Gel Analyzer (x-axis indicating the x coordinate, y-axis indicating the opacity per unit length). The percentage opacity was then enumerated by using this solution: (Raw Opacity of the Sample Before Clearing) / (Raw Opacity of the

Sample After Clearing). The percentage opacity of each sample was interpreted as the clearing efficiency of the sample.

Calculating Artifacts and DAPI Staining Efficiency

The artifacts (auto fluorescence or scatter phenomenon due to microscopic lasers) of individual sample were assumed to be proportional to the brightness of 3 channels' color. Also the DAPI staining efficiency of individual sample was assumed to be proportional to the brightness of DAPI signal. The 3 channels' color and the brightness of DAPI signal was quantified as the product of signal intensity and the area of the signal, and the "IntDen" value (the product of Area and Mean Gray Value) of Image J was used to derive this quantified value. In other words, the "IntDen" value of each sample was interpreted as the artifacts and the staining efficiency of the sample.

Results

Comparing Clearing Efficiency of Fresh and Cadaver Samples

Fresh samples required 4 hours and cadaver samples required 40 hours to obtain 80% transparency (20% opacity) (Figure 1A). Quantification was not feasible beyond 5% of transparency in fresh samples, due to dissociation and unstable quantification (Figure 1B). Both fresh and cadaver samples showed slight swelling. Yellow color persisted even after clearing in cadaver

sample (Figure 1C). Taken together, these results show that the fresh samples are superior in clearing efficiency than the cadaver samples in terms of time and quality.

Comparing Artifacts Level of Fresh and Cadaver Samples

In fluorescence imaging, “signal to noise” is important because it can cause real signals to become embedded among noises. Artifacts (auto fluorescence or scatter phenomenon due to microscopic lasers) interfere image acquisition. Particularly, since human tissues have more auto fluorescence than mouse tissues, auto florescence should also be considered in imaging research. Our study compared the artifacts of fresh sample with cadaver sample.

With the same clearing time of two samples, the degree of artifacts was quantified. Both fresh and cadaver samples removed lipids from the ETC chamber for 14 hours. Comparing the fresh samples with the cadaver samples (Figure 2A), the cadaver samples have higher artifacts. However, when the clearing time is fixed, the clearing level of the two samples is different (since the cadaver samples take longer to become transparent).

With the same clearing efficiency of two samples, the degree of artifacts was quantified. Fresh and cadaver samples were both set to 20% opacity (Figure 2B). The fresh samples and the cadaver samples showed various aspects of artifacts (Figure 2C). The level of artifact (integrated density) tended to increase with increasing opacity (Figure 2D). Cadaver samples showed significantly higher artifact level compared with fresh samples

($P<0.05$) (Figure 2E). The above results conclude that, the fresh samples have less artifacts than the cadaver samples.

Comparing Staining Efficiency of Fresh and Cadaver Samples

To compare the staining efficiency between fresh and cadaver samples, we stained DAPI and GFAP according to 4DP.

Comparing the staining results of two samples with same clearing times, no DAPI staining observed in cleared cadaver samples (Figure 3A).

To investigate if similar phenomenon is observed in conventional sectioned slide (not cleared), we performed DAPI staining in both conventionally sectioned cadaver sample and conventionally sectioned fresh sample. Using 30um thick sectioned slide sample, no DAPI staining was observed in cadaver samples, but DAPI stating was observed in fresh samples (1-2 um thickness sample and 30 um thickness sample) (Figure 3B). GFAP staining was well observed in both groups. (Figure 3C, D).

Comparing the staining results of two samples with a same clearing efficiency of about 20%, no DAPI staining observed in cadaver samples (Figure 3A). On the other hand, fresh samples were sufficient stained with DAPI. GFAP staining was well observed in both groups (Figure 3E, F). We measured the depth of GFAP staining to quantitatively evaluate the penetration efficiency. There was no significant difference between fresh samples (67.86um) and cadaver samples (65.85um) (Figure 3G). The degree of fixation (cadaver vs. fresh) of the sample was not related to the antibody penetration efficiency.

Research in our study indicates that the fresh sample has faster clearing speed, less artifacts, and better DAPI staining than the cadaver sample. However, the penetration depth of the antibody was the same for both samples. Therefore, we conclude that it is more efficient to use fresh samples for tissue clearing study (Table 3).

Postmortem Interval (PMI) and Staining Quantity

Large amount of heterogeneity was observed regarding staining quantity. Postmortem interval (PMI), which is the interval between time of death and time of tissue collection, could be an important determinant of staining quantity. PMI showed a tendency toward negative correlation with staining quantity (r^2=0.3,P=0.2), although statistically not significant (Figure 4).

Penetration Depth of DAPI Staining in Fresh Human Brain

DAPI (4′,6-diamidino-2-phenylindole) is a chemical dye that stains nucleic acid. DAPI staining is known to penetrate efficiently from superficial to deep tissue because it has nanoscale size. However, the exact depth of DAPI staining over time has not been studied precisely especially in human brain tissue. We stained the human cerebral cortex with DAPI by serial time schedule (1hr, 2hr, 4hr, 8hr, 16hr and 32hr).

After analyzing up to 600um, it was observed that when DAPI over 16hr was incubated, DNA staining was more than 500um depth (Figure 5A). Since the intensity of the laser is lowered and

the brightness of the fluorescence is not uniform as the image is deepened, the depth comparison is post-processed with the optimum brightness and contrast of each stage. We also confirmed that not only the depth but also the fluorescence brightness of the sample surface can be improved by incubation for a long time (Figure 5B). For a more accurate comparison, the surface image of the sample was quantified using Image J's integrated density function and the heat map was drawn (Figure 5C).

These results suggest that DAPI can be imaged more than 500um when incubated for more than 16hr, and the brightness of the surface is brightened by longer incubation time.

Comparing the Antibody Staining Methods (4DP, 11DP, 4DP-C and 1DA)

The antibody staining is crucial for brain research, because it enables specific protein quantification and imaging. We conducted a comparison between different staining methods using fresh samples (since fresh sample were more suitable for tissue clearing research in the above results).

We compared the staining efficiency of the 4DP, 11DP, 4DP-C and 1DA. (Table 3) Each staining method was compared with several aims such as staining time, antibody penetration depth, staining method's complexity, and level of tissue contamination and cost (Figure 6A).

Staining time was the shortest for 1DA (total procedure time = 4 days). In the results of antibody penetration depth, 11DP showed a trend of deeper staining compared with 4DP (4DP: 65um vs

11DP: 125um) (Figure 6B, C). Furthermore, 4DP-C showed significantly deeper staining compared with 4DP (P=0.006) (4DP: 65um vs. 11DP: 125um vs. 4DP-C: 160um) (Figure 6C, D). Although statistically not significant (n=1), 1DA showed highest efficiency (Figure 6C, D).

For the complexity of the staining method, all of the passive staining methods are relatively easy because all methods do not require special devices. Using 1DA is most difficult because of using electrophoresis device and making the gel for staining. For the cost, 1DAis most expensive because of commercially available electrophoresis device (Table 5).

Visualizing 3D Structure of Human Brain

Using tissue clearing technique, the image of human brain astrocyte (GFAP), nucleus (DAPI) and blood vessel (Lectin) was visualized (Figure 6A). In a previous study, human brain astrocyte showed more complex structure compared with rodent astrocyte. 18 Similarly, the human brain astrocyte in the present study showed extremely complex structure (Figure 6B). The blood vessel was observed up to a depth of about 800um and the blood vessel branch structure was satisfactorily visible (Figure 7C).

Discussion

We developed the optimal tissue clearing and staining protocol for human brain samples. Fresh samples are more suitable than

cadaver samples, for tissue clearing experiments. We could determine a superior staining method by comparing staining time, antibody penetration depth, level of method complexity, and cost, among various staining methods.

To our knowledge, this is the first human brain clearing study to quantify and compare the clearing and staining protocols. A recent study reported preliminary tissue clearing results for human brain [13]. Both this study and the present study used fresh and cadaver samples. However, the previous study investigated staining methods for fresh sample and cadaver samples without quantification and direct comparison. The present study vigorously quantified the time, depth and efficiency of antibody and chemical staining. The previous study used only traditional buffer. However, the present study investigated various staining methods (traditional buffer, commercial buffer kit, and electrophoresis device) and provided technical comparisons. Other difference was that the clearing method was different. The previous study used OPTIclear. The present study used X-CLARITY. Other previous studies reported several preliminary human clearing images [2]. However, these studies did not quantify or compare methods. The number of samples used was relatively smaller than the present study (previous studies = 1-5 vs. the present stud = 15 samples). The previous study used only biobank samples. However, since we performed the sample harvest and preparation stage in our lab, the present study could directly investigate the importance of human brain sample preparation for human clearing study.

We speculate that difference in clearing efficiency between fresh and cadaver samples could be resulted from difference in fixation

time. The prolonged fixation time of cadaver samples could be the cause of delayed clearing time (about 2 years fixation for cadaver and about 1 week fixation for fresh sample). Prolong fixation time could cause over-crosslinking of molecules with methylene bridge, which could delay the removal of lipids (clearing mechanism).

Cadaver samples showed higher artifact compared with fresh samples. The artifact could be result from auto fluorescence or scatter phenomenon. Auto fluorescence is assumed to be the same in both samples. Scatter phenomenon occurs more in cadaver samples since the tissue remains yellow after clearing. We speculate that the increased cross linking could cause increased scatter phenomenon.

It is assumed that the difference in artifacts generated when the fresh and cadaver samples are made transparent is also caused by various reasons. When the clearing time was fixed, the cadaver sample was less cleared at the same time, so the artifacts were larger due to the opacity of the tissue. However, when the clearing efficiency was fixed, the cadaver sample showed more artifacts. In particular, the cadaver sample was observed to remain brown yellow when tissue clearing technique was applied [2, 13]. We think this phenomenon could cause these artifacts. But the reason why cadaver samples persisted retain yellow color remains to be discovered.

The greatest difference between fresh and cadaver samples was the DAPI staining. When the tissue was frozen sectioned and stained, DAPI staining was not observed for the cadaver samples. There could be several reasons for lack of DAPI staining in cadaver samples. DNA could be damaged or lost

during the long fixation time for various mechanisms. In previous studies using cadaver sample, they showed DAPI staining [2]. The main reason and solution for this DAPI staining problem needs further investigation.

In the case of cadaver sample, it is difficult for the researcher to participate in the fixation procedure because the sample is obtained according to the procedure for the donor body for research. The time taken from postmortem to fixation (PMI; postmortem interval) might be different. For fresh samples the PMI are approximately in 48-80 hours and, for cadaver samples, PMI are unknown. It might be longer than 80 hours.

In regard to antibody staining, both cadaver and fresh samples showed similar GFAP staining pattern and depths. But there appeared to be slight differences in the amount of antigen due to antigen masking by excessive formaldehyde protein crosslinks [17]. However, quantitative analysis with Image J did not seemed to be no difference. This is presumed that the artifacts of the cadaver sample effects the quantitative analysis. Future research with better control of artifacts would be required to compare the antibody staining efficiency between fresh and cadaver samples.

Staining quantity will be determined by antigen quantity, antigen quality, antibody penetration, antibody-antigen affinity and etc. Longer PMI could result in protein degradation and bacterial decomposition. These could decrease antigen quantity and antigen quality. Baesed on our results, human brain samples with PMI over 60 hours would not be recommended for immunohistochemical staining.

There are several limitations. Firstly, the present study used only GFAP antibody. We tried NeuN and Neurofilament antibodies for

several times. However, we could not obtain sufficient amount of results to support the aim of the present study. Secondly, the present study used only cortex. There could be heterogeneity between brain regions. Especially, cortical and subcortical tissues could have different aspects (lipid contents, cell density and etc.). There could be a distinct different optimal method for subcortical tissue. The present study's optimized protocols could not be generalized to whole brain. Thirdly, our study did not obtain ideal depth of 3D imaging like the previous studies. In the present study, 800um image was the deepest. Fourthly, we did not use the other tissue clearing technique and other staining method. Fifthly, some results could not be concluded because the number of samples was not sufficient. Lastly this study is based on the results of observations. The mechanism of these results requires further investigations. However, based on wealth of experiences, we are confident to suggest the optimal methods for human brain tissue clearing, to the best of our knowledge.

In conclusion, we have successfully visualized human brain 3D microstructure with optimized clearing and staining methods. The present study provided and compared various cleared specific and protocols for human brain clearing and staining. It is the most optimized human brain imaging strategy for tissue clearing. We recommend using fresh human brain samples and the specialized commercial staining methods. The most optimized human brain clearing method of the present study will open new paradigm for disease diagnosis and human brain research.

Reference

1. Chung, K. & Deisseroth, K. CLARITY for mapping the nervous system. Nature Methods 10, 508, doi:10.1038/nmeth.2481 (2013).
2. Lai, H. M. et al. Next generation histology methods for three-dimensional imaging of fresh and archival human brain tissues. Nature communications 9, 1066, doi:10.1038/s41467-018-03359-w (2018).
3. Harrison, C. H. et al. A novel method to visualise the three-dimensional organisation of the human cerebral cortical vasculature. Journal of anatomy 232, 1025-1030, doi:10.1111/joa.12805 (2018).
4. Fowler, J. L. et al. Three-Dimensional Analysis of the Human Pancreas. Endocrinology 159, 1393-1400, doi:10.1210/en.2017-03076 (2018).
5. Azaripour, A. et al. Three-dimensional histochemistry and imaging of human gingiva. Scientific reports 8, 1647, doi:10.1038/s41598-018-19685-4 (2018).
6. Tinne, N. et al. Three-dimensional hard and soft tissue imaging of the human cochlea by scanning laser optical tomography (SLOT). PloS one 12, e0184069, doi:10.1371/journal.pone.0184069 (2017).
7. Vigouroux, R. J., Belle, M. & Chedotal, A. Neuroscience in the third dimension: shedding new light on the brain with tissue clearing. Molecular brain 10, 33, doi:10.1186/s13041-017-0314-y (2017).
8. Hama, H. et al. ScaleS: an optical clearing palette for

biological imaging. Nat Neurosci 18, 1518-1529, doi:10.1038/nn.4107 (2015).
9. Yang, B. et al. Single-cell phenotyping within transparent intact tissue through whole-body clearing. Cell 158, 945-958, doi:10.1016/j.cell.2014.07.017 (2014).
10. Nojima, S. et al. CUBIC pathology: three-dimensional imaging for pathological diagnosis. Scientific reports 7, 9269, doi:10.1038/s41598-017-09117-0 (2017).
11. Chung, K. et al. Structural and molecular interrogation of intact biological systems. Nature 497, 332, doi:10.1038/nature12107 (2013).
12. Ando, K. et al. Inside Alzheimer brain with CLARITY: senile plaques, neurofibrillary tangles and axons in 3-D. Acta Neuropathol 128, 457-459, doi:10.1007/s00401-014-1322-y (2014).
13. Liu, A. K. et al. Bringing CLARITY to the human brain: visualization of Lewy pathology in three dimensions. Neuropathol Appl Neurobiol 42, 573-587, doi:10.1111/nan.12293 (2016).
14. Costantini, I. et al. A versatile clearing agent for multi-modal brain imaging. Scientific reports 5, 9808, doi:10.1038/srep09808 (2015).
15. Lee, E. et al. ACT-PRESTO: Rapid and consistent tissue clearing and labeling method for 3-dimensional (3D) imaging. Scientific reports 6, 18631, doi:10.1038/srep18631 (2016).
16. Burns, J. A. et al. Choice of fixative is crucial to successful immunohistochemical detection of phosphoproteins in paraffin-embedded tumor tissues. J Histochem Cytochem 57, 257-264, doi:10.1369/jhc.2008.952911 (2009).

17. Fowler, C. B. et al. Elevated hydrostatic pressure promotes protein recovery from formalin-fixed, paraffin-embedded tissue surrogates. Lab Invest 88, 185-195, doi:10.1038/labinvest.3700708 (2008).
18. Oberheim, N. A., Wang, X., Goldman, S. & Nedergaard, M. Astrocytic complexity distinguishes the human brain. Trends Neurosci 29, 547-553, doi:10.1016/j.tins.2006.08.004 (2006).

Table 1 Fresh sample lists and clinical information used in the experiment

Fresh Sample ID	Sex	Age	Height	Weight	Cause of Death	Postmortem Intervals(hr)
F1	Male	41	179	86	CKD	>72
F2	Male	78	162	52	MI	48
F3	Male	57	171	66	Hypothermia-	48
F4	-	-	-	-	-	
F5	Male	65	173	66	MI	80
F6	Female	67	166	70	Drowning	72
F7	Female	26	163	48	Arrhythmia	70
F8	Male	65	166	68	Hypertension	48

Table 2 Cadaver sample lists and clinical information used in the experiment

Cadaver Number	Sex	Birth Year	Death Year
C1	Male	1932	2016
C2	Male	1951	2016
C3	Male	1944	2016
C4	Male	1940	2016
C5	Male	1941	2016
C6	Male	1935	2016
C7	Female	1946	2016
C8	Male	1939	2016

Table 3 Four different staining methods

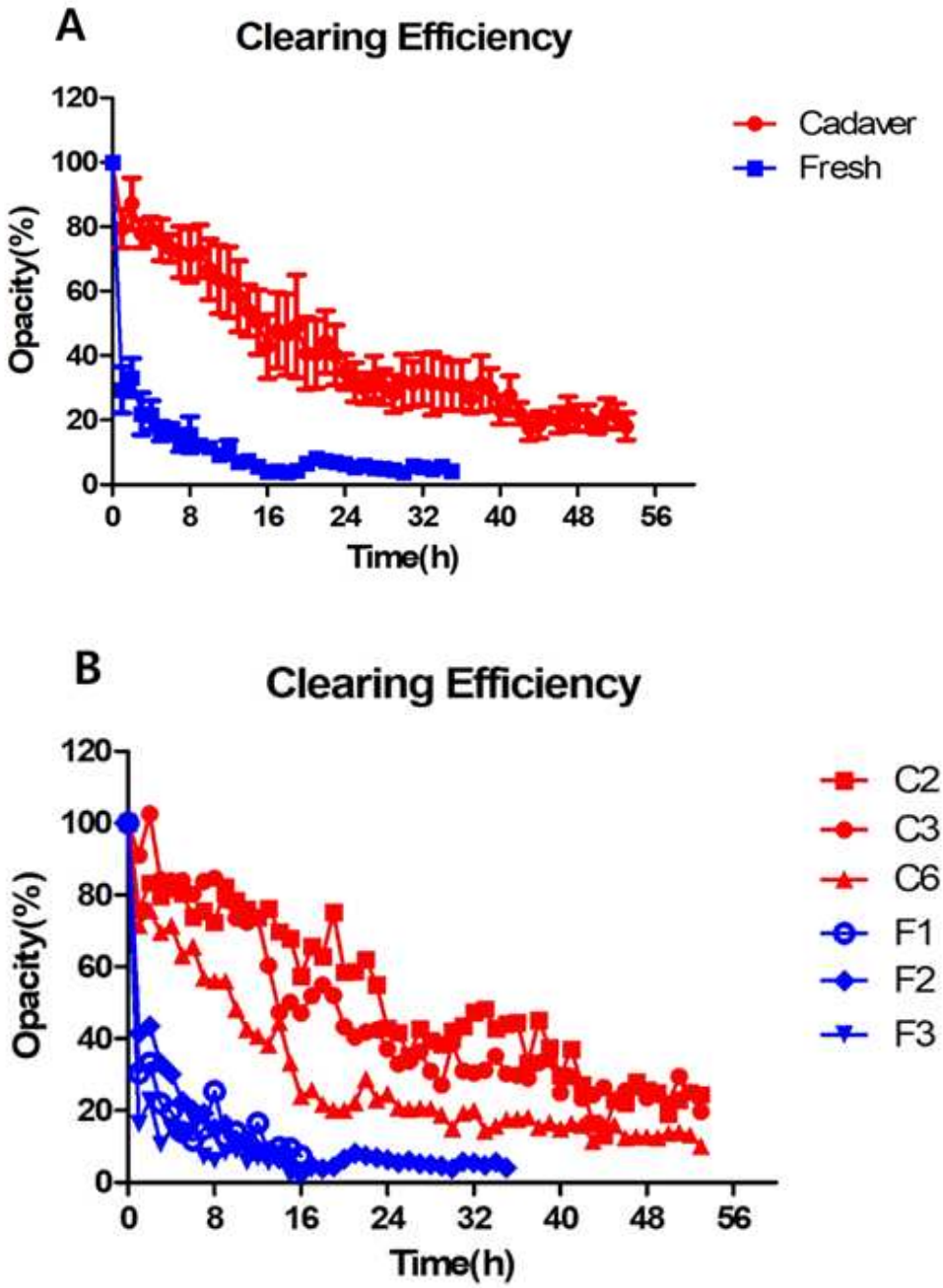
Passive/Active staining	Staining Method	Blocking	Primary antibody	Washing	Secondary antibody	Washing
Passive Staining	4days incubation in PBS (4DP)	Donkey 3%+0.01% Triton-X 4hr	GFAP 1:500 + 4days	PBS 2days	Rabbit 488 1:500 DAPI 1:1000 (405) Lectin 1:500 (594) + 4days	PBS 2days
	11days incubation in PBS (11DP)	Donkey 3%+0.01% Triton-X 4hr	GFAP 1:500 + 11days	PBS 2days	Rabbit 488 1:500 DAPI 1:1000 (405) Lectin 1:500 (594) + 11days	PBS 2days
	DeepLabel kit (4DP-C)	Permeabilization buffer 1day	GFAP 1:500 in antibody dilution buffer + 4days	PBS 2days	Rabbit 488 1:500 DAPI 1:1000 (405) Lectin 1:500 (594) in antibody dilution buffer + 4days	PBS 2days
Active Staining	perfectSTAIN (1DA)	Saponin buffer 10min Washing (RT) 10min 10% BSA 30min	GFAP 1:500 in staining buffer 90min (100mA) + 1day incubation	PBS 30min	Rabbit 488 1:500 DAPI 1:1000 (405) Lectin 1:500 (594) in staining buffer 90min (100mA) + 1day incubation	PBS 30min

Table 4 Comparison results of fresh and cadaver samples

	Fresh	Cadaver
Clearing Time (until 20% opacity)	4hr	40hr
Artifacts	Low	High
Antibody Penetration Depth (Ave)	67.86um	65.85um
DAPI Staining	Sufficient	Insufficient

Table 5 Comparison results of staining method

	Passive staining Active staining			Active staining
	4days incubation in PBS (4DP)	11days incubation in PBS (11DP)	DeepLabel kit (4DP-C)	perfectSTAIN (1DA)
Staining Time for Primary Antibody	4days	11days	4days	1day
Antibody Penetration Depth (Ave)	65um	125um	160um	700um
Complexity	+	+	+	++
Tissue Contamination	++	+++	+	+
Cost	+	+	++	+++


A
Clearing Efficiency
Opacity(%)
Time(h)
Cadaver
Fresh
B
Clearing Efficiency
Opacity(%)
Time(h)
C2
C3
C6
F1
F2
F3

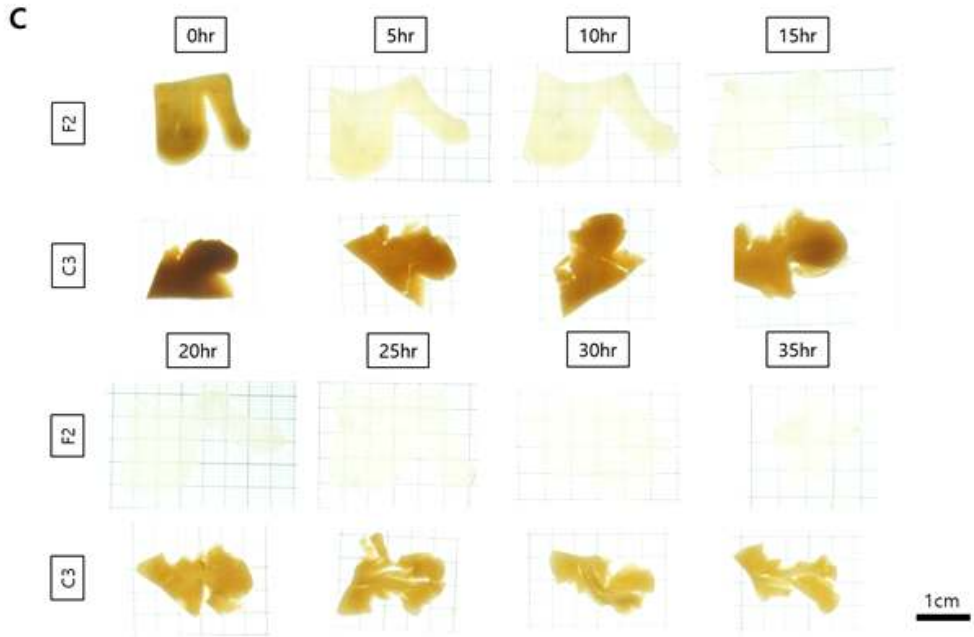
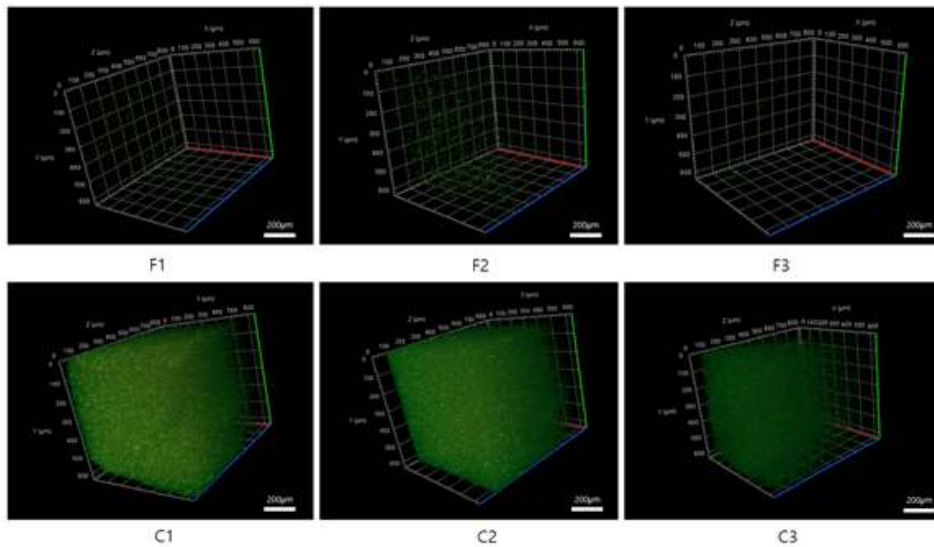
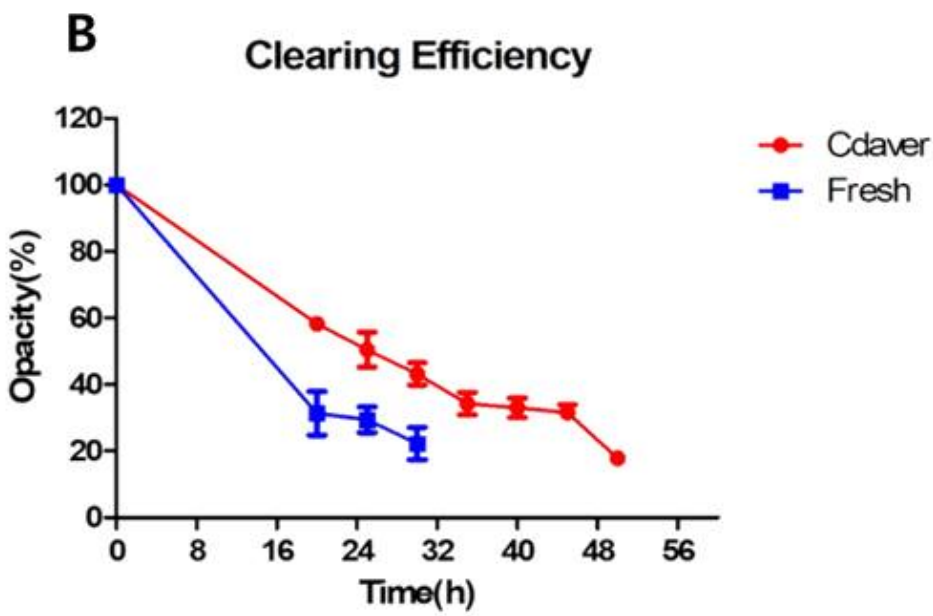


Figure 2 Comparing Clearing Efficiency of Fresh and Cadaver Samples. (A) Average graph of clearing efficiency of fresh and cadaver samples by time. (B) Individual graph of clearing efficiency of fresh and cadaver samples by time. (C) Picture of fresh and cadaver samples by clearing time.

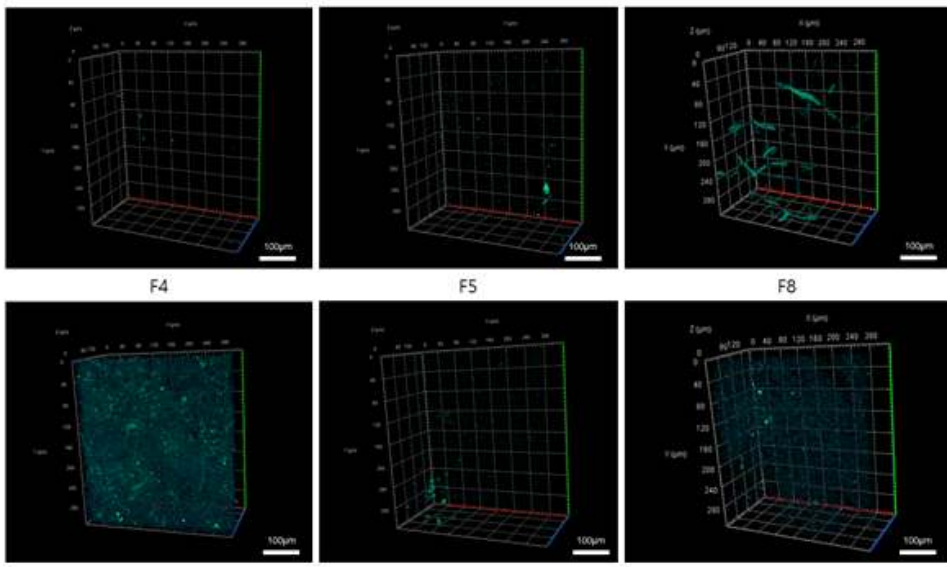
A


F1
F2
F3
C1
C2
C3

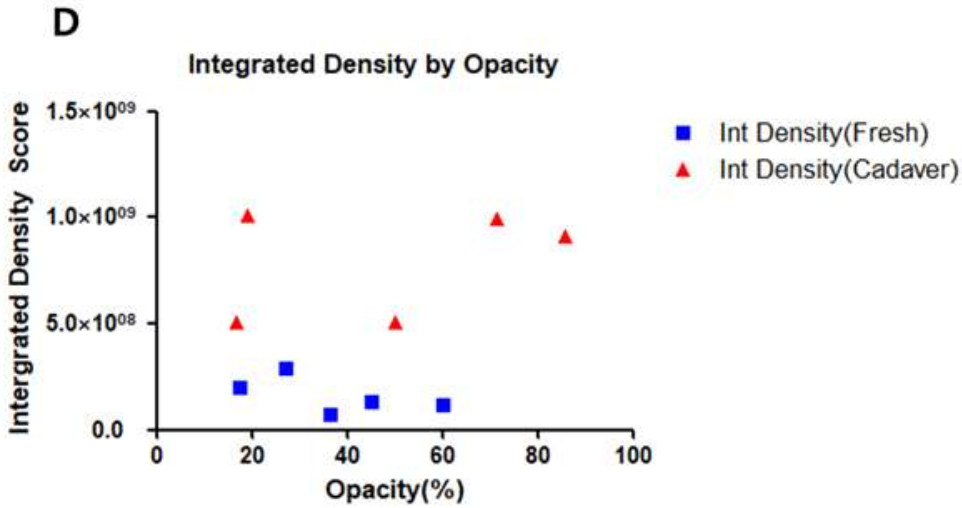
B


Clearing Efficiency
Opacity(%)
120
100
80
60
40
20
0
0
8
16
24
32
40
48
56
Time(h)
Cdaver
Fresh

C


F4
F5
F8
C5
C7
C8

D


Integrated Density by Opacity
Intergrated Density Score
1.5×10^09
1.0×10^09
5.0×10^08
0.0
0
20
40
60
80
100
Opacity(%)
Int Density(Fresh)
Int Density(Cadaver)

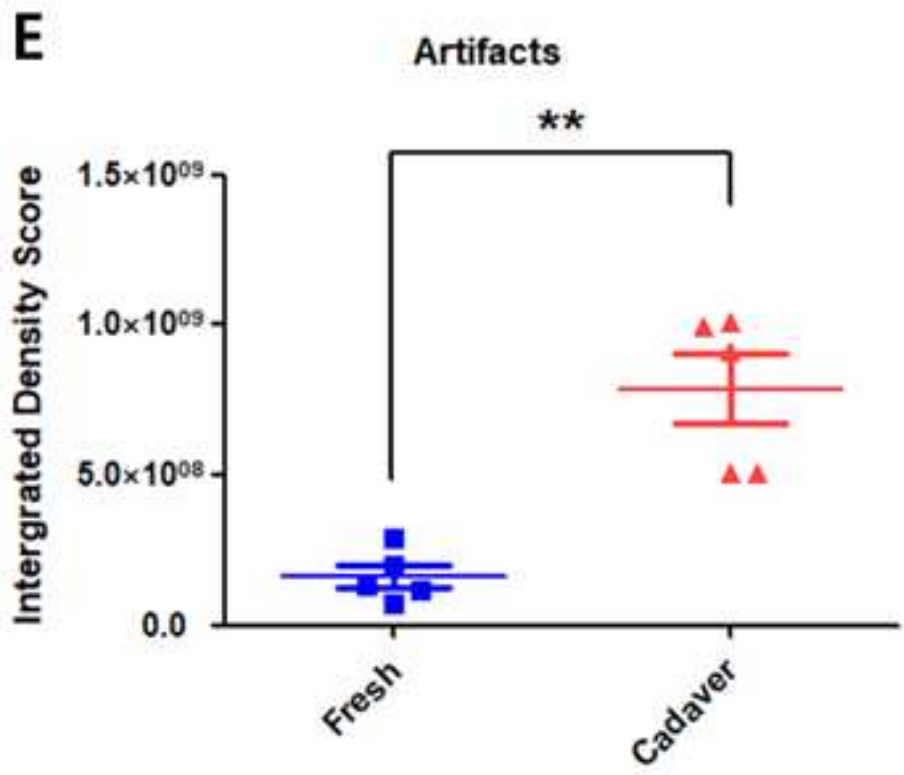
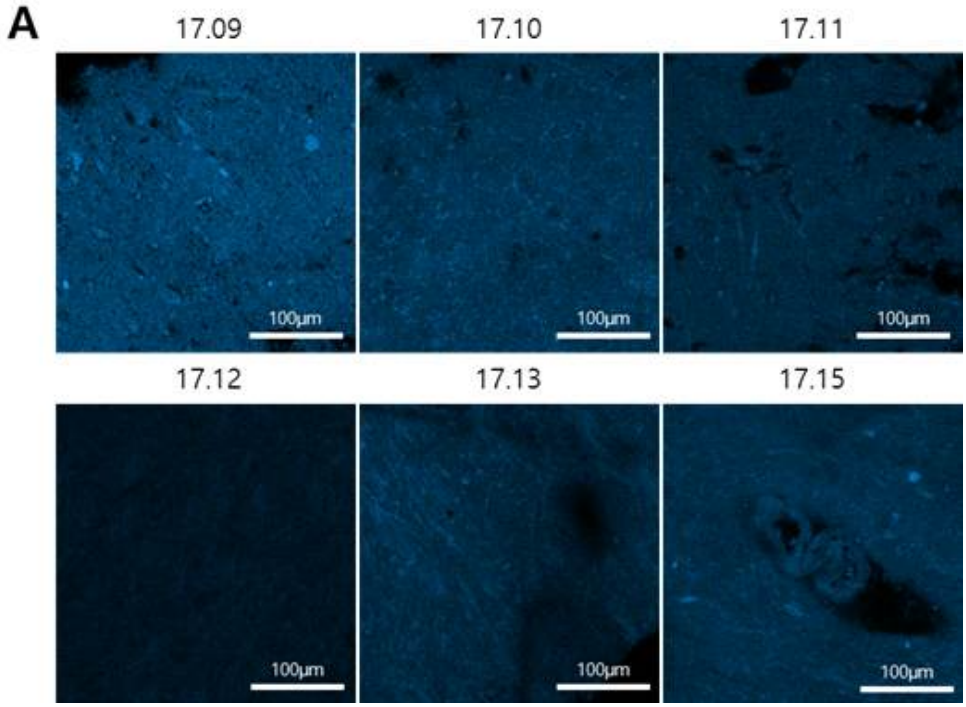
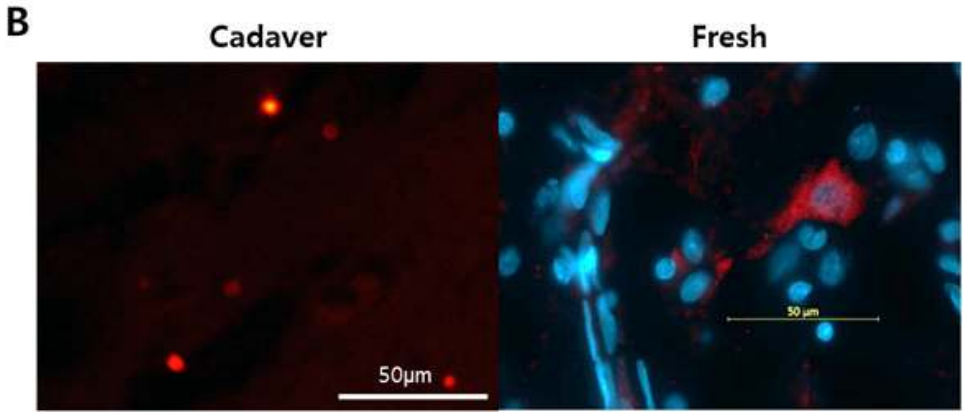
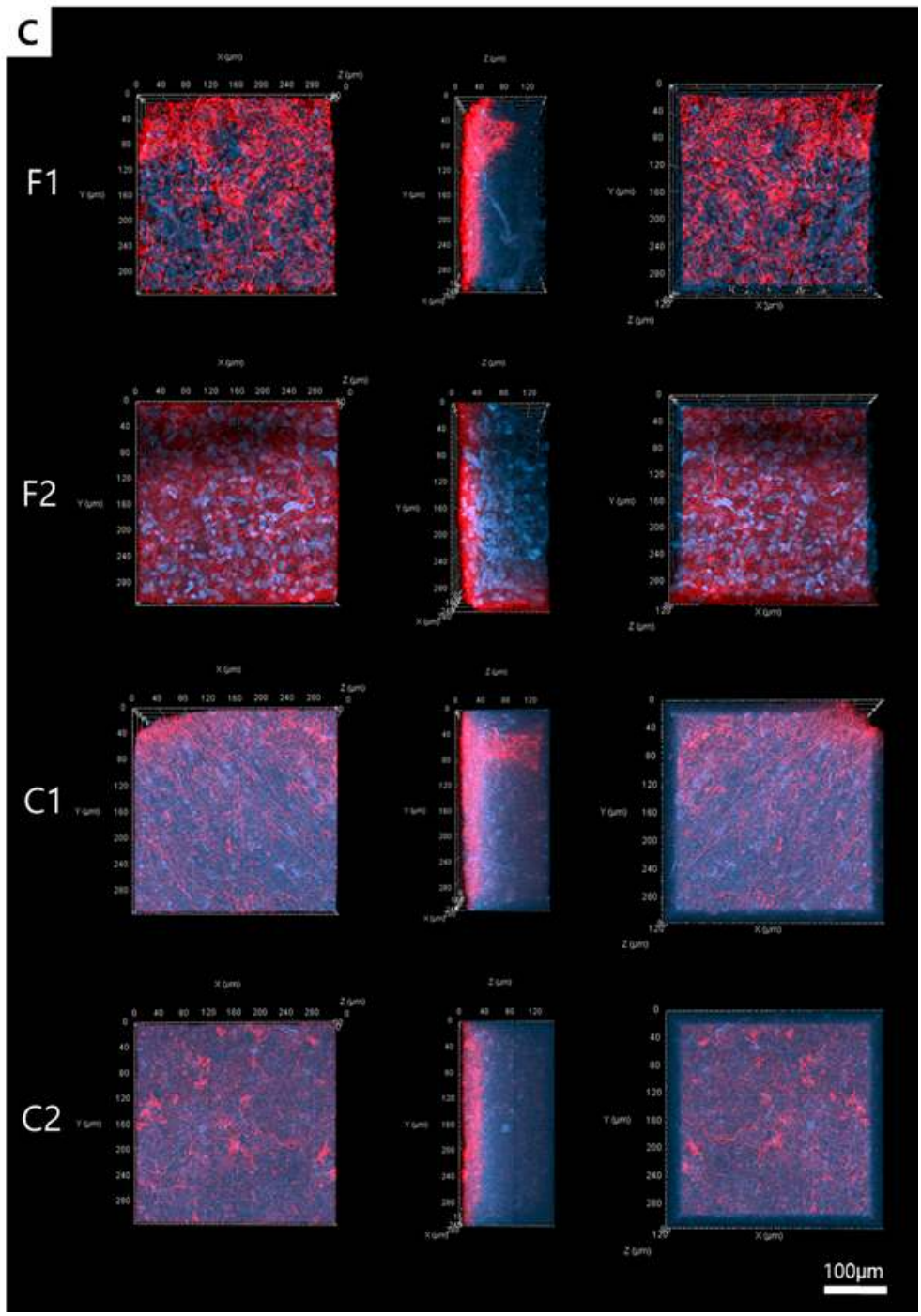
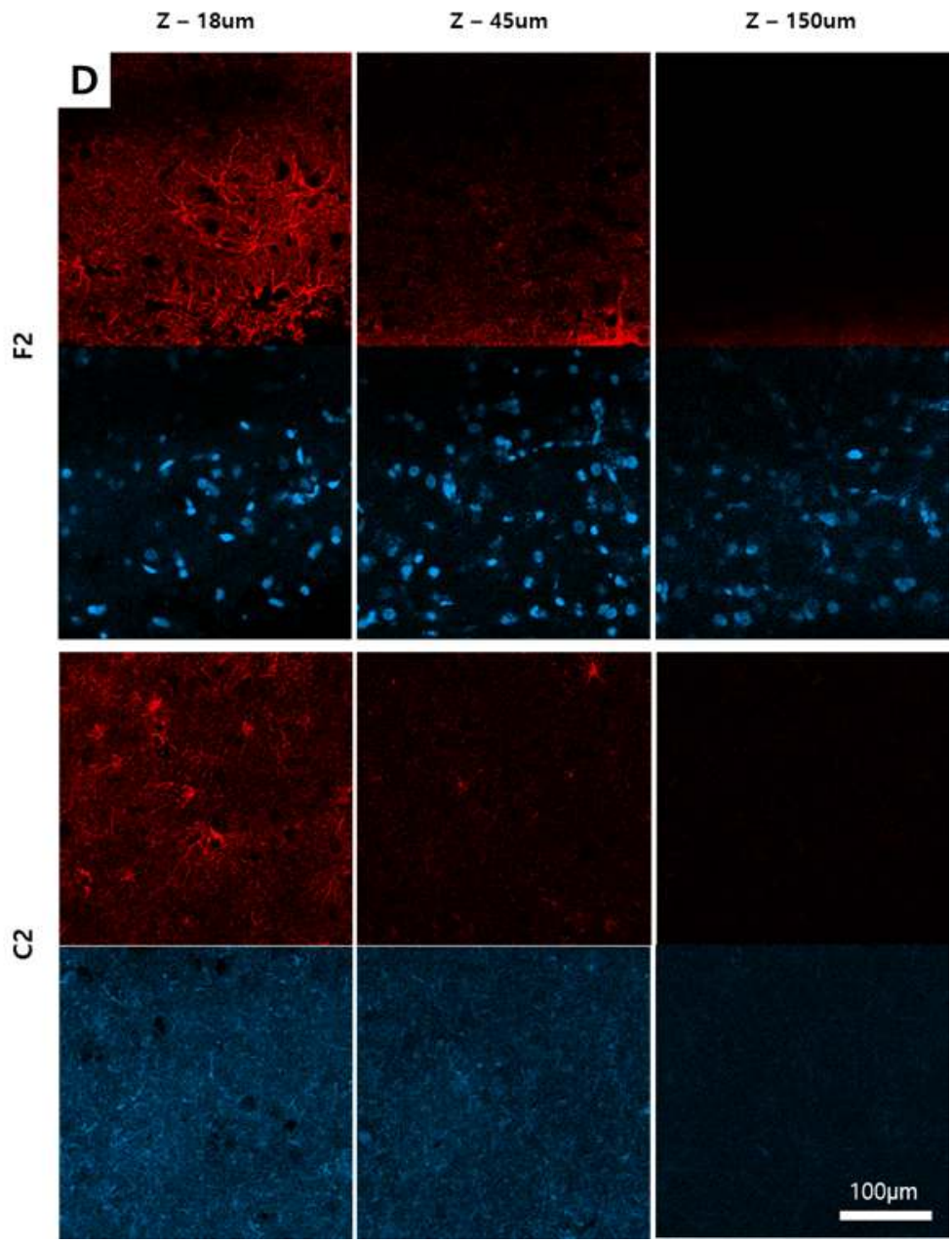


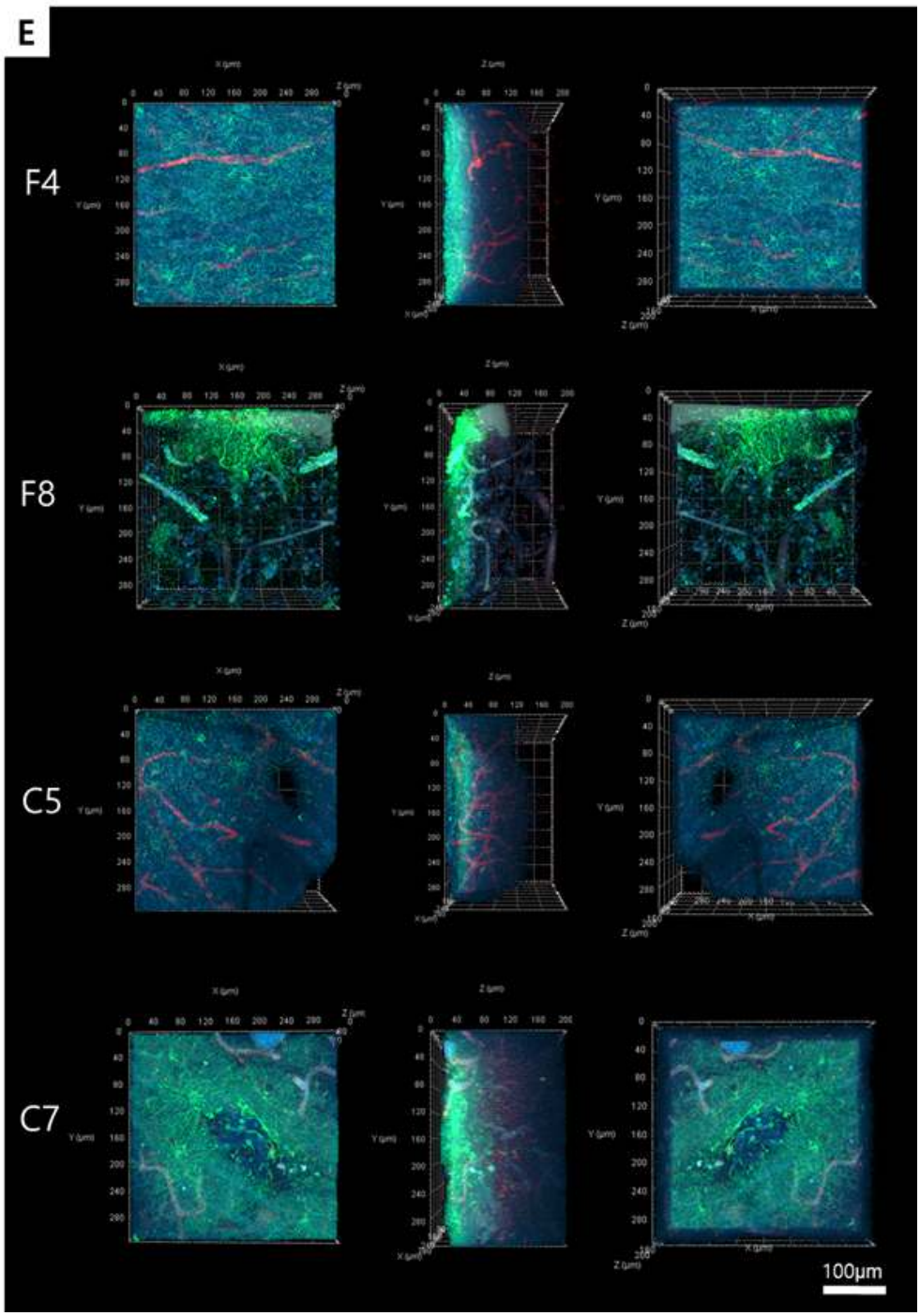
Figure 2 Comparing Artifacts level of Fresh and Cadaver Samples (A) Artifacts of fresh and cadaver samples. All images taken from confocal microscope (LSM 800), Laser wavelength 561nm:0.2%/488nm:0.18%, Detector Gain 700V, Detector Digital Gain 2.0 (B) Transparency depending on clearing time about figure 2c samples. (C) Artifacts of fresh and cadaver samples. All images taken from confocal microscope (LSM 800), Laser wavelength 561nm:0.2%/488nm:0.2%/405nm:0.14% Detector Gain 750V, Detector Digital Gain 1.0 (D) Integrated density depending on opacity. (E) Artifacts difference of fresh and cadaver samples (P=0.004).

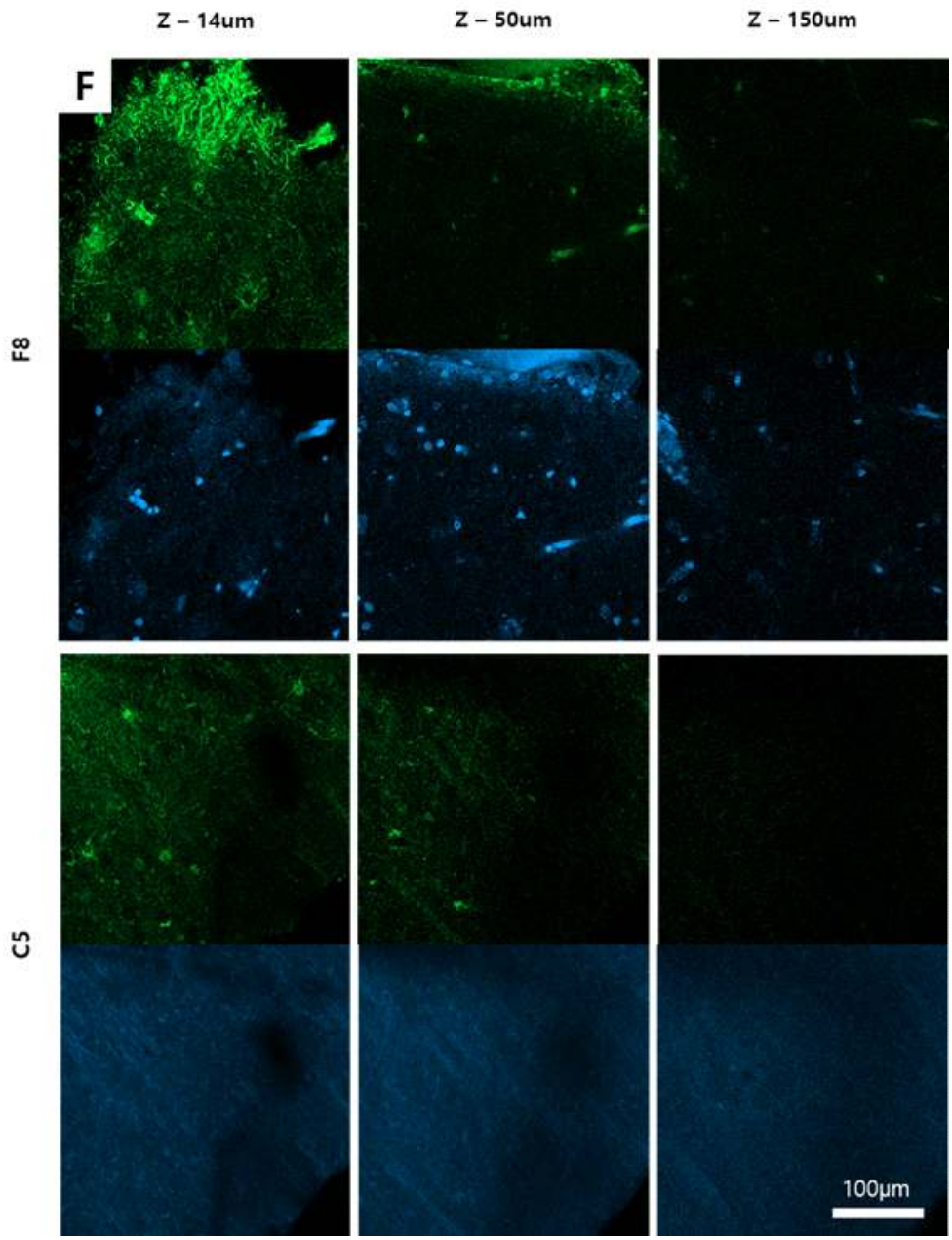

A
17.09
17.10
17.11
100μm
100μm
100μm
17.12
17.13
17.15
100μm
100μm
100μm


B
Cadaver
Fresh
50μm
50 μm


C
F1
F2
C1
C2
100μm


Z – 18um
Z – 45um
Z – 150um
D
F2
C2
100μm


E
F4
F8
C5
C7
100μm


Z – 14um
Z – 50um
Z – 150um
F
F8
C5
100μm

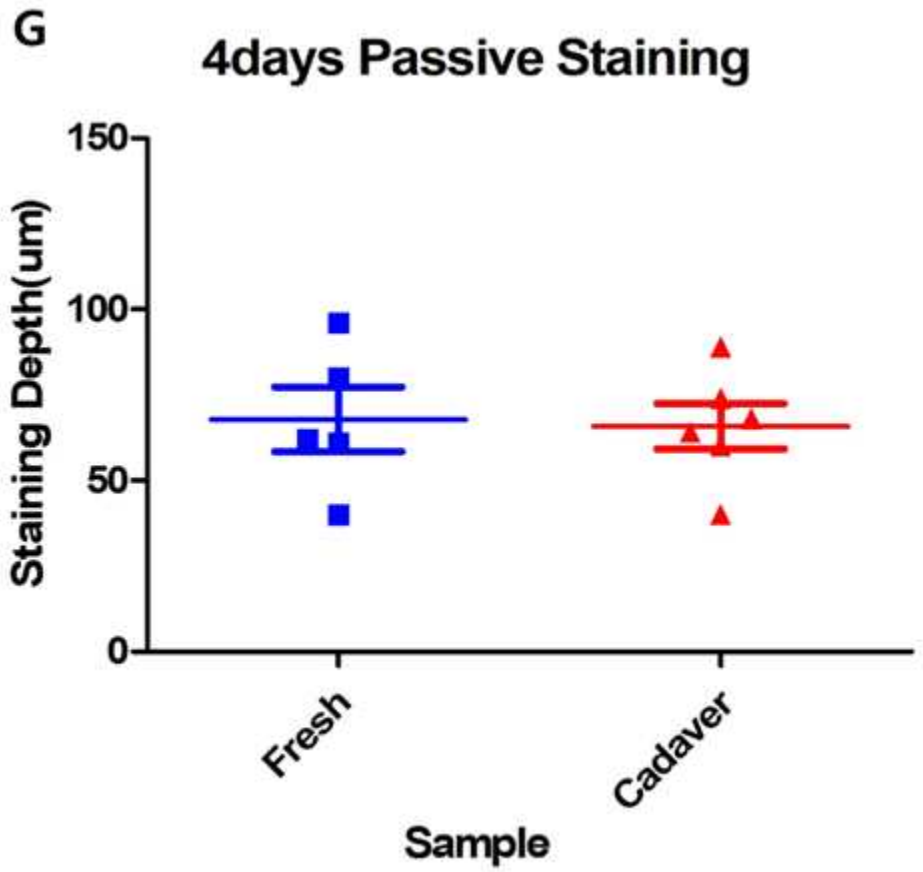


Figure 3 Comparing Staining Efficiency of Fresh and Cadaver Samples (A) DAPI staining results of cadaver samples. (B) Sectioned image of fresh and cadaver samples. (C) 4 days passive staining result in 3D with same clearing times (Red: GFAP, Blue: DAPI). (D) 4 days passive staining result in 2D with same clearing times. (E) 4 days passive staining result in 3D with same clearing efficiency (Red: Lectin, Green: GFAP, Blue: DAPI). (F) 4 days passive staining result in 2D with same clearing efficiency (Red: Lectin, Green: GFAP, Blue: DAPI). (G) The depth of GFAP staining in fresh and cadaver samples.

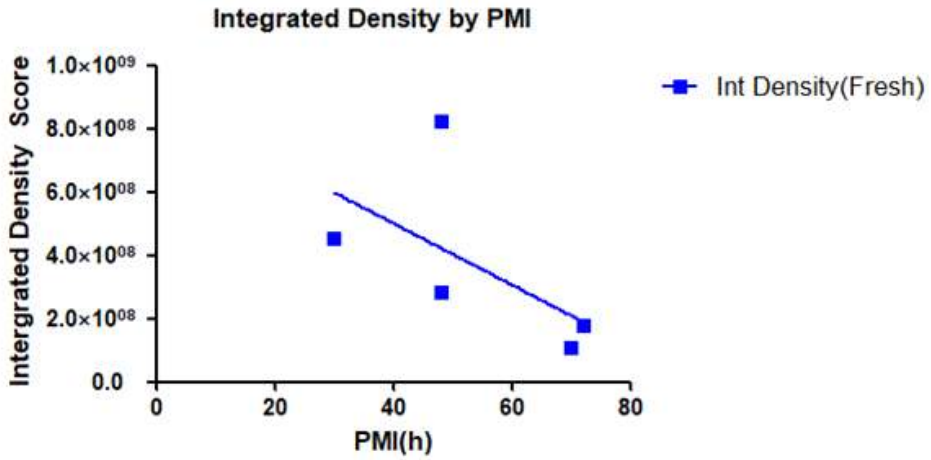
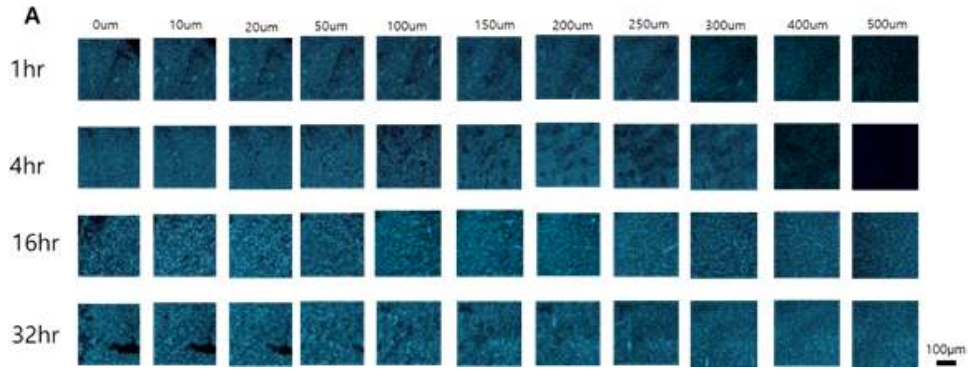
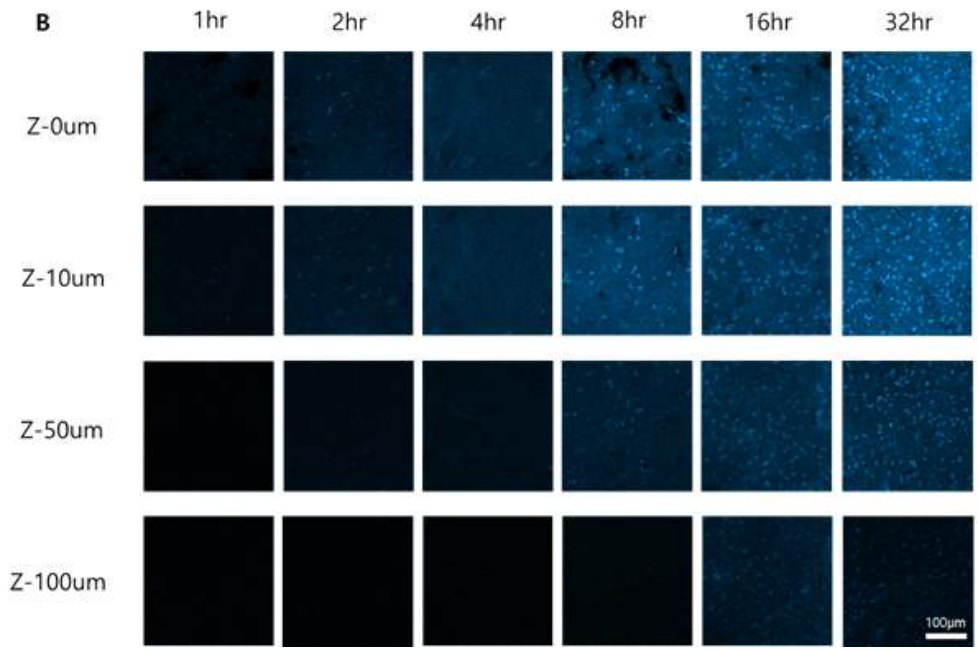


Figure 4 Amount of Antigen According to Postmortem Intervals. (r^2=0.3, P=0.2)

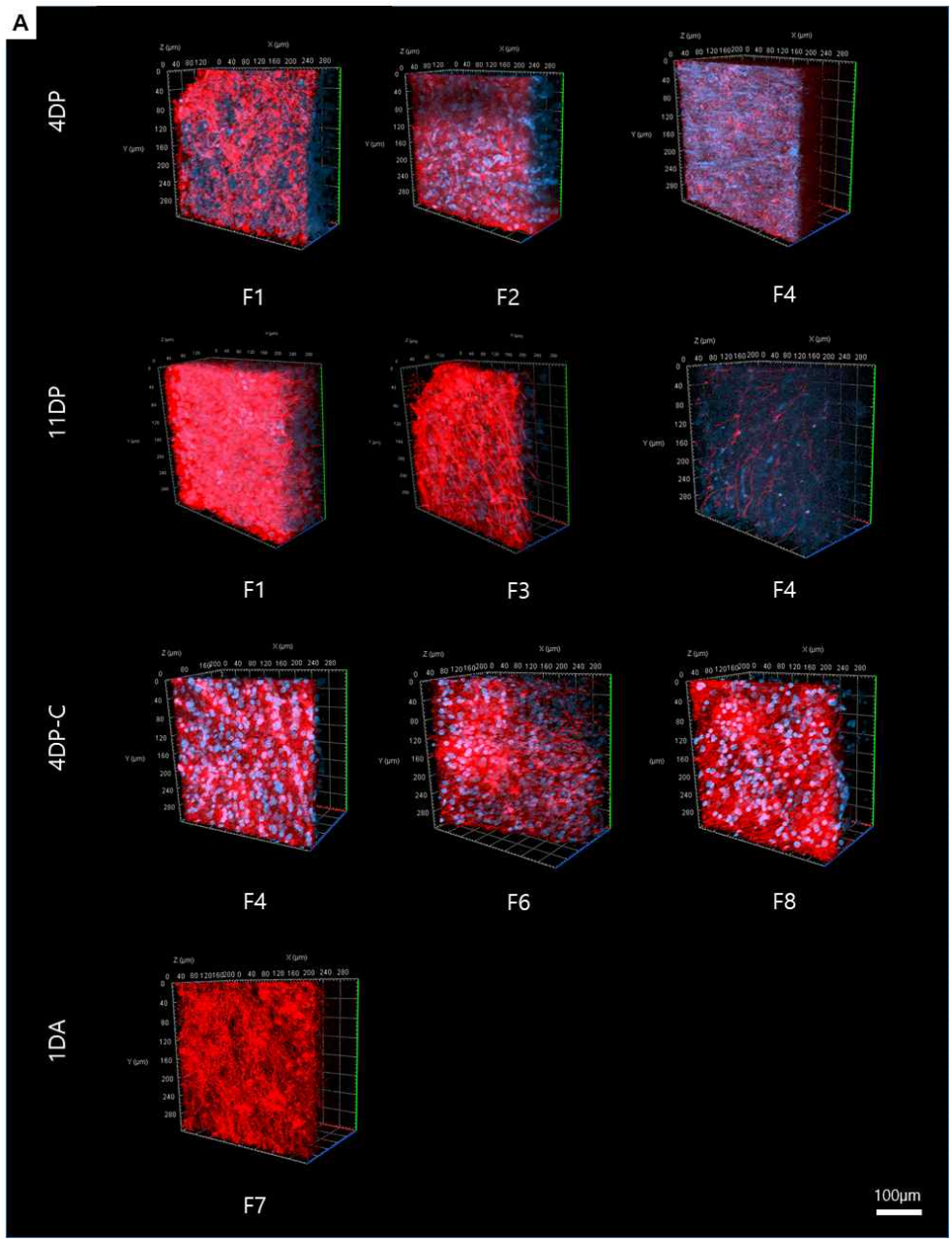

A
0um
10um
20um
50um
100um
150um
200um
250um
300um
400um
500um
1hr
4hr
16hr
32hr
100μm

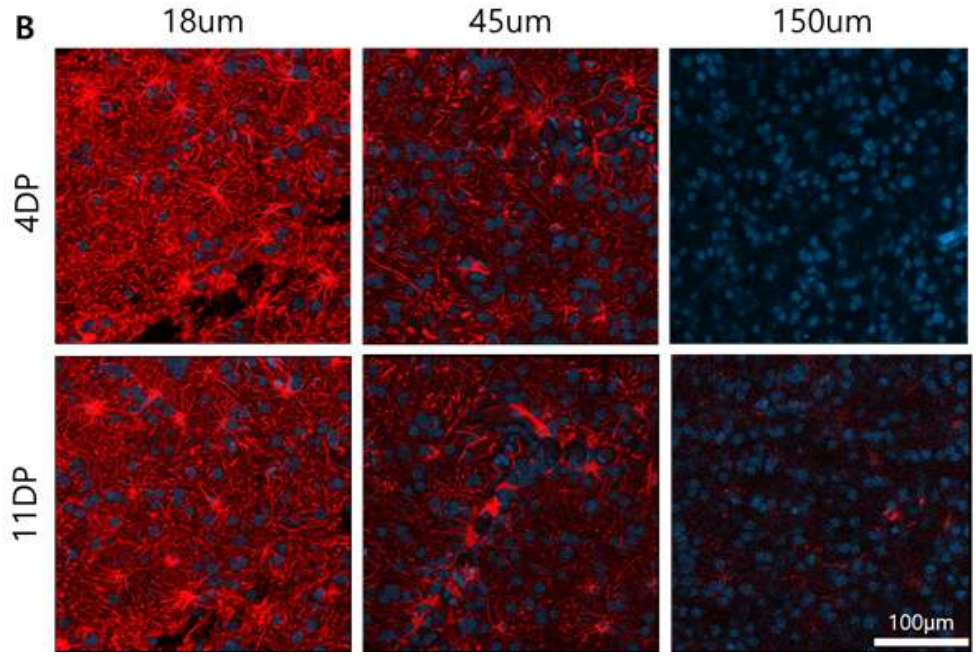

B
1hr
2hr
4hr
8hr
16hr
32hr
Z-0um
Z-10um
Z-50um
Z-100um
100μm

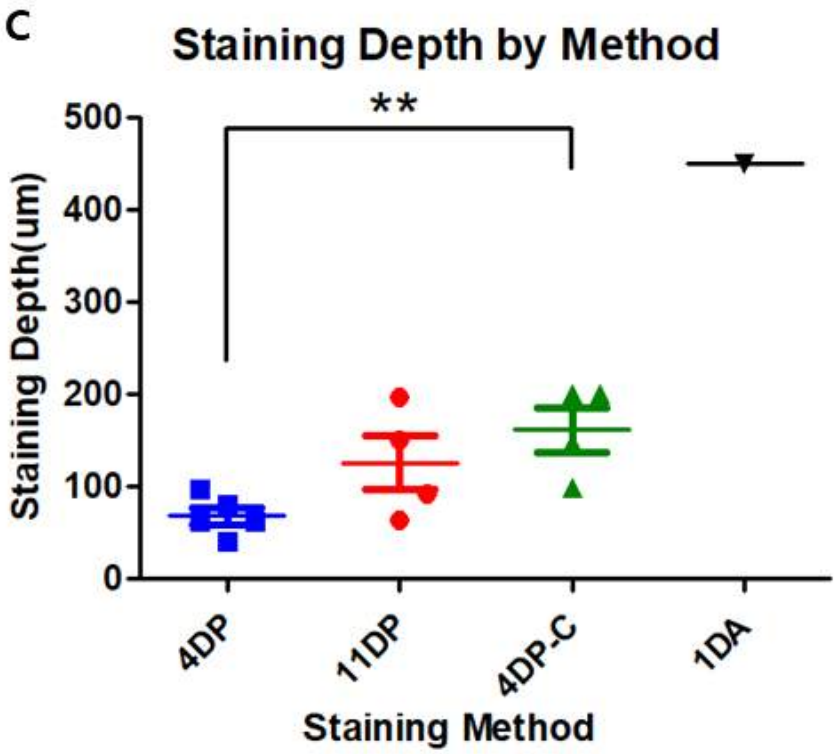
C

	1hr	2hr	4hr	8hr	16hr	32hr
0um	495.012	888.727	1289.71	1567.19	1909.22	2518.89
10um	412.491	849.987	1240.55	1927.82	1823.88	2461.23
50um	157.331	420.785	552.871	966.513	1361.64	1411.79
100um	184.82	143.537	183.192	273.463	873.091	583.737

Figure 5 Penetration Depth of DAPI Staining in Fresh Human Brain (A) The results of the DAPI staining set to the optimal values for each plane. (B) The results of the DAPI staining set to the same values for each plane. (C) Heatmap of staining efficiency depending on time and depth.


A
4DP
F1
F2
F4
11DP
F1
F3
F4
4DP-C
F4
F6
F8
1DA
F7
100μm


B
18um
45um
150um
4DP
11DP
100μm


C
Staining Depth by Method
**
500
400
300
200
100
0
Staining Depth(um)
4DP
11DP
4DP-C
1DA
Staining Method

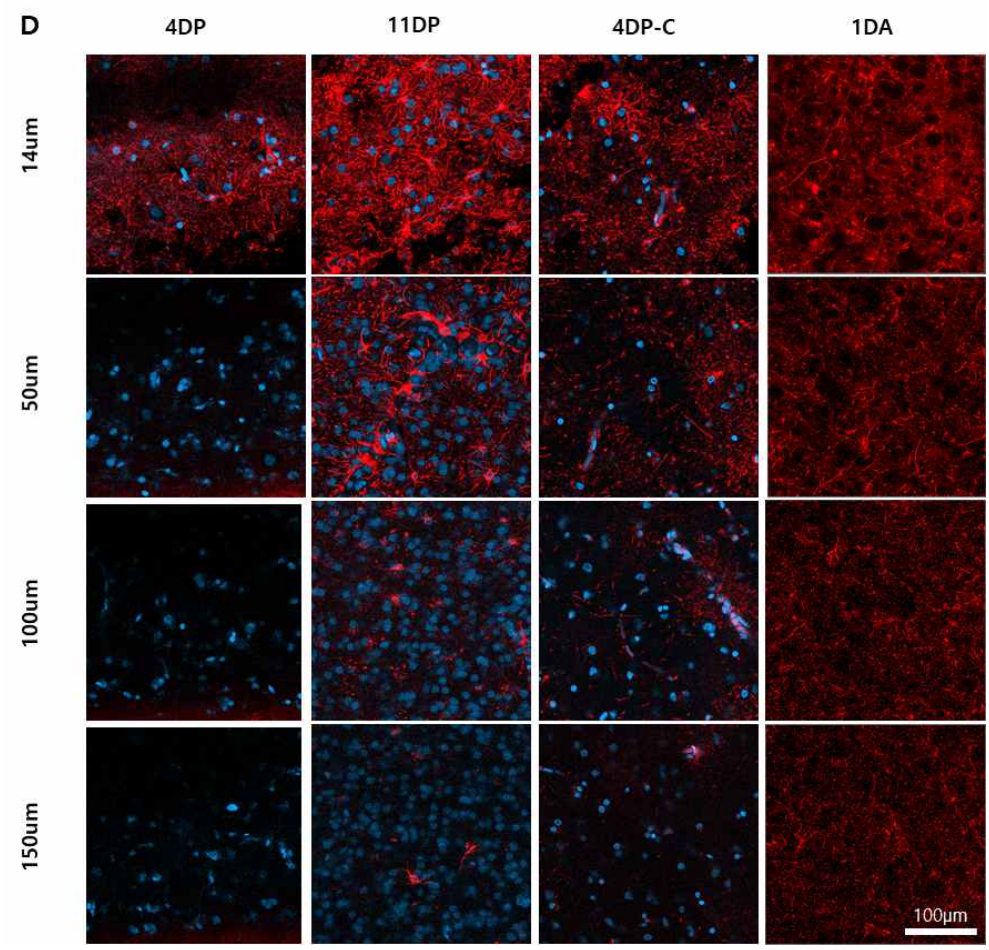
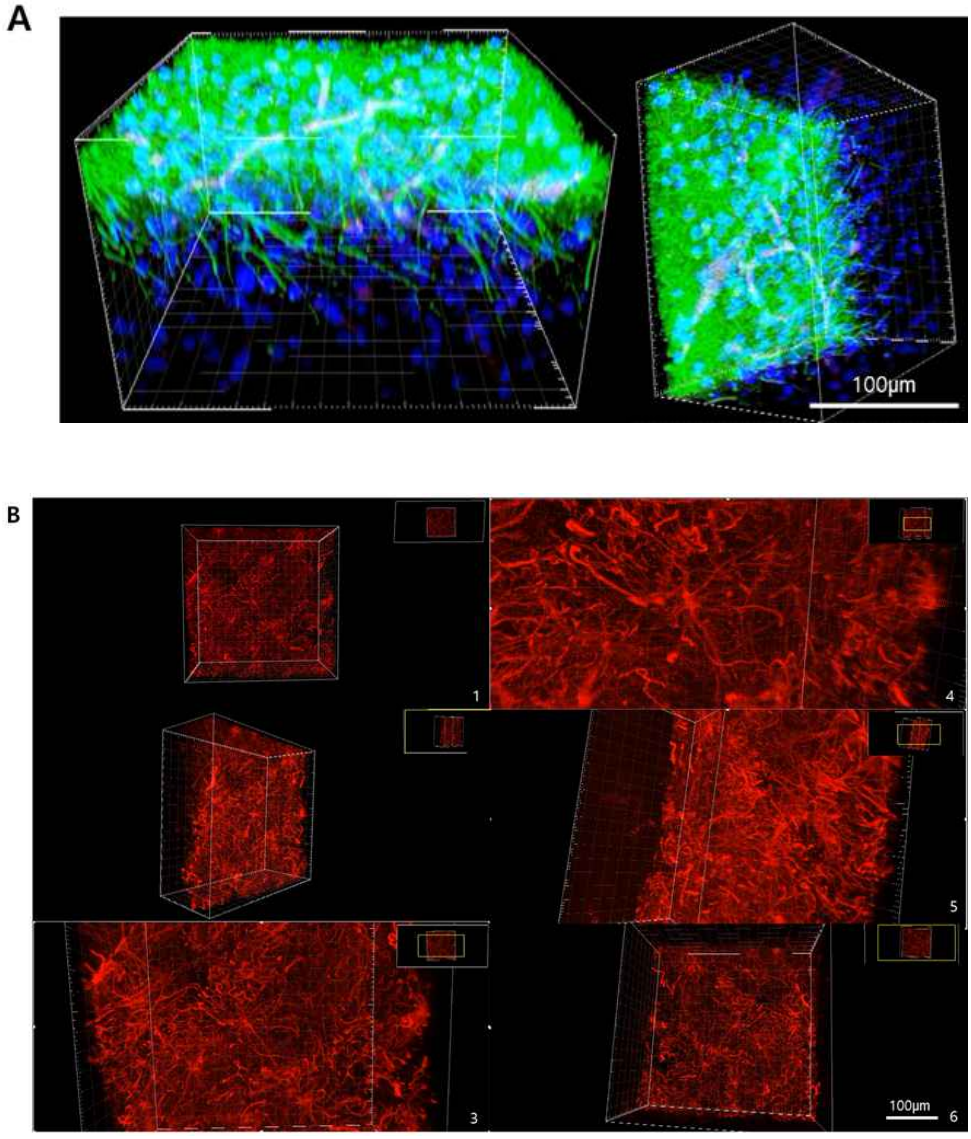


Figure 6 Comparing the staining method. (A) Image of human brain samples. (B) Fresh sample (F2) staining results for 4DP and 11DP (Red: GFAP, Blue: DAPI). (C) Antibody penetration depth according to staining method. (D) Fresh sample (F4) staining results 4DP, 11DP and 4DP-C (Red: Lectin, Blue: DAPI, Green: GFAP).


A
100μm
B
1
4
5
3
100μm
6

C

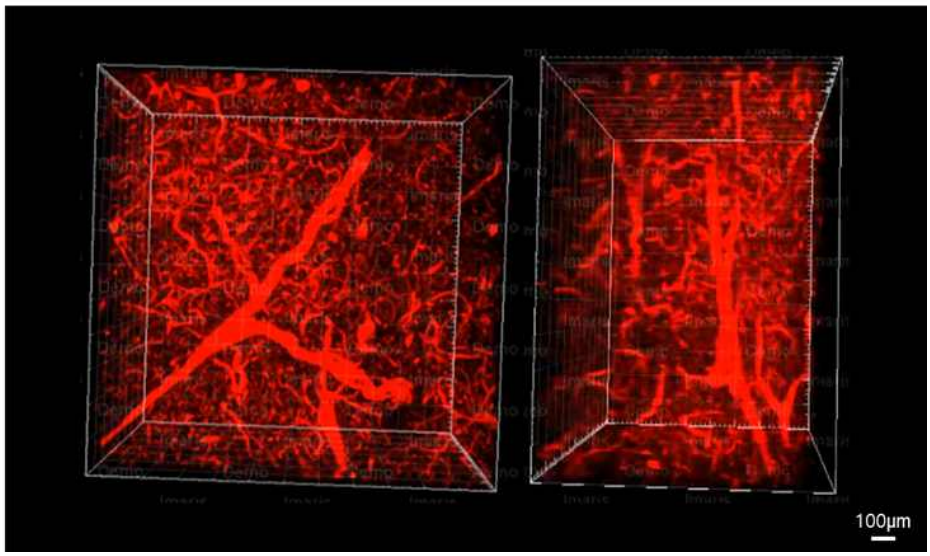


Figure 7 Visualizing 3D Structure of Human Brain (A) Structure of human brain (Red: Lectin, Green: GFAP, Blue: DAPI). (B) Human astrocyte image. (C) Human blood vessel.

요약

오늘날, 조직 투명화 기법은 신경과학연구에 널리 사용되고 있다. 근래에는 조직 투명화 기법을 사용하여 설치류 전체 뇌의 신경 연결까지 확인할 수 있을 만큼 기술이 발달했다. 하지만 사람 뇌에 조직 투명화 기법을 적용한 예는 많지 않기 때문에 사람 뇌 연구를 위한 최적화된 조직 투명화 및 염색 방법이 없다. 이 논문에서는 사람 뇌 연구에 사용할 수 있는 최적화 된 조직 투명화 기법과 면역 염색 방법을 제안하고자 하였다.

본 연구진은 두 종류의 사람 뇌 샘플을 사용하여 연구를 진행하였다. 첫번째로는 부검 시에 얻을 수 있는 신선한 뇌 샘플이고, 두번째로는 의과대학에 교육 및 연구용으로 기증된 뇌 샘플이다. 연구용 기증 뇌 샘플의 경우 약 2년간 고정되었고, 신선한 뇌 샘플의 경우 약 일주일간 고정되었다. 이 연구에서는 전기 영동 장치를 사용한 조직 투명화 기법을 위의 두 샘플에 적용하고, 시간에 따른 투명화 정도, 이미징 시 발생하는 artifacts, 핵 염색의 밝기 정도를 Image J로 정량화 하였다. 염색은 사람 뇌 조직을 4일, 11일 동안 항체와 화학 염색약을 희석한 PBS에 담그는 방식으로 진행되었다. 또한 시중에 판매하는 염색 키트와 전기 장치를 사용한 염색 방법을 도입하였다. 각 염색 방법은 염색 시간, 항체 침투 깊이, 염색 방법의 난이도, 조직 오염도와 비용의 항목에서 비교 되었다. 모든 이미징은 공초점 현미경을 사용했다.

신선한 뇌 샘플은 연구용 기증 뇌 샘플에 비해 투명화 효율이 높았다. 80%가량 투명화 되는 데에 신선한 뇌 샘플은 4시간, 연구용 기증 뇌 샘플은 40시간이 소요됐다. 또한 신선한 뇌 샘플은 연구용 기증 뇌 샘플에 비해 더 적은 artifacts를 보였다. 항체 염색에 관해서는 신선한 뇌 샘플과 연구용 기능 뇌 샘플이 비슷한 항체 침투 깊이를 보였다. 신선한 뇌 샘플을 사용하여 핵 염색을 했을 때 16시간 이상 담가두면 500um깊이까지 염색 되었고, 표면 밝기도 강해졌다. 여러 염색 방법을 비교한 결과 4

일동안 염색한 샘플은 11일동안 염색한 샘플보다 항체 침투 깊이가 얕았다. 또한 11일 동안 염색한 샘플보다 시판되는 염색 키트를 사용했을 때 항체 침투 깊이가 깊었다. 그리고 통계적인 비교는 불가능했지만 (n=1) 시판되는 염색 키트보다는 전기 장치를 사용한 염색 방법이 항체 침투 깊이가 깊었다.

본 연구는 성공적으로 사람의 3차원 뇌 구조를 보여주었다. 사람 뇌 샘플을 3차원으로 이미징 하기 위해서는 신선한 뇌 샘플을 사용하고, 시판하는 염색 키트를 사용하는 것이 가장 최적화된 방법이다. 만약 특수 기기 사용이 가능하다면 전기 장치를 사용한 염색 방법도 시도해볼 가치가 있을 것 같다. 결론적으로 본 연구는 사람 뇌 연구에서 사용할 수 있는 최적화 된 조직 투명화 기법과 염색 방법을 제안하였고, 추후 질병 진단이나 사람 뇌 연구에 적용 가능한 새로운 패러다임이 될 것이다.

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학　　번: 2017-25849