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Review

Tissue optical clearing methods for microscopy: A review of their application in neuroscience

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Abbreviations:

3DISCO – three-dimensional imaging of solvent-cleared organs
BABB – benzyl alcohol and benzyl benzoate
CHAPS – 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CLARITY – Clear lipid-exchanged acrylamide-hybridized rigid imaging/immunostaining/ in situ hybridization-compatible tissue-hydrogel
CT – computed tomography
CUBIC – clear, unobstructed brain imaging cocktails and computational analysis
ECi – optical clearing using ethyl cinnamate
EDTA – ethylenediaminetetraacetic acid
EGFP – enhanced green fluorescent protein
ETC – electrophoretic tissue clearing
fDISCO – fluorescence-preserving three-dimensional imaging of solvent-cleared organs
FFPE – formalin-fixed paraffin-embedded
FRUIT – fructose and urea-based clearing
GFP – green fluorescent protein

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Abstract

Recent advances in microscopy have enabled cellular-resolution imaging of thick tissue samples or even whole organs. The natural opacity of organs and tissues acts as a barrier to light penetration and must be removed to visualise structures of interest on a three-dimensional scale. Tissue optical clearing methods achieve sample transparency while also preserving fluorescently labelled epitopes. This innovative approach to sample preparation effectively enhances traditional histological sections and, with the aid of light sheet microscopy, enables optical sectioning and three-dimensional reconstruction of entire organs, even whole brains. Light sheet microscopy of optically cleared brain samples is a valuable method in neuroscience that is used in neuro-oncology, traumatic brain injury, ischemic brain injury, and neurodegenerative disease research.

In this review, we describe tissue optical clearing methods used to achieve optical transparency in brain samples. This quickly developing field has a significant potential for producing cutting-edge uses in neuroscience research.

INTRODUCTION

Tissue cutting and preparation of histological samples on glass slides has always been a standard approach when analysing morphology in the fields of anatomy, histology, embryology, pathology, and many other medical and biological disciplines. Cutting through FFPE (formalin-fixed paraffin-embedded) samples allows not only physical sectioning of organs but also light propagation through thin sections of tissue that are opaque in their native state *(1)*. Because a tissue histological section only reveals two dimensions, with a third dimension reduced to the section thickness, the only way to reconstruct a complete three-dimensional structure is to collect a series of successive sections. Serial section imaging, immunolabeling, and cutting, on the other hand, take a long time and are frequently hampered by tissue artifacts. The three-dimensional reconstruction of large samples based on serial sections necessitates advanced image processing and carries the risk of distortions and spatial data loss. Imaging of whole organs or large tissue samples is likely the only method for accurate three-dimensional reconstruction, and the brain is an excellent example of an organ that is difficult to reconstruct using tissue sections. It is nearly impossible to track all neuron projections and blood vessels using two-dimensional tissue slices and merging them together *(2)*. Because the samples in neuroscience are three-dimensional and relatively large, researchers have long sought a technique that could be used to image thick samples or even the entire brain. The development of the confocal microscope,

which could remove all out-of-plane fluorescence and imaging only fluorescence from the focal plane, was a significant breakthrough for visualising thicker samples. Even though two-photon microscopy has exceptional penetration abilities, it cannot image the entire brain because it cannot penetrate tissue deeper than 1 mm. Aside from the short working distance of the objectives, there are several physical light properties that cause problems when imaging large samples. Multiple structures (e.g., pigment lipofuscin and haemoglobin from residual blood if tissue is poorly perfused) in the brain can absorb incoming light *(3)*. Most biological samples natural blurriness is caused by inhomogeneity in refractive index (RI) within tissue components, which causes light to scatter. This discrepancy in the RI values of water, lipids, and proteins leads to light scattering and tissue opaqueness *(3,4)*.

However, light can be replaced by other imaging modalities and modern neuroimaging techniques, such as magnetic resonance imaging (MRI), positron emission tomography (PET), or computed tomography (CT), which have become indispensable methods in the field of brain research and for diagnosing pathological conditions in humans. Although these methods are quick, can be used *in vivo*, and provide insight into the entire brain (even in the relatively large human brain), their spatial resolution is still insufficient to compete with light microscopy and depict morphological characteristics at the cellular level *(3)*.

Light sheet microscopy was developed, which was a breakthrough because it allowed for the high-resolution imaging of structures of interest in large organ parts, entire organs, or even the entire organism, like a mouse *(5)*. This type of microscopy is used for visualization of fluorescently labeled structures that come from endogenous expression of fluorescent proteins, immunohistochemistry, or injection of fluorescent dyes. Confocal or two-photon microscopes are commonly used for imaging thicker sections, whereas light sheet fluorescence microscopy does not have thickness limitations, but it can only be used for larger samples and is currently limited by the working distance of the used objectives. However, before these samples can be imaged, they must go through a series of preparation steps to ensure that they are completely transparent. Tissue optical clearing methods, which will be discussed further, are required for imaging large samples using light sheet microscopy. Some biological samples are naturally transparent and can be imaged without being cleared. For instance, it is possible to observe the development and function of organs in zebrafish embryos *in vivo* without the use of hazardous chemicals *(6)*.

TISSUE OPTICAL CLEARING METHODS

Tissue optical clearing methods were evolving and progressing while light sheet fluorescence microscopy was improving. Although most of the tissue clearing methods have been developed in the last 30 years, Spalteholz recorded the first tissue clearing method more than a century ago. It was based on a standard histological method that entails dehydrating the sample in an increasing gradient of alcohol and matching refractive indices with a benzyl benzoate and methyl salicylate mixture *(7)*. Over 30 different tissue clearing methods have been described in the literature since then. According to Silvestri's most recent proposal, modern tissue clearing techniques can be divided into four main categories: organic solvent-based clearing techniques, aqueous clearing techniques, hyperhydrating solution clearing techniques, and tissue transformation techniques *(1)*. The suggested classifica-

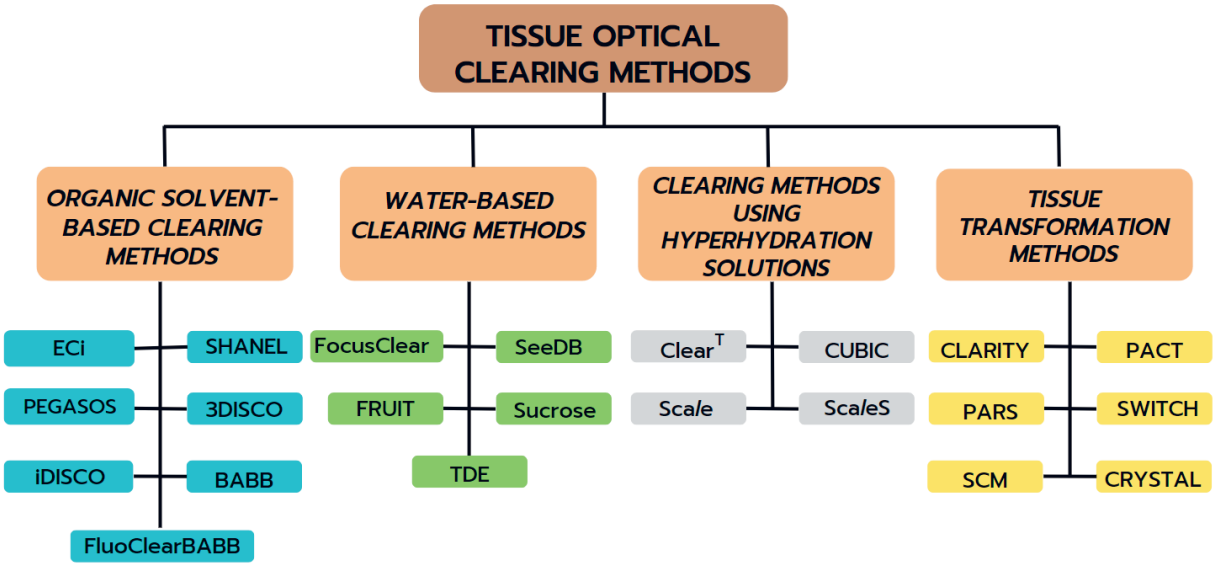

Figure 1. *Schematic representation of the most used tissue clearing methods (adapted according to 1,2)*

tion is applicable to the most popular tissue clearing techniques (Figure 1). All these techniques aim to make the samples transparent enough to be imaged with light sheet microscopy and then post-processed for three-dimensional visualization and reconstruction. A good clearing method should make tissue completely transparent (matching RI within the tissue to minimize light scattering) while preserving tissue integrity and endogenous fluorescence or fluorescence from conjugated antibodies in the whole organ immunolabeling process. The sample size, which depends on the tissue clearing method used, affects the volumetric analysis of cleared samples. Tissue shrinkage when using organic solvent-based clearing methods or tissue expansion when using aqueous-based clearing methods is frequently inhomogeneous and must be considered during the three-dimensional reconstruction process *(3)*.

ORGANIC SOLVENT-BASED CLEARING METHODS

Organic solvent-based clearing methods are the most used in neuroscience research because they achieve the highest level of tissue transparency. The development and implementation of new organic chemicals and procedures allowed the replacement of harsh and toxic chemicals as well as improved endogenous fluorescence preservation. They typically consist of two distinct steps: dehydration and lipid solubilization, with additional lipid solubilization and clearing steps needed for refractive index matching *(2)*. Although BABB (benzyl alcohol and benzyl benzoate)-based tissue clearing methods are still the most effective for achieving tissue transparency, they use a corrosive and toxic combination of chemicals. These techniques allowed the visualization of GFP (green fluorescent protein)-positive neurons and dendritic trees in isolated mouse hippocampus CA1 regions *(8)*. In addition, autofluorescence from cleared mouse and fruit fly embryos is utilized for species anatomical analysis following BABB clearing *(8)*. The FluoClearBABB method, which employs *tert*-butanol as a dehydrating agent and maintains a pH of around 9.5, was introduced as an improvement to the BABB method regarding fluorescence quenching. When *tert*-butanol was used as the dehydrating agent instead of ethanol, 79.2% of the initial fluorescence of EGFP (enhanced GFP)-labelled *E. coli* cells was maintained *(9)*. When imaging cortical neurons in cleared Thy1-GFP rat brain slices with FluoClearBABB for clearing, similar results of endogenous fluorescence preservation were obtained (10). Pigments that normally reside in neuronal tissue can cause imaging issues when working with the entire brain because they absorb light. To remove these pigments (e.g., lipofuscin and blood residues), bleaching chemicals must be used while endogenous fluorescence is preserved. In only one week of clearing, the polyethylene glycol (PEG) associated solvent system (PEGASOS) tissue clearing method achieved three positive effects: pigments were removed in the decolorization step using N,N,N',N'-tetrakis(2-hydroxypropyl)ethylenediamine (Quadrol), endogenous fluorescence was preserved using *tert*-butanol under alkaline conditions and stepwise dehydration steps, and a completely pure and transparent brain sample was obtained using the method *(11)*. Furthermore, some of the samples were cleared without the brain being removed from the skull. The entire mouse head was thoroughly cleaned using an immersion technique, and the skull was decalcified using a 20% water solution of ethylenediaminetetraacetic acid (EDTA). Using the isolectin GS-IB4 dye, neuron somas and axons in the cortex, cerebral peduncle, midbrain, and hippocampus, as well as the whole-brain vasculature, were visualized at an appropriate resolution *(11)*. Most of the organic solvent-based tissue clearing methods employ toxic and hazardous chemicals for final clearing, RI matching, and imaging. Many research facilities and laboratories, however, lack the necessary conditions for placing the light sheet microscope in a fume hood or a well-ventilated room. That is why alternative tissue clearing methods that reduce exposure to harmful chemicals would be advantageous. Recently, a non-toxic ethyl cinnamate-based (ECi) clearing protocol was introduced. When studying three-dimensional reconstruction of multiple neurons in 80-day-old sparsely labelled cerebral organoids, researchers achieved antibody staining for GFP-positive cells as well as excellent transparency with ethyl cinnamate *(12)*. The most popular clearing techniques come from a large family of tissue optical clearing techniques called 3DISCO (three-dimensional imaging of solvent-cleared organs) *(13)*. For the adult mouse brain, clearing takes just one day. It is compatible with many labelling techniques, such as transgenic fluorophores and antibody labelling, and it results in good tissue transparency for the visualization of neurons. The 3DISCO method has the drawback of using toxic, flammable, and volatile chemicals for imaging and clearing, such as tetrahydrofuran and dibenzyl ether *(13)*. Other DISCO alternative protocols, such as immunolabeling DISCO (iDISCO) (14), iDISCO+ (15), DISCO with superior fluorescence-preserving capability (fDISCO) (16), and ultimate DISCO (uDISCO), were developed using this technique as their foundation (17). Most of the previously mentioned tissue optical clearing techniques have been improved for use with samples from both human and animal subjects, but they remain demanding to clear and label. Recently, a new clearing protocol that is specifically intended to clear human organs was created (18). The small-micelle-mediated human organ efficient clearing and labelling (SHANEL) protocol uses CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) detergent for tissue permeabilization and nanobodies for labelling. Successful imaging of blood vessels (lectin), microglia (Iba1 antibody), and neurons (Neurotrace Nissl stain) in aged human brain slices was achieved using SHANEL *(18)*.

WATER-BASED TISSUE OPTICAL CLEARING METHODS

The search for fluorescent, protein-friendly, and non-toxic chemicals for clearing and imaging led to the development of water-based tissue clearing methods. Most aqueous-based tissue clearing methods exhibit tissue swelling and expansion caused by water molecules entering the brain tissue. Because all the final clearing solutions have high RI values (ranging from 1.42 to 1.48), they are commonly referred to as "high-RI aqueous solution clearing methods" *(3)*. Organ transparency is lower when compared to organic-based methods, but it is still adequate for microscopy imaging. Transparency is achieved through one of three methods: removal of lipid followed by hydration of the sample to lower the RI of the remaining tissue components; passive immersion in a solution with a RI matched to the tissue; or active or passive removal of lipid followed by immersion in a RI-matched medium *(2)*. A commercially available solution called FocusClear was used to clear neuronal circuits in an invertebrate (*Drosophila melanogaster*) to visualize N-methyl-D-aspartate glutamate receptors (NMDAR) in the ring glands and brain *(19)*. However, because of the lower optical clearing potential, the clearing potential remains questionable for imaging thicker tissue samples *(20)*. 2,2-thiodiethanol (TDE), which mixes well with water and can be used, in a straightforward, inexpensive, quick, and non-toxic protocol, to create a variety of solutions with RI values ranging from 1.33 (pure water) to 1.52 (pure TDE). Also, the hippocampal slices of the Thy1-YFP-H mouse show fluorescence from YFP-expressing neurons, which is used to examine the dendritic spines *(21)*. Clearing mouse brain slices using See Deep Brain (SeeDB) is another popular aqueous-based tissue clearing technique. Clearing of fixed samples only requires a few days, and endogenous fluorescence is preserved with barely perceptible changes in sample volume. Because this method does not use detergents or organic solvents, the plasma membrane and myelin remain intact and can be used for labelling with lipophilic dyes. The permeabilization of tissue is, however, limited without the application of detergents, and the antibody staining is therefore not applicable (the penetration of antibodies is around 100–250 µm in depth in adult mouse brain). To avoid tissue browning and autofluorescence when incubating at higher temperatures, tissue is immersed in an ascending gradient of fructose solutions (20, 40, 60, 80, and 100%), and final clearing is achieved in SeeDB reagent consisting of 80.2% fructose in water and 0.5% thioglycerol *(22)*. SeeDB fructose solutions have a high viscosity, which makes clearing large samples difficult due to their poor penetration into tissue. FRUIT is a modified SeeDB method that, in addition to fructose, uses urea in cocktail solutions (fructose and urea-based clearing). Cocktails with varying amounts of urea and fructose exhibit less viscosity and have a higher clearing potential. The FRUIT method, due to its low viscosity, can clear the entire adult rabbit brain using an arterial perfusion protocol. Additionally, clearing is superior to SeeDB and adequate for imaging of, for example, cortical pyramidal neurons with two-photon microscopy *(23)*. The last clearing method worth mentioning uses a high-RI aqueous solution of sucrose. The architectonics of neurons, glia cells, and angioarchitecture can be reconstructed using millimeter-thick slices of neocortex *(24)*.

CLEARING METHODS USING HYPERHYDRATION SOLUTION

Immersion of whole organs in urea and urea-like chemicals causes tissue hyperhydration, which improves the penetration of clearing reagents into the tissue to achieve greater transparency *(25)*. Another strategy for achieving tissue transparency involves first removing lipids from the brain with detergents like sodium dodecyl sulphate (SDS), saponin, or Triton X-100 *(26)*, then immersing and becoming hyperhydrated in highly concentrated urea solutions of a tissue-matching RI in the range of 1.38 to 1.48 *(2)*. This group's first clearing technique, called Sca*l*e, uses 4 moldm^{-3} urea for clearing, 10% glycerol to prevent excessive hydration and limit tissue expansion, and 0.1 percent Triton X-100 to remove lipids. In this manner, the endogenous fluorescence is preserved while clearing out the entire adult mouse brain and mouse embryo. YFP-positive neurons in cleared tissue are used for the visualization and reconstruction of neuronal populations and projections at subcellular resolution in cortical, callosal, hippocampal, and neurogenic populations. Although this method is quick, non-toxic, and inexpensive, the disadvantage is that the clearing takes two weeks *(27)*. Sca*l*eS, in which sorbitol is used instead of glycerol, is an improved version of Sca*l*e. Sorbitol, as a sugar alcohol, has tissue clearing properties like urea, but in comparison to glycerol, it preserves the original sample volume better by compensating for hyperhydration with a highly concentrated urea solution *(28)*. Results of tissue clearing using hyperhydration methods encourage further study of chemicals (polyalcohols and detergents) with the potential to clear larger tissue samples and whole organs while preserving fluorescence and speeding up the process. CUBIC (clear, unobstructed brain imaging cocktails and computational analysis) is the most widely used technique for treating hyperhydration. The Sca*l*e protocol served as the foundation for the development of CUBIC reagents. For cleaning and imaging mouse brain samples, CUBIC also employs a combination of aminoalcohols. The first step in the CUBIC protocol is lipid removal with reagent 1 (named Sca*l*eCUBIC-1 in honour of Sca*l*e), which is made from urea, N,N,N',N'-tetrakis(2-hydroxypropyl)ethylenediamine (Quadrol), and polyethylene glycol mono-p-isooctylphenyl ether/Triton X-100. The inclusion of Quadrol in a delipidation reagent allowed for

the clarification of highly pigmented organs because it also functions as a decolorization chemical. In the second step, RI matching and transmittance are increased using reagent 2, which is made up of 2,2',2''-nitrilotriethanol, polyethylene glycol mono-p-isooctylphenyl ether/Triton X-100, urea, and sucrose. These brain samples are suitable for imaging with single-cell resolution using various fluorophores. The multicolour imaging of fluorescent proteins or immunostained adult mouse brains is also possible with CUBIC. Despite sharing numerous chemicals with Sca*l*e, the CUBIC protocol increases brain transparency more quickly *(29)*. Clear^{T} is another hyperhydration protocol that, unlike the others, does not use lipid removal. This method uses an ascending gradient of formamide in PBS for clearing, and it takes two days to clear a whole postnatal or adult mouse brain. Since the Clear^{T} method reduces GFP intensity, immunolabeling techniques cannot be used with it. As a result, a similar Clear^{T2} protocol was developed that, in addition to formamide, uses PEG to stabilise protein conformation. Using Clear^{T} and Clear^{T2} together improved the ability to see cell morphology and connections in both neuronal and non-neuronal tissues *(30)*.

TISSUE TRANSFORMATION METHODS

When using a high concentration of detergent solution, it is common to lose native proteins from the sample and damage the tissue's structure. The use of urea, which is highly hydrophilic, causes protein denaturation. Hyperhydrating techniques have a dual effect that reduces endogenous protein fluorescence while also causing epitope hindering, which affects immunohistochemical labelling, especially for large samples. Alternative methods for preserving proteins in samples include cross-linking them with covalent bonds and creating a rigid protein mesh within the tissue. Proteins and nucleic acids are thus stabilised, and tissue can be cleared further. Preservation is frequently accomplished by immersing the sample in an acrylamide or bisacrylamide solution, which transforms it into a tissue-hydrogel matrix hybrid. This result has led to the term "hydrogel embedding tissue clearing methods" being applied to the techniques we'll discuss in more detail *(1-3,26)*. Clear Lipid-exchanged Rigid Imaging, Immunostaining, and In Situ Hybridization Compatible Acrylamide Hybridized PFA (paraformaldehyde), acrylamide, bisacrylamide, VA-044 (non-nitrile, cationic water-soluble azo polymerization initiator), and PBS (phosphate buffered saline) are all used in the tissue-hydrogel (CLARITY) method. The tissue (e.g., whole mouse brains) are incubated in this solution for three days after being isolated. The temperature is increased to 37 °C to start the polymerization process. Lipids from the hydrogel-encased brains are washed out using electrophoresis for two days in an SDS-containing buffer. Rather than removing lipids through passive diffusion of SDS micelles, which would take months (as in Sca*l*e clearing), CLARITIY uses an active, electrophoresis-assisted process. The process of actively removing lipids through electrophoresis is known as electrophoretic tissue clearing (ETC). Following this procedure, the sample can be trimmed to the desired dimensions and immunolabeled. Imaging of long-range projections, local circuit wiring, cellular relationships, subcellular structures, protein complexes, nucleic acids, and neurotransmitters is possible using CLARITY on mouse brain and human brain samples *(31)*. Although electrophoresis accelerated clearing, some detrimental effects on the tissue were observed. The magnitude of the electromagnetic field had a significant impact on tissue transparency and resulted in heat-induced tissue damage and browning. Because of this, the passive CLARITY technique (PACT) was developed to lessen these adverse effects. Yang and his team introduced the PACT method, which is essentially CLARITY without the electrophoresis step to reduce its adverse effects and embedding in RI-matching solution (RI = 1.46), which is used for imaging or long-term sample storage. For whole-body or large organ clearing combined with immunolabeling, a new method called PARS (perfusion-assisted agent release *in situ*) is proposed, which uses PACT clearing reagents and animal vasculature to deliver hydrogel forming monomers and clearing solutions directly to the desired organ. All the procedures described above are compatible with preserving fluorescent proteins present in the sample *(32)*. SWITCH (system-wide control of interaction time and kinetics of chemicals) is a clearing method that works well with human tissue and allows multiple (more than 20) steps of immunohistochemistry on large tissue samples. Two different buffer types (SWITCH-OFF and SWITCH-ON) are used after tissue fixation. The first allows antibodies and chemicals to enter the tissue but prevents interactions between them and tissue macromolecules. This ensures an even distribution of clearing chemicals and labelling antibodies. The SWITCH-OFF buffer has the opposite effect and stimulates tissue reactivity to clearing agents and antibodies. The visualisation and quantitative analysis of entire myelinated fibre tracts are adequate after clearing using this technique. The ability to conduct proteomic analysis and protein expression profiling using common analytical techniques like MALDI-MS and LA-ICP-MS on the same samples after clearing is another advantage of the SWITCH clearing method *(33)*.

CONCLUSION

Tissue clearing techniques are expanding the field of cellular and sub-cellular resolution imaging by allowing the observation of entire organs or large samples using contemporary microscopy techniques, such as light sheet fluorescence microscopy. More than 30 different tissue optical clearing methods have been developed, and the number is still expanding because of advancements in chemistry and microscopy. In this review, we aimed to

present an overview of tissue clearing methods used for research in the field of neuroscience. By outlining their relative benefits and drawbacks, we hope to provide readers with a solid foundation from which to choose the best approach. The obvious potential of optical clearing methods is still in progress and evolving. Because further significant innovations and improvements in clearing methods are still to be expected, particularly in terms of understanding the underlying chemistry, each application should be able to find its ideal clearing protocol.

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