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Chapter 21

3D imaging in the postmortem human brain with CLARITY and CUBIC

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Abstract

Recent innovations in tissue clearing and imaging technologies have enabled us to analyse biological systems directly in three-dimensions using thick samples. In this review, we discuss two of these recently reported tissue-clearing technologies (CLARITY and CUBIC) that are compatible with archival formalin-fixed human brain materials that have been fixed in formalin for a long period of time. We will discuss the pros and cons of these two technologies, examples of visualisation of Alzheimer neuropathological hallmarks and the exact protocols that we regularly use in the laboratory.

INTRODUCTION

Recent developments in tissue-clearing methodologies facilitate the study of three-dimensional (3D) structures in thick biologic tissues. These new methods are highly valuable alternatives to the classic 3D analyses that were based on a series of thin sections. Two-dimensional (2D) pictures of these sections were stacked to form the 3D image. The new techniques provide a direct 3D view of the anatomic structure under study. In the central nervous system, the neurons extend their processes in various directions to construct neuronal networks. 3D imaging in such a complex organ provides profound information on the network connections at both local and regional levels. Combining tissue-clearing technologies with immunostaining has enabled visualization of protein localizations in the three dimensions of thick biologic samples. However, postmortem human tissues have often been fixed for months or decades and are not easily clarified and/or immunostained.

We have tested several tissue-clearing methods on human postmortem tissues and have previously reported that CLARITY (clear lipid-exchanged acrylamide-hybridized rigid imaging/immunostaining/in situ hybridization-compatible tissue-hydrogel) (Chung et al., 2013) is a valid method to visualize lesions of Alzheimer disease (AD) and axonal processes (Ando et al., 2014). We also successfully tested another tissue-clearing method, CUBIC (clear, unobstructed brain imaging cocktails and computational analysis) (Susaki et al., 2014), and found that CUBIC is also compatible with human postmortem tissues that have been fixed for a long period of time (for more than 5 years). This review summarizes how we used these two methods (CLARITY and CUBIC) on human postmortem brain tissues from patients with AD, and how we applied these methods to the study of brain lesions in AD: intracellular neurofibrillary tangles composed of tau and extracellular amyloid plaques constituted of amyloid ß (Aß). There are, no doubt, other efficient methods of clarifying human postmortem samples, but we restricted our review to the two techniques that we have regularly used.

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TISSUE-CLEARING METHODS

Biologic tissues lack transparency because they are made of structures that scatter the light. Light scattering occurs at the interface between two contiguous structures or elements with different refractive index. Packed lipid bilayers are mostly responsible for the refractive index heterogeneity of biologic samples. Removing the lipids from the tissue is one of the most common approaches to make the samples transparent. As a bonus, lipid removal makes the proteins more accessible to antibody or other molecular probes in aqueous solution.

The tissue-clearing methods can be divided into two large categories: those based on organic solvents and thus that require an initial step of dehydration of the tissue, and those that are performed in aqueous solutions (for more information on mechanisms of action of each component in these techniques, see review by Richardson and Lichtman, 2015). The first category uses organic solvents to solubilize lipids and extract them from the sample. This method has been used for more than a century to clarify microscopic sections. The second category uses aqueous solutions of concentrated detergents to create lipid micelles that may passively leave the sample (Sca/e, CUBIC, CLARITY-passive clarity technique (PACT)) or be actively drawn out of it by an electric current (active CLARITY technique using electrophoretic tissue clearing (ETC); see detailed methods below).

Organic solvent-based tissue clearing

Several new methods using organic solvent have been recently described: BABB (Dodt et al., 2007), 3DISCO (Erturk et al., 2012a, b, 2014), iDISCO (Renier et al., 2014), and uDISCO (Pan et al., 2016). While organic solvents with high refractive index are efficient for optical clearing of entire rodent brains, there are some disadvantages to their use, e.g., they may cause fluorescence quenching and tissue shrinkage and the solvents are quite toxic.

Tissue clearing in aqueous solutions

The aqueous clearing methods can be divided into several groups, such as simple immersion and passive or active lipid removal, followed by hydration or refractive index matching. There are a number of tissue-clearing techniques, including sucrose (Tsai et al., 2009), SeeDB (Ke et al., 2013), FocusClear (Chiang et al., 2002), ClearT (Kuwajima et al., 2013), FRUIT (Hou et al., 2015), CUBIC (Susaki et al., 2014, 2015), whole-body CUBIC (Tainaka et al., 2014), CLARITY (Chung et al., 2013; Tomer et al., 2014), CLARITY-PARS and CLARITY-PACT (Poguzhelskaya et al., 2014; Yang et al., 2014), FASTClear (Liu et al., 2016b), Sca/eA2 and Sca/eU2 (Hama et al., 2011), Sca/eS (Hama et al., 2015), and 2,2-thiodiethanol (TDE) (Staudt et al., 2007; Aoyagi et al., 2015; Costantini et al., 2015).

Several of these techniques are adequate to visualize intrinsically fluorescent proteins in transgenic animals expressing fluorescent proteins, such as GFP, YFP, RFP, though some are not compatible with immunolabeling (e.g., SeeDB is more adequate for visualization of GFP than immunostaining) (Ke et al., 2013). Some of the techniques also bleach fluorescent proteins and are not adequate for long-term storage (Ke et al., 2013; Hama et al., 2015).

We tested several tissue-clearing protocols applied after immunostaining of 500-µm-thick postmortem human AD tissue slices. We could not observe a conclusive signal in the depth of such slices immunostained for Alzheimer lesions (tau and Aß) and postclarified with 3DISCO, ScaleA2, or SeeDB (Ando et al., 2014). CLARITY-PACT (Fig. 21.1) (Chung et al., 2013; Yang et al., 2014; Liu et al., 2016a) or active CLARITY technique (Chung et al., 2013) and CUBIC (Susaki et al., 2014) (Fig. 21.2), combined with immunostaining, provided the most contrasted images of protein aggregates in human postmortem AD brains at depths deeper than 200 µm.

The difficulties in immunostaining thick postmortem human brain sections may be related to long fixation times, since some samples are fixed for more than 2 months, and sometimes for decades in formol, and to the poor antibody penetration that may not make it into the depth of these thick tissue sections. 3DISCO is not compatible with human postmortem thick tissues due to very limited antibody penetration (Ando et al., 2014). Several pretreatments with dimethyl sulfoxide render the more recently reported iDISCO compatible with immunostaining in rodent brains (Renier et al., 2014). iDISCO is a valid method to detect Alzheimer lesions in transgenic mouse models and in frozen tissues of postmortem human AD brains (Liebmann et al., 2016). Further tests are needed to determine if the method is compatible with human postmortem brain samples after a long period of fixation.

The recently reported Sca/eS technology was proved to be efficient in clearing frozen human tissues thawed and postfixed with 4% paraformaldehyde (PFA). Effective antibody diffusion was achieved by applying pretreatments prior to immunostaining (Hama et al., 2015). The original technique was responsible for marked tissue swelling. For instance, Sca/eA2 makes use of Triton X, glycerol, and urea. Triton X removes membrane lipids, glycerol causes dehydration without altering tissue structure, and urea facilitates the penetration of the tissue by the detergent Triton X. Hama and

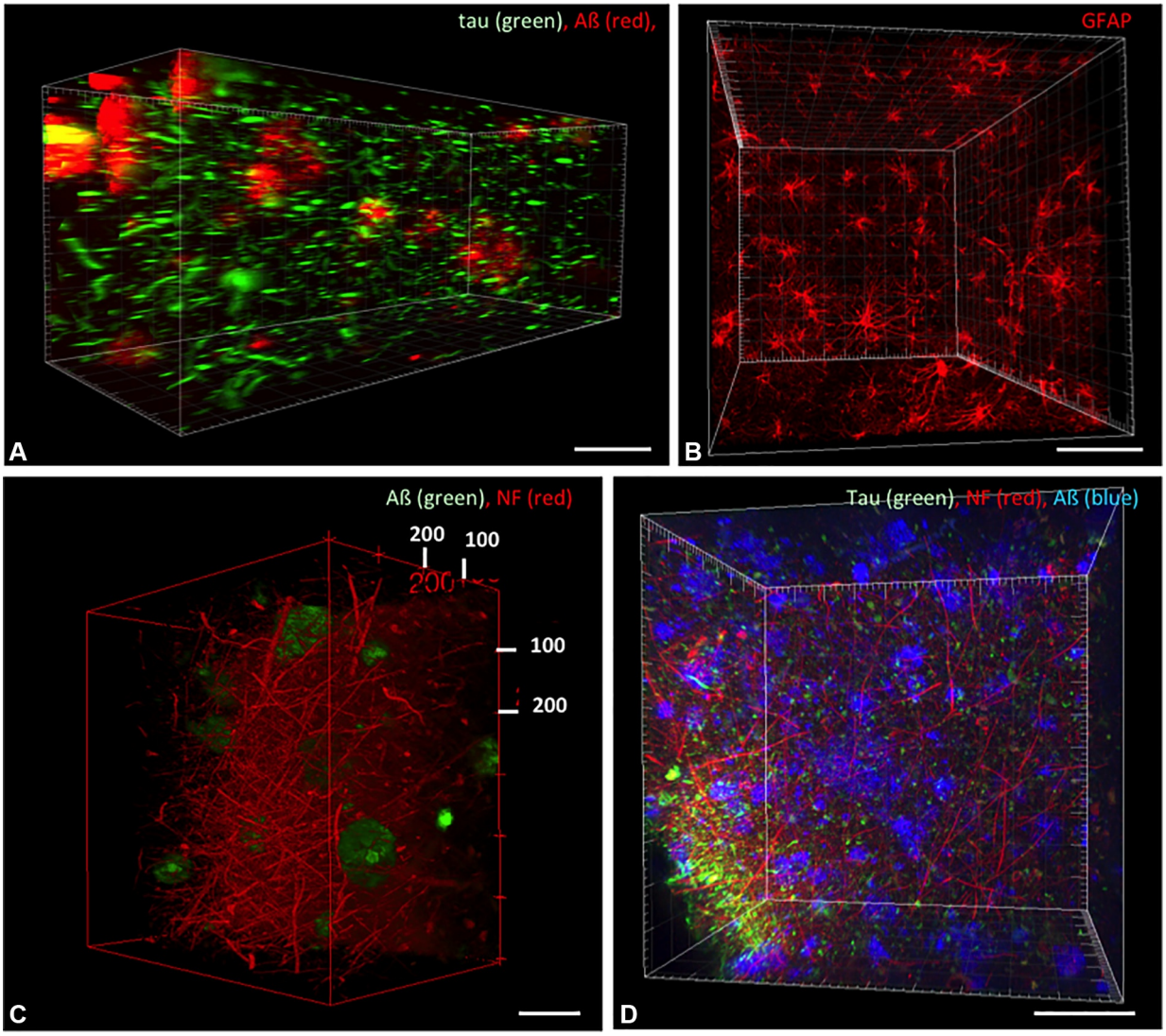

Fig. 21.1. Immunostaining of CLARITY-clarified 500-µm-thick slices from human Alzheimer disease (AD) postmortem frontal cortex. (**A**) Human AD frontal cortex tissue immunostained for Aß (4G8, red) and for tau (B19, green). Stack depth of 379 µm; step size = 1 µm. Stack photos were taken with an upright confocal microscope equipped with a 20 × water immersion objective. (**B**) Human AD frontal cortex tissue immunostained for glial fibrillary acidic protein (GFAP: red). Stack photos were taken with an upright confocal microscope equipped with a 20 × water immersion objective. Depth 864 µm; step size = 1µm. (**C**) Human AD frontal cortex tissue immunostained for Aß (4G8, green) and neurofilament (NF) (2F11, red). Stack photos were taken with a two-photon microscope equipped with a 20 × water immersion objective. Stack depth of 401 µm; step size = 1 µm. (**D**) Human AD frontal cortex tissue immunostained for tau (B19, green), NF (2F11, red) and Aß (biotin-labeled 4G8, blue). Stack photos were taken with an upright confocal microscope equipped with a 20 × water immersion objective. Stack depth of 418 µm; step size = 1 µm. Scale bar, 100 µm.

colleagues (2015) modified the original method to avoid tissue swelling by adjusting the quantity of sorbitol and glycerol (the latter inducing dehydration and thus causing tissue shrinkage). It remains to be tested whether Sca/eS is compatible with human brain samples after long formalin fixation. A simple protocol for postmortem brain clarification named FASTClear has been recently reported (Liu et al., 2016b). FASTClear is a simplified version of CLARITY-PACT, free of hydrogel embedding, but the tissues need to be fresh without prolonged formaldehyde fixation. FASTClear needs to be further optimized for archival formalin-fixed human brain materials (Liu et al., 2016b).

The clearing step results in homogenization of the tissue refractive index, that is, however, higher (with values between 1.38 and 1.48) than the refractive index of air (1.0). The refractive index of the mounting medium and of the immersion medium placed between the

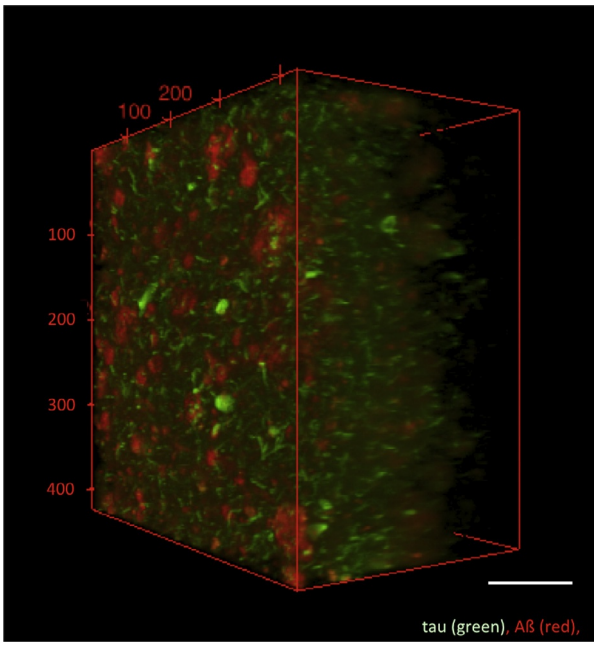

Fig. 21.2. Immunostaining of CUBIC-clarified 500-µm-thick slices from human Alzheimer disease postmortem brain frontal cortex. Human Alzheimer disease frontal cortex tissue immunostained for Aß (6E10, red) and for tau (B19, green). Stack depth of 264 µm; step size = 1 µm. Stack photos were taken with a two-photon microscope equipped with a 20× air objective. Scale bar, 100 µm.

specimen and the microscope objective have then to be adjusted to be close to the refractive index of the tissue to prevent light scattering at their interfaces.

OVERVIEW OF CUBIC

CUBIC is a simple clarification method (Susaki et al., 2014) that has three steps, as shown in Figure 21.3: (1) clarification of fixed samples with CUBIC1; (2) immunostaining with primary and secondary antibodies; and (3) refractory index homogenization with CUBIC2 (Susaki et al., 2014). CUBIC is much simpler and faster than CLARITY-PACT (Fig. 21.3). CUBIC is a modification of the original Sca*l*e method, in which urea at a high concentration was the basic component (Hama et al., 2011). Urea, however, makes the sample swell by increasing its water content. To counteract that effect, glycerol was added to the mixture in Sca*l*eA2 which also contained Triton X-100, a nonionic detergent. In the CUBIC1 solution glycerol was replaced by an amino alcohol, N,N,N',N'-tetrakis(2-hydroxypropyl) ethylenediamine, that does not quench the fluorescent signal and probably solvates anionic phospholipids by its cationic amino group (Susaki et al., 2014). Reagent 2 contains a high concentration of sucrose that increases the refractive index. The CUBIC method does not cause obvious tissue swelling in postmortem human brain tissues fixed

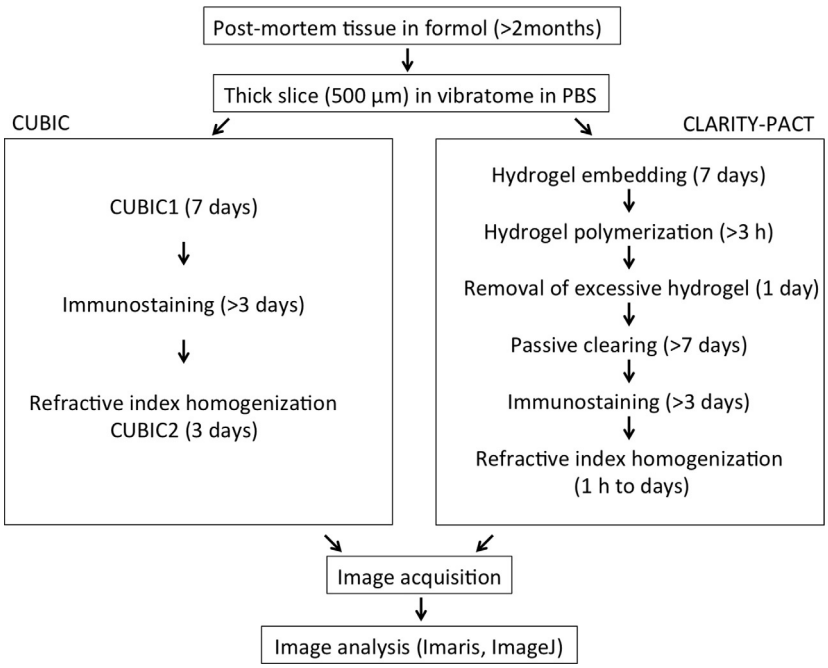

Fig. 21.3. Overview of the steps in the CLARITY-PACT and CUBIC techniques. The CLARITY technique requires more steps and longer experimental time, but can retain intrinsic proteins better than the CUBIC technique. *PBS*, phosphate-buffered saline.

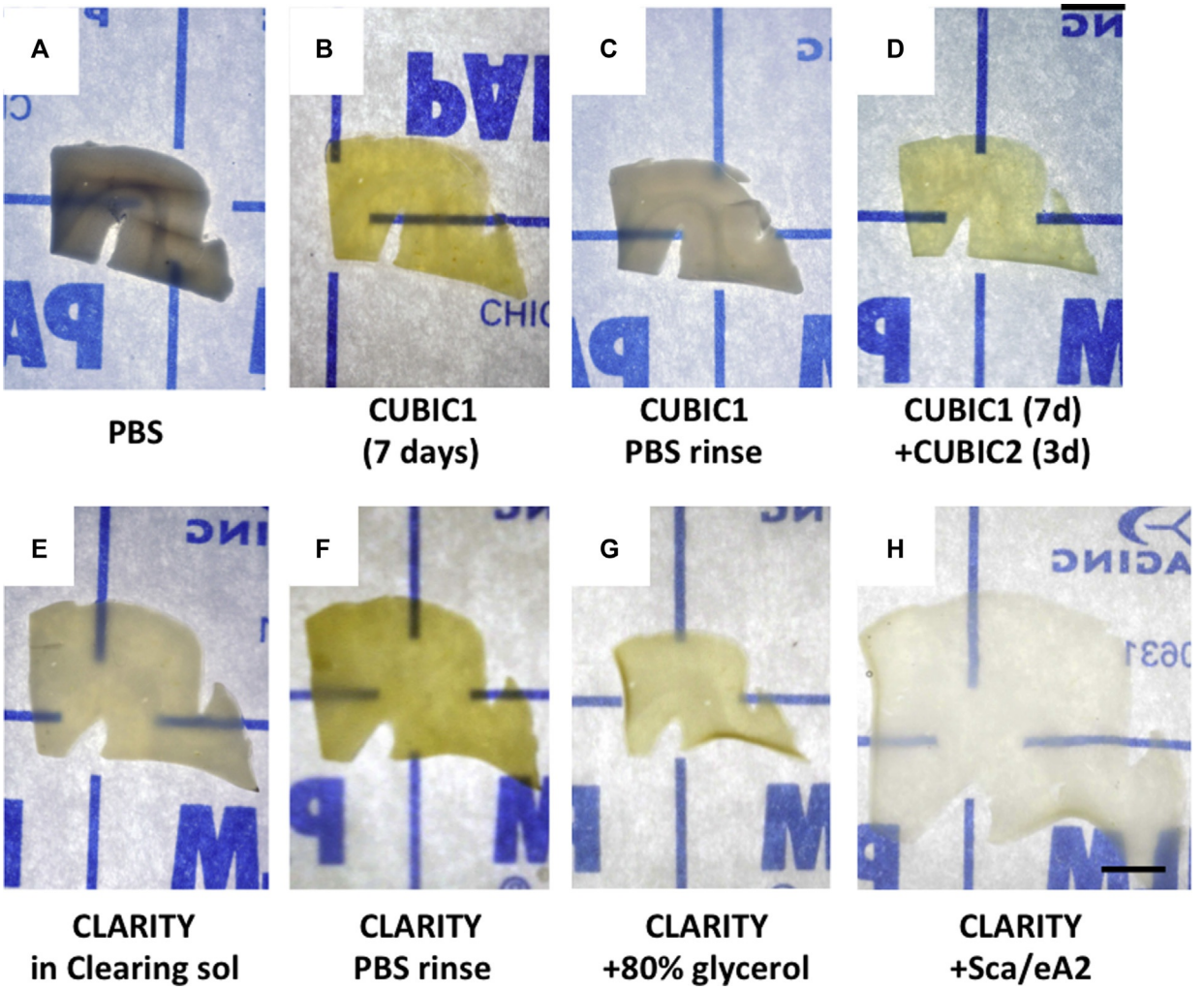

Fig. 21.4. Representative photos of adjacent postmortem human brain slices (500 μm thick) kept in phosphate-buffered saline (PBS) (**A**), incubated for 7 days in CUBIC1 (**B**), then rinsed with PBS (**C**) and subsequently incubated with CUBIC2 for 3 days (**D**). Alternatively the section may be embedded in CLARITY hydrogel and passively cleared for 20 days in clearing solution at 37°C (**E**), then rinsed in PBS (**F**) and incubated with refractory index-matching solutions such as 80% glycerol (**G**) or Sca/eA2 (**H**). A severe tissue expansion was observed in a CLARITY-clarified tissue slice incubated in Sca/eA2. Scale bar, 5 mm.

for a long time (e.g., for more than 2 months to years) (Fig. 21.4A–D).

OVERVIEW OF CLARITY

The experimental steps for the CLARITY-PACT method in postmortem human tissues are shown in Figure 21.3. In CLARITY, the biologic samples are first embedded in a hydrogel that will keep the morphology of the structure despite removal of the lipids, which takes place in a second step. The sample is initially left in a solution containing acrylamide, PFA, phosphate-buffered saline (PBS), and a thermo-sensitive initiator. The solution is kept at low temperature to inhibit polymerization of acrylamide.

PFA generates covalent bonds between the acrylamide monomers and the tissue proteins. The solution is degassed and the polymerization is initiated by heating. As a result, the proteins of the tissue are fixed to the hydrogel, while lipids (that are not fixed by PFA) may still be removed. The excess hydrogel is then peeled off. It is a delicate step and the fragile slices have to be handled with care. Hands have to be protected with gloves as always with human material but also because unpolymerized acrylamide is toxic. Very intricate structures such as hippocampus can be easily destroyed in this process.

It might be helpful to dissect small pieces of tissues (e.g., the dentate gyrus) prior to hydrogel embedding. The tissue is then made transparent by removing the

lipids (Chung and Deisseroth, 2013; Chung et al., 2013; Yang et al., 2014), which is not only useful for transparency but also facilitates the penetration of the molecular probes (Tomer et al., 2014). Lipids are extracted by sodium dodecyl sulfate (SDS), an anionic detergent. Hydrogel-embedded samples are either passively clarified at 37°C by a clearing solution composed of SDS and boric acid or actively clarified in an electric field or by ETC. Negatively charged lipid micelles are more efficiently removed from the tissue in an electric field than by passive lipid removal. Passive clarification of human postmortem tissue slices requires 7–14 days depending on the tissue thickness and brain region. To accelerate passive clarification, higher temperature up to 50–60°C can be used (Yang et al., 2014).

Myelin is the most abundant lipid and the myelin content of the sample is one of the important factors determining the time required for clarification. Hippocampus and substantia nigra are rapidly clarified while brainstem regions caudal to the midbrain can never be made fully transparent by the CLARITY passive clearing method (Liu et al., 2016a). The length of the fixation time affects negatively tissue-clearing efficiency (Liu et al., 2016a) and the authors noticed that the clarified human brain tissues fixed for months were less rigid than tissues fixed for more than 2 years. For rodent brains, clarification can be accelerated by reducing the concentration of acrylamide (from 4% to 2%) in hydrogel solution, as described in the CLARITY-PACT protocol (Yang et al., 2014). However, this renders the human tissues more fragile and can make them collapse without accelerating clarification and is the reason why altering the original method in this way is not recommended in human postmortem tissues (Liu et al., 2016a).

Clarified tissues can be subsequently immunostained. An extended incubation time (days to weeks) is critical for good immunoglobulin penetration (Yang et al., 2014). After immunostaining, tissues can be postfixed with 4% PFA in PBS to properly preserve the antibody labeling (Ando et al., 2014; Hama et al., 2015).

CUBIC AND CLARITY: THE PROS AND CONS

The removal of lipids, either by an electric current in CLARITY with ETC or by detergents in passive CLARITY-PACT or CUBIC, is associated with a detrimental loss of some tissue proteins. In CLARITY, the protein loss is 8–13%. The hydrogel in this method probably prevents additional loss because protein loss is significantly higher in CUBIC, at 24–41% (Chung et al., 2013; Liu et al., 2016a). Insoluble or aggregated proteins, such as those observed in neurodegenerative diseases, e.g., Aβ and tau, are highly insoluble and naturally more resistant to solubilization in the tissue. In that context, the simpler method CUBIC may be preferred to the more complex method CLARITY. Also, cytoskeletal proteins (e.g., neurofilaments, glial fibrillary acidic protein) are highly insoluble and can be visualized by these two techniques. For more soluble or labile proteins, such as neurotransmitters or membrane-bound proteins, CLARITY may be a better choice as the hydrogel can trap the protein in the sample.

REFRACTIVE INDEX AND MICROSCOPE OBJECTIVES

As already mentioned, light scattering is the essential factor that explains the opacity of the tissues. Removal of the lipids increases the homogeneity of the tissue refractive index. The tissue section is placed on a glass slide, immersed on the slide in a mounting medium, and cover slipped. The tissue refractive indices are first fixed by the clearing procedure, then by the mounting medium. The refractive indices of mammalian tissues after fixation and clearing are higher (between 1.38 and 1.48) than those of water (1.33) or of air (1.0) (see review by Marx, 2014). To avoid light scattering, the refractive index of the mounting medium must be as close as possible to the refractive index of the tissue itself. For the CUBIC method, the recommended mounting medium is CUBIC2 (see Appendix 21.2).

For the CLARITY-PACT method, refractive index homogenization can be achieved by various mounting media, including 80% or 85–87% glycerol (Chung et al., 2013; Tomer et al., 2014), FocusClear – a commercial product of unknown composition (Chung et al., 2013), Sca/eA2 – a solution containing urea, Triton X, and glycerol (see step 7 of Appendix 21.1) (Hama et al., 2011), or 47% TDE in 0.01M PBS (Costantini et al., 2015). We regularly use ScaleA2 as the mounting medium for CLARITY-PACT human tissues. ScaleA2 provides the best clarification but causes marked tissue swelling (Fig. 21.4H) (Ando et al., 2014; Liu et al., 2016a) that can reach 150–180% in one dimension.

We have noticed the amount of swelling depends on the tissue fixation time (shorter fixation leads to more significant swellings) and on the degree of tissue clarification (longer clarification leads to more severe swellings). To avoid such tissue swellings, Liu et al. (2016a) recommend 47% TDE in phosphate buffer instead of PBS as the mounting medium for CLARITY-PACT human tissues in order to obtain transparency without overt tissue swelling.

Yang et al. (2014) reported a protocol with Histodenz, a nonionic X-ray contrast compound developed specifically as a density gradient medium. It is used to modify the refractive index of the mounting medium in a solution

called refracting index-matching solution (RIMS). The same group has also developed a more affordable mounting medium made of a sorbitol solution (sRIMS), but it is less efficient than RIMS (Yang et al., 2014). While RIMS has been used with success in transgenic mouse tissues with fluorescent protein expression (Yang et al., 2014), it is not recommended for human postmortem tissues that have been clarified with the CLARITY protocol (Liu et al., 2016a).

The microscope objectives that have been designed specifically for the new clearing methods are all immersion objectives. The refractive index of air (1.0) is too far away from the tissue refractive index. Therefore the objectives have a long working distance, i.e., the distance between the focal plane and the objective front edge, in order to permit the analysis of thick samples. Leica and Olympus both commercialize CLARITY-optimized objectives (Tomer et al., 2014).

AUTOFLUORESCENCE

Generally, autofluorescence is increased by aldehyde fixatives such as PFA. Quenching of autofluorescence with glycine (see appendices, steps on immunostaining) or sodium borohydride to reduce reactive aldehyde groups helps to reduce signal/noise ratio (Baschong et al., 2001). Furthermore, aged postmortem brain tissues contain intracellular granules called lipofuscins that are residues of lysosomal digestion. Lipofuscins are yellow to brown pigments in visible light and show autofluorescence under ultraviolet illumination with a wide emission spectrum. There are other inherently fluorescent molecules in biologic tissues such as collagen and elastin. Tissue clearing does not remove these autofluorescence background signals. However, the interfering effect of autofluorescence depends on the intensity of the specific signal. Autofluorescence is less pronounced when using a high-affinity antibody and a sensitive detection method that result in an intense and specific immunostaining signal. Enhancement of signal intensity will also allow a shorter acquisition time. To increase the signal-to-noise ratio, it may be beneficial to use sensitive systems of amplification (such as biotin-streptavidin) to improve the specific immunolabeling signal.

3D IMAGING

Confocal, two-photon, or light-sheet fluorescent microscope (LSFM, aka ultramicroscope) may also be used to obtain thin optical sections of thick clarified samples (Keller and Ahrens, 2015). In LSFM, the light sheet illuminating the sample is confined to the focal plane. This technique dramatically reduces the image acquisition time and bleaching of fluorescence, but requires excellent transparency of the sample.

Our current protocol for clarification of postmortem human brains, with CLARITY or CUBIC, uses 500-µm-thick tissues. Such sections can be analyzed with a confocal or two-photon microscope. Image acquisition with LSFM is more difficult because it is often difficult to fasten the clarified samples, which are especially soft and fragile, to the holder and to keep them flat. Since the samples are very soft, they vibrate easily during image acquisition. These technical problems can be partially resolved by embedding the cleared samples in 2% low-temperature melting agarose gel (see Appendix 21.1, step 8).

IMAGE PROCESSING AND ANALYSIS

The raw images are collected in TIFF format and the 3D reconstruction of the images can be visualized using open-source software such as Fiji (Image J, NIH, http://fiji.sc/Fiji), Vaa3D (http://home.penglab.com/proj/vaa3d/home/index.html), XuvTools (www.xuvtools.org), Tera Stitcher (http://abria.github.io/TeraStitcher/), or commercially available software products such as Imaris (Bitplane) or Neurolucida (MBF Bioscience, www.mbfbioscience.com/cleared-tissue-samples).

IMMUNOSTAINED TISSUE CONSERVATION

After image acquisition in mounting medium (e.g., CUBIC2 for CUBIC method and ScaleA2 or 80% glycerol for CLARITY-PACT method), the immunostained tissue samples should be returned to PBS supplemented with 0.13% sodium azide. It is not recommended to store the samples in Sca/eA2 solution that contains a high concentration of urea, a chaotropic agent. In our experience, the immunolabeling signal is lost within a couple of days when the immunostained sample is kept in Sca/eA2 (unpublished observation). After immunolabeling, a postfixation with PFA/PBS can help to retain the labeling. The postfixed immunostained samples may be kept in PBS supplemented with sodium azide at 4°C for a long time (we confirmed that the immunolabeling can be retained for more than 8 months). The sample has to be reincubated with the mounting medium prior to imaging. The tissue may, however, become more fragile and may easily collapse. Because the fluorescence tends to fade with time, it is recommended that the images are acquired immediately after immunostaining and refractive index homogenization.

REPROBING CLARIFIED TISSUES

Antigen reprobing was described in the original article of Chung et al. (2013) and demonstrated in a detailed review on the CLARITY technique by Tomer et al.

(2014). After the first immunolabel, the clarified tissue is incubated at 60°C in a clearing solution of boric acid and SDS. A second round of immunostaining can then be performed. To our knowledge reprobing after CLARITY has not been reproduced by any other research groups. Liu et al. (2016a) recently reported that some residual fluorescent signals remained in immunolabeled human postmortem tissues clarified by CLARITY.

PERSPECTIVES

There are a number of technical challenges ahead that will need to be addressed in order to improve the clarification of tissues. For example, it will be necessary to overcome the limitation of sample volume, to facilitate antibody penetration, to improve imaging in three dimensions, and to develop ways to manage the massive information-processing challenges provided by the acquired images.

Clarification of large tissues

We work on human postmortem brain samples with 500 µm thickness for efficient clearing. However, clarification of formalin-fixed human brain tissues (Chung et al., 2013; Ando et al., 2014; Costantini et al., 2015; Liu et al., 2016a) has been done with samples of various thicknesses, including 500-µm-thick (Chung et al., 2013; Ando et al., 2014), 2-mm-thick (Costantini et al., 2015), 3-mm-thick (Liu et al., 2016a), 3–5-mm-thick frozen tissue blocks (Hama et al., 2015), and 0.125 cm^2 (e.g., 5 × 5 × 5 mm cube) frozen tissue block (Liebmann et al., 2016). When CLARITY-PACT is used, larger samples require longer clarification but prolonged clarification increases tissue swelling.

There may be a limit to the use of passive tissue clarification (Chung et al., 2013). It may be better to apply active clarification with ETC to thicker samples. We tested ETC clearing with a hand-made system that we assembled according to the protocols of Chung that are well described on their website, http://clarityresourcecenter.org (Chung et al., 2013). However, this protocol presented several difficulties and we noticed that the clearing solution eroded the system materials. Fortunately an ETC system named X-CLARITY is now commercially available (logos biosystem, http://logosbio.com/x_clarity/x_clarity/features.php). Since ETC can significantly shorten the time of clarification, it consequently reduces tissue yellowing and swelling.

Antibody penetration into the tissue

The antibodies do not reach the core of the sample when tissue section is too thick. The deepest immunostaining obtained in a human brain sample is currently 1 mm from the surface. This was achieved in a large specimen surgically removed from a patient with drug-resistant epilepsy due to hemimegalencephaly. The result was obtained with a parvalbumin antibody applied after CLARITY. The sample was secondarily incubated in a solution of 47% TDE in PBS to improve refractive index homogeneization (Costantini et al., 2015). To our knowledge imaging deeper than 1 mm has not been performed in formalin-fixed human tissue.

A technical breakthrough will be necessary in order to overcome this limitation. For instance, more efficient antibody penetration may be achieved by electroimmunofluorescence staining techniques that deliver antibodies into the tissues by electrophoresis (Liu and Kao, 2009). In addition, immunoglobulins of lower molecular weight, e.g., Fab fragments, single-chain camelid antibodies, and nanobodies, may facilitate antibody penetration in thick samples (see review by Holliger and Hudson, 2005). For example, an in vivo study has shown that a single-domain camelid-derived VHH antibody can cross the blood–brain barrier and penetrate cellular membranes in live mice after tail intravenous injections (Li et al., 2012). These emerging technologies may dramatically facilitate immunolabeling on large thick tissues in the future.

Imaging large tissue blocks

High-throughput µm-scale imaging of large volumes of tissue is necessary to analyze the neural networks in the brain. Confocal and two-photon microscopes have limited working distance. Currently LSFM appears to be the system best adapted to rapidly collect the large amount of information obtained either with fluorescent antibodies or with proteins made fluorescent by transgenesis (Mullenbroich et al., 2015). The fluorescent signals are collected in a plane perpendicular to the thin sheet of light confined into the focal plane of the detection lens (Huisken and Stainier, 2009). LSFM significantly reduces the time of image acquisition and fluorescent bleaching, and enables excellent resolution at high penetration depth. It however requires the samples to be adequately clarified. Some of the problems associated with limitation of signal acquisition at depth can be partially overcome by shrinkage of samples using uDISCO, as recently described (Pan et al., 2016).

Analyzing massive information on three-dimensional network

This last challenge is to provide a system large enough to store and analyze the large number of serial pictures. The analysis of the neural connections, or of the

so-called connectome, will require further interdisciplinary expertise on neuroinformatics and computer sciences.

The advancs in tissue-clearing methods, image acquisition, and data analysis will promote a better understanding of the neural networks and how pathologic processes can alter them.

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APPENDIX 21.1. CLARITY PROTOCOLS

CLARITY protocols have been described in detail in the original articles (Chung and Deisseroth, 2013; Chung et al., 2013; Tomer et al., 2014) as well as in other articles reporting the use of CLARITY techniques (Yang et al., 2014; Poguzhelskaya et al., 2014; Liu et al., 2016a). Our protocols were based on the original found in the website devoted to CLARITY (http://clarityresourcecenter.org). We modified the CLARITY-PACT protocol slightly to adapt it to postmortem human tissue slices of 500 μm thickness.

Step 1: incubation with hydrogel solution

1. Formalin-fixed brain tissues are rinsed with PBS 1 × and sliced with a vibratome into 500-μm-thick sections in PBS. Each slice is kept in PBS in a 40-mL flat-bottom tube (VWR, 216-2689) or in a 50-mL Falcon tube.
2. The reagents of the hydrogel solution are prepared on ice under the fume hood: 4% acrylamide, 0.25% VA-044 (the azo-initiator used to start the polymerization), 4% PFA, 1 × PBS (Table 21.1A). To avoid heat-induced polymerization, all the components need to be kept on ice. If PFA is prepared from powder, its pH should be kept at 7.4–7.5 for an optimal acrylamidepolymerization. Prepare 5–10 mL of hydrogel solution per 500-μm slice of tissue.
3. The slices are incubated in unpolymerized hydrogel solution at 4°C with gentle agitation for 7 days. That delay is necessary for a good penetration of the solution in the tissue.

Step 2: degassing hydrogel solution

After 7 days of passive diffusion of polymerized hydrogel, the tissue needs to be degassed to remove the oxygen (oxygen prevents efficient polymerization of

Table 21.1

(A) 40 mL CLARITY hydrogel solution

Final concentration	Add	Product	References
4%	4 mL	Acrylamide 40%	Bio-Rad 161-0140
0.25%	0.1 g	Temperature-triggered initiator	Wako VA-044
1 ×	4 mL	10 × PBS	Euromedex
4%	10 mL	16% PFA	Electron Microscopy Sciences 15710-S
QSP 40 mL	22 mL	milliQ water	

Hydrogel solution can be aliquoted and kept at –20°C.
PBS, phosphate-buffered saline; PFA, paraformaldehyde.

Table 21.1

(B) 5 L CLARITY clearing solution

Final concentration	Add	Product	References
200 mM	61.83 g	Boric acid	Sigma B7901 1 kg
4%	200 g	SDS	Sigma L3371 500 g
To pH 8.5	40–50 mL	6M NaOH	120 g NaOH in 500 mL H_2O
QSP 5 L		milliQ water	

CLARITY clearing solution can be kept at room temperature.
SDS, sodium dodecyl sulfate.

acrylamide) and the air in the tubes needs to be removed and replaced with nitrogen.

1. Place the tubes in a desiccator.
2. Leave the caps slightly open for gas exchange.
3. Before desiccation, make sure that nitrogen can be sent to the desiccator by turning on the valve of the nitrogen tank and by moving the desiccator chamber valve to the nitrogen inlet.
4. First, turn on the vacuum pump to remove air from the desiccation chamber. The chamber is efficiently vacuumed if the lid is firmly closed and cannot be moved from the chamber. Keep the pump turning for 10 minutes.
5. Switch the vacuum off. Turn on the valve of the nitrogen tank and switch the desiccator chamber valve to send the nitrogen gas into the chamber.
6. Once the desiccation chamber fills with nitrogen, the lid can be easily removed. Open the chamber to close the caps of the tubes tightly. Then turn off the valve of the nitrogen tank.

Step 3: hydrogel polymerization

1. Incubate the tightly closed tubes at 37–40°C with a gentle agitation for 3–48 hours until the hydrogel is polymerized.
2. Once the hydrogel is polymerized, the tube is placed at room temperature under a fume hood. With gloved hands, the hydrogel-embedded tissue slice is delicately taken out of the tube. The excess hydrogel is peeled off with a paper towel.
3. The slice is incubated in the clearing solution (200 mM boric acid, 4% SDS, pH 8.5) for 24 hours at room temperature with gentle agitation to remove small pieces of hydrogel. (See Table 21.1B for CLARITY clearing solution composition.)

Note: Prepare clearing solution under a fume hood. SDS is toxic and an irritant to skin and the respiratory system. The solution can be made and stored for a long time (> 6 months) at room temperature.

Step 4a: passive CLARITY tissue clearing

The tissue slices are left in at least 30 mL of clearing solution and incubated with gentle agitation at 40°C for 2 weeks till they become transparent. The temperature can be increased up to 60°C to accelerate clarification (Yang et al., 2014). The clearing solution needs to be changed every 3 days.

Step 4b: active clearing with ETC

The tissue slices are placed in an ETC chamber (http://clarityresourcecenter.org) for 48–72 hours. A constant voltage (40 V) is applied across the tissue samples during the delipidation process. The temperature of the SDS reagent can be increased up to 60°C to accelerate the clarification process. The clearing solution needs to be changed every 24 hours.

Step 5: rinsing clearing solution

1. Rinse several times with 40 mL PBS with gentle agitation at room temperature for 0.5–1 day. A complete removal of SDS is critical for immunostaining and imaging. The slices that are not immediately immunostained are stored in 1× PBS supplemented with 0.13% sodium azide at room temperature or at 4°C (the tissue transparency is temporarily lost in PBS).
2. The tissue slices can be cut into smaller pieces to save the quantity of antibody necessary for subsequent immunolabeling. For instance, a tissue slice approximately 5 × 5 × 0.5 mm thick requires 100–200 µL of antibody solutions.

Step 6: immunostaining of clarified tissue slices

For an efficient antibody diffusion, the immunostaining is carried out at 37–40°C with gentle agitation for several days to weeks.

1. For Aß detection, antigen retrieval pretreatment can be done with 100% formic acid for 3 minutes on CLARITY-clarified tissues (optional). The slice is then rinsed with PBS three times for 30 minutes. Tissues clarified for more than 20 days are more fragile and should not be pretreated with formic acid.
2. For blocking nonspecific binding of antibodies, incubate the tissue slices with 10% normal goat serum (NGS) in PBST (PBS 1 × supplemented with Triton X-100 to reach the concentration of 0.1% and sodium azide to reach the concentration of 0.13%) for a few hours. One tissue section is placed in a 2-mL Eppendorf tube. To sufficiently cover the tissue block, more than 200 µL of solution is necessary.
3. Incubate the tissue slices with gentle agitation at 37°C for more than 24 hours (ideally 5 days) with primary antibodies diluted in 1% NGS and 0.13% sodium azide in PBST (starting dilution at 1/100).
4. Rinse with PBST three times in total for 6 hours at 37°C.
5. Incubate for more than 24 hours (ideally 5 days) with secondary fluorescent antibodies (starting dilution at 1/100) in PBST containing 1% NGS and 0.13% sodium azide at 37°C.
6. Rinse with PBST three times in total over 6–12 hours at 37°C.
7. A postfixation is useful to attach the antibody molecules to their target. The tissue slices are incubated in PBS containing 4% PFA for 15 minutes at room temperature with agitation.
8. Rinse with PBS three times for 10 minutes at room temperature with agitation.
9. Glycine helps to quench autofluorescence: incubate with 0.2 M glycine in PBS (1.5 mg/mL) for 15 minutes at room temperature with agitation.
10. Rinse for 10 minutes three times with PBS at room temperature with agitation.

Step 7: refractive index homogenization

The day before imaging, the tissues are incubated overnight at 4°C or for a few hours at room temperature with a mounting medium that will match the refractive index of the tissue.

1. Prepare solution for refractive index matching such as ScaleA2 (4 M urea, 0.1% Triton X-100, 10% w/w glycerol) (Hama et al., 2011) or 80–87% glycerol in water (Chung et al., 2013; Tomer et al., 2014). The compositions for refractory index-matching solutions are shown in Table 21.1 (Table 21.1C for ScaleA2 and 21.1D for 80% glycerol).

The original CLARITY protocol used FocusClear (CellExplorer labs) or 85% glycerol (85 mL glycerol and 15 mL water). The incubation time is between 1 hour and a few hours for refractive index matching for thin tissues (less than 1–2 mm). Longer incubation (such as 2 days) may cause tissue yellowing and swelling and is not recommended. After imaging, the tissue sections need to be returned to PBS and can be stored in the dark at 4°C.

Step 8: imaging at microscope

For image acquisition at an upright confocal microscope, prepare one glass slide, adhesive putty, one glass coverslip, and some drops of mounting medium

Table 21.1

(C) 1 L ScaleA2

Final concentration	Add	Product	References
4 M final	240.24 g	Urea	Wako catelog no. 217-615 or any
0.1% (w/v)	1 mL	Triton X-100	Nacalai tesque catelog no. 25987-85, Japan or any
10% (w/w)	100 g	Glycerol	Sigma catelog no. 191612 or any
	QSP 1 L	milliQ water	

ScaleA2 can be kept at room temperature.

Table 21.1

(D) 10 mL 80% glycerol

Final concentration	Add	Product	References
80% (v/v)	8 mL	Glycerol	Sigma catelog no. 191612 or any
QSP 10 mL	2 mL	milliQ water	

(e.g., Sca/eA2). Place the tissue slice on to the glass slide and gently drop mounting medium on to the tissue. To fasten the coverslip on the slide, place four dots of adhesive putty that are as thick as the section to act as glue and spacer (Poguzhelskaya et al., 2014). Water immersion objectives generally provide longer working distance, and thus image deeper into the tissue.

Glass-bottom Petri dishes can be used for image acquisition in an inverted microscope (in single-photon or multiphoton confocal mode). The tissue slice is placed in a glass-bottom dish with some drops of mounting medium. A glass coverslip can be gently (to avoid air bubbles) placed on to the tissue to keep it flat. Cover the Petri dish to avoid evaporation.

For LSFM acquisition, the tissue may need to be embedded in low-melting-point agarose gels of 0.2–5% (recommended starting dilution 2%). If not, the thick tissue slice may not stay flat on the sample holder.

Agarose embedding

This protocol is compatible with both CLARITY and CUBIC-clarified samples (Silvestri et al., 2012).

1. 0.2–5% low-melting-point agarose gel (starting concentration, 2%) is mixed with PBS 1 × in a microwavable Pyrex bottle.
2. Heat shortly in microwave till the solution becomes transparent.
3. Pour into a Petri dish. Cool down for 3 minutes at room temperature.
4. Place samples on to the agarose solution in the Petri dish.
5. Cool down till polymerization for 10 minutes at 4°C.
6. Remove excessive agarose gel around the tissue.
7. Place on to a holder adequate for LSFM.

APPENDIX 21.2. CUBIC PROTOCOL IN HUMAN POSTMORTEM TISSUE SECTIONS

The protocol of CUBIC is well detailed in the original articles (Susaki et al., 2014, 2015; Tainaka et al., 2014) and we slightly modified CUBIC2 to prevent microbial contamination. It is recommended to prepare the reagents CUBIC1 and CUBIC2 immediately before use. The reagents should be degassed before use to avoid the production of air bubbles in the tissues.

Step 1: tissue clarification by lipid removal

1. Slice the formalin-fixed samples into 500-μm-thick slabs in PBS using a vibratome.
2. Prepare reagent 1 (CUBIC1) composed of 25% w/v urea, 25% w/v N,N,N',N'-tetrakis(2-hydroxypropyl) ethylenediamine, 15% w/v Triton X-100 (Table 21.2A) immediately before use.
   (a) In a glass beaker with a magnetic stirrer, weigh 7.5 grams N,N,N',N'-tetrakis (very viscous) with a flat spatula, then 7.5 grams urea in powder. Add 10.5 mL water and stir with moderate heating (up to 35°C) till the solution becomes transparent.
   (b) Cool down at room temperature.
   (c) Add 4.5 grams of Triton X. Stir at room temperature.
   (d) Degas in a desiccator.
3. Incubate the tissue slices in CUBIC1 at 37°C with gentle agitation till they are transparent (e.g., for 500-μm-thick human brain, 5–7 days). Larger blocks require longer incubation time. CUBIC1 solution needs to be changed every 3–4 days with freshly prepared and degassed solution.

Table 21.2

(A) 30 g CUBIC1

Final concentration	Add	Product	References
25% (w/v)	7.5 g	Urea	Wako catelog no. 217-615 or any
25% (w/v)	7.5 g	N,N,N',N'-tetrakis(2-hydroxypropyl) ethylenediamine	Tokyo Chemical Industry, T0781, Japan
35% (w/w)	10.5 g	milliQ water	
15% (w/w)	4.5 g	Triton X-100	Nacalai Tesque, catelog no. 12697-45, Japan or Sigma catelog no. T9284, T8787, T8532 or Tokyo Chemical Industry catelog no. P0873

After confirming that all the chemicals were dissolved, cool down, and then add Triton X-100.
5–10 mL per slice needs to be prepared.
CUBIC1 needs to be freshly prepared for each experiment.

4. Wash in PBS several times at room temperature with gentle shaking to remove CUBIC1 (0.5–1 day).
5. The tissue section can be cut into smaller pieces (e.g. 5 × 5 × 500 μm) before immunostaining to save antibodies.

Step 2: immunostaining

Keep the tubes protected from light after secondary antibody labeling.

1. For Aß staining, pretreat tissue sections for 3–15 minutes with 100% formic acid (optional). Rinse well with 2 mL PBS for 10 minutes three times with gentle agitation at room temperature.
2. For blocking nonspecific binding of the antibodies, the tissue sections are gently agitated at 37°C in a PBST (PBS 1 × supplemented with Triton X-100 to reach the concentration of 0.1%) solution containing 10% NGS and 0.13% sodium azide.
3. Incubate the tissue sections with primary antibodies in PBST supplemented with 1% NGS and 0.13% sodium azide for more than 24 hours (ideally for 5 days) at 37°C with gentle agitation.
4. Wash the tissue sections in PBST several times at 37°C with gentle agitation for 0.5–1 day.
5. Incubate the tissue sections with secondary antibodies conjugated with fluorescent tag in PBST supplemented with 1% NGS and 0.13% sodium azide for more than 24 hours (ideally for 5 days) at 37°C with gentle agitation.
6. Wash in PBST several times at 37°C with gentle agitation for 0.5–1 day.
7. A postfixation is useful to attach the antibody molecules to their target. The tissue slices are incubated in PBS containing 4% PFA for 15 minutes at room temperature with agitation.
8. Rinse with PBS three times for 10 minutes at room temperature with agitation.
9. Glycine helps quench the autofluorescence: incubate with 0.2 M glycine in PBS (1.5 mg/mL) for 15 minutes at room temperature with agitation.
10. Rinse with PBS 3 × 10 minutes at room temperature with agitation.

Step 3: refractive index homogenization with CUBIC2

1. The tissue sections are first immersed in 20% (w/v) sucrose in PBS and then degassed in a desiccator with a vacuum pump for 10 minutes. The tissue sections need to be further incubated for 1 hour at room temperature with gentle agitation in 20% sucrose till the tissues sink down.
2. Prepare CUBIC2 solution composed of 50% (w/v) sucrose, 25% (w/v) urea, 10% (w/v) triethanolamine / 2,2',2'-nitrilotriethanol, 0.1% sodium azide, and 0.1% (v/v) Triton X-100 (Table 21.2B).
   (a) In a glass beaker with a magnetic stirrer, add 15 grams sucrose, 7.5 grams urea and 4.5 mL milliQ level H_2O. Stir while heating (around 35–50°C) till the solution becomes transparent.
   (b) Add 3 grams of triethanolamine and 0.03 gram sodium azide. Stir.
   (c) Cool down at room temperature.
   (d) Add 30 μL Triton X-100. Stir.
   (e) Degas in a desiccator.
3. The tissue is then immersed in degassed CUBIC2 for 2–7 days. Further immersion increases the transparency but causes tissue swelling.

Table 21.2

(B) For 30 g CUBIC2

Final concentration	Add	Product	References
50% (w/v)	15 g	Sucrose	Nacalai Tesque catelog no. 30403-55 or any
25% (w/v)	7.5 g	Urea	Nacalai Tesque catelog no. 30904-45 or any
10% (w/v)	3 g	Triethanolamine (2,20,20'nitrilotriethanol)	Sigma catelog no. 90279 or Wako, catelog no. 145-05605 or any
0.1 wt%	0.03 g	Sodium azide	Sigma catelog no. S2002 or any
15 wt%	4.5 g	milliQ water	
0.1% (v/v)	30 μL	Triton X-100	Nacalai Tesque, catelog no. 12697-45, Japan or Sigma catelog no. T9284, T8787, T8532 or Tokyo Chemical Industry catelog no. P0873

After confirming that all the chemicals were dissolved, cool down and then add Triton X-100.
CUBIC2 (5–10 mL per slice) needs to be freshly prepared for each experiment.

Step 4: samples will be imaged in CUBIC2

See Step 8 of Appendix 21.1 for slide preparation.

Materials needed for CLARITY

Equipment

Vibratome that can make slices thicker than 500 μm
Vacuum pump
Desiccation chamber
Nitrogen supply
Agitator at 37°C

Materials necessary for CUBIC

Equipment

Vibratome that can make slices thicker than 500 μm
Vacuum pump
Desiccation chamber
Agitator at 37°C

Materials needed for slide preparation (confocal imaging)

For an upright microscope, adhesive putty, glass slides, glass coverslips

For an inversed microscope, a glass-bottom Petri dish, glass coverslips.

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