Research Article

Basics and Applications of Tissue Clearing Technology

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Abstract

The recent growth of tissue clearing technology supports the dissemination and generalization of three-dimensional (3D) observations of complex biological tissues. Since many clearing protocols have been proposed, understanding the basic principles of tissue clearing is essential for understanding their technical characteristics and selecting the appropriate one. Practical 3D tissue staining protocols that can stain whole large tissue samples have also been reported. In addition to these clearing and staining reagents, resources such as light-sheet microscopy and software required for 3D imaging and data analysis have been commercialized and open sourced in recent years. These trends suggest that this technology will become a more standard basic technology in biomedical research, clinical medicine, and drug discovery fields.

Keywords: Tissue clearing, light-sheet fluorescent microscopy, three-dimensional imaging, image analysis, three-dimensional tissue staining

Introduction

Due to its complex three-dimensional (3D) structure, biological tissue should primarily be analyzed by 3D information. The optical microscope has a desirable resolution that allows cells and subcellular structures to be resolved in the tissue. However, due to the opacity of the biological tissue, traditional histology does not allow direct observation of internal structures using the optical microscope. Therefore, microscopic tissue observation in conventional histology is performed by cutting and preparing many sliced sections. The tissue clearing technique is a histological method that has shown rapid technological maturity in recent years. Tissue clearing renders light to pass through the target tissue and thus allows 3D observation of the entire sample without physical slicing. This review presents basic knowledge of recent tissue clearing techniques and their representative applications.

Mechanism and procedure of tissue clearing

Tissue clearing is both an old and new technology. The earliest tissue clearing applications by Lundvall in Sweden and Spalteholz in Germany in the early 1900s were an attempt to visually observe the 3D construction of tissues.[1,2] More than half a century after their early trials, Dent reported a 3D observation case of frog embryos using Murray's clear (a mixture of benzyl alcohol and benzyl benzoate, called BABB), an improved clearing reagent over Spalteholz's one.[3] This study was followed by Tuchin's physical investigation in the 1990s and Chiang's insect tissue clearing reagents in the early 2000s. Finally, in 2007, Dodt published whole-organ/body 3D imaging by combining BABB-cleared tissue with a light-sheet microscope (discussed below).[4] This epoch-making work triggered the development of subsequent tissue clearing technology.

Modern tissue clearing technologies include three categories as follows:[5,6]

(1) a method using organic solvents (hydrophobic reagents),
(2) a method using water-soluble compounds (hydrophilic reagents), and
(3) a method combined with a robust fixation method using artificial gels (hydrogel tissue chemistry).

BABB and DISCO reagents are representative of group (1). While these reagents achieve high clearing efficiency, issues such as safety and relatively low signal preservation of fluorescent proteins should be considered. Group (2) includes variations of protocols according to the experimental purposes. Our CUBIC reagents fall into this category. Group (3) includes protocols represented by CLARITY, which impart resistance to physicochemically severe conditions such as high temperature and strong tension to biological tissues. This operation improves the preservation of the molecules and allows multiplex labeling. These three groups were a convenient categorization method based on the background of tissue clearing development. However, several recent integrated protocols such as PEGASOS and SHANEL combine the advantages of each group. Therefore, a recent review proposed a way to understand and classify tissue clearing more comprehensively.[7]

Tissue clearing can be achieved by (1) the reduction of light scattering inside the tissue, and (2) the reduction of light absorption inside the tissue.

Step (1) is the process for the tissue to be infiltrated into a reagent having similar optical properties as the biological materials (mainly proteins), which suppresses light scattering and diffraction inside the tissue. Because the refractive index (RI) is considered a representative index for the optical properties considered here, this step is called refractive index matching (or RI matching). Several protocols aim to lower the macroscopic RI of tissue by swelling.[8–10] However, more complicated physicochemical processes such as suppression of light scattering due to structural changes in fibrous proteins are also assumed.[11] Therefore, the principle of "RI matching" has not been completely elucidated.

Organic solvents such as BABB and dibenzyl ether, and various water-soluble chemicals such as sugars, alcohols, and aromatic amides can achieve RI matching. Occasionally, RI matching is combined with additional steps for achieving higher final transparency. Delipidation is the step to remove lipids that have a high RI and form light-scattering structures such as vesicles. Decalcification removes bone hydroxyapatite. Organic solvents such as alcohols and tetrahydrofuran, nonionic surfactants such as Triton X-100, and ionic surfactants such as SDS and CHAPS are used for delipidation. EDTA, a representative chelating agent, is effective for decalcification.

Step (2), so-called decolorization and depigmentation, aims to remove tissue pigments such as heme and melanin that absorb light. Removal of heme is crucial for efficient clearing because the compound is particularly abundant in the body and can absorb visible light shorter than the red wavelength range. Decolorization methods had been limited to harsh treatments such as bleaching. However, we found that amino alcohols enable efficient heme removal under mild conditions without protein denaturation.[12] Melanin is a complex biopolymer synthesized from tyrosine. So far, there is no efficient way to remove this pigment gently, like amino alcohols for heme. Therefore, bleaching with hydrogen peroxide is usually adopted. Depigmentation of melanin is essential for observing pigmented tissues such as in the eyes.[13] However, it should be noted that bleaching eliminates fluorescent protein signals.

Figure 1 shows a general tissue clearing process incorporating the mechanisms of steps (1) and (2). Fixed tissue is first treated with delipidation/decolorization/decalcification reagents, followed by immersion into an RI matching reagent, which finally renders the sample transparent.

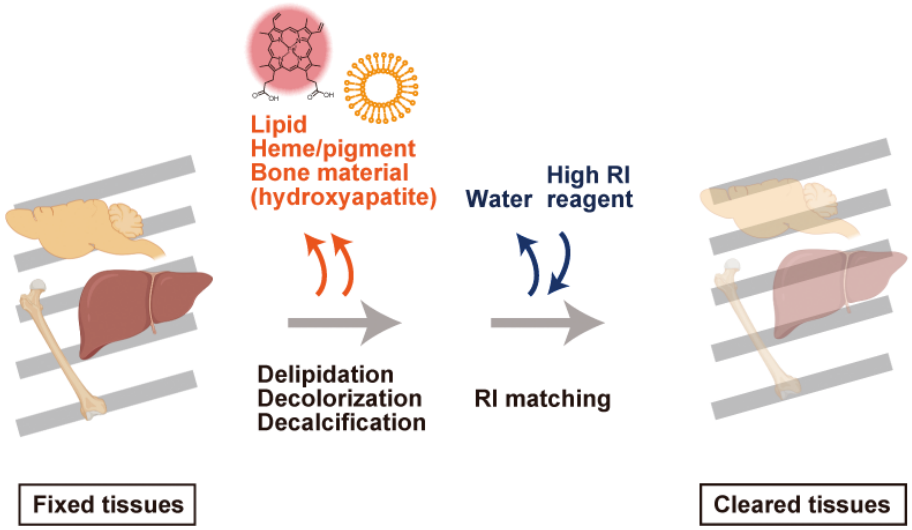

Figure 1. A general procedure of tissue clearing. Part of the figure was created with BioRender.com

We screened the tissue clearing ability of more than 1600 water-soluble compounds and invented a series of CUBIC tissue clearing reagents.[14,15] CUBIC-L has strong delipidation and decolorization abilities due to an amino alcohol, *N*-butyldiethanolamine, and a non-ionic detergent, Triton® X-100. CUBIC-R is a highly efficient RI matching reagent composed of two types of aromatic amines. CUBIC-P contains 1-methylimidazole, which has strong decolorizing activity, in addition to the composition of CUBIC-L. Perfusion of this reagent allows decolorization from the whole body of the animal. CUBIC-B for bone decalcification is composed of EDTA and imidazole. These CUBIC reagents can clear almost all rodent organs. For the delipidation of primate specimens, including human tissues, CUBIC-HL containing 1,3-bis(aminomethyl)cyclohexane and a 1,2-hexanediol solution[16] is additionally provided. Tokyo Chemical Industry has commercialized these CUBIC reagents. End users can quickly deploy the quality-guaranteed CUBIC products in their experiments.

Three-dimensional imaging and image analysis of cleared tissue

3D observation of cleared samples can be performed with prevalent confocal and multiphoton microscopes. However, light-sheet microscopy is further used for faster 3D imaging of large cleared samples (**Figure 2**). Light-sheet microscopy creates an optical section in a sample with excitation light spread out in a sheet shape. This configuration makes it possible to collect the z-stack of 2D images by moving the generated optical sections. Therefore, light-sheet microscopy achieves high-speed 3D imaging. On the other hand, this device presupposes a transparent subject. Therefore, light-sheet microscopy could not be applied without achieving advanced tissue clearing technology.

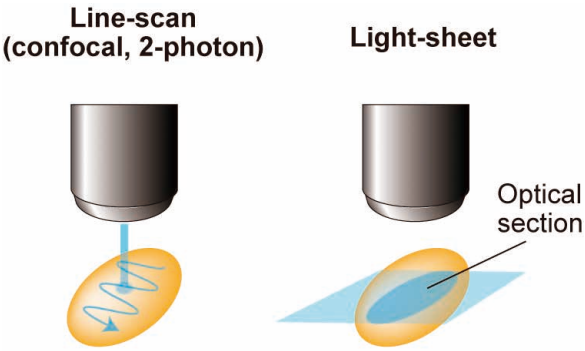

Figure 2. Microscopy for 3D imaging

While prevalent line-scan microscopes can be used for observing a cleared sample, there are issues such as signal leakage from outside the sectioning plane and long imaging time due to the line-scan operation. Since light-sheet microscopy excites only the acquiring cross-section with sheet-shaped illumination light, it enables high-speed 3D observation, avoiding the issues of line-scan microscopes.

Recently, several companies such as Miltenyi Biotec (LaVision Ultramicroscopy), 3i (Cleared Tissue LightSheet), and Bruker (LCS SPIM) have commercialized light-sheet microscopy for cleared tissues. In addition, a group at the University of Zurich has released mesoSPIM, an open-source light-sheet system for cleared tissues[17] (https://mesospim.org/). In the future, cheaper light-sheet systems that non-expert end-users can easily handle will further contribute to the spread of tissue clearing technology.

The experimenter needs to visualize the cell or tissue structure of interest by appropriate labeling. Genetic tools such as transgenic and knock-in animals, or viral vectors, are representative labeling methods. Histological staining with small dyes and antibodies is also an option. However, in such 3D tissue staining, the staining probe requires penetrating the tissue until it binds to the target. It is generally difficult to obtain uniform staining inside the tissue with a simple extension of the conventional 2D staining. The insufficient penetration even of small molecules suggests that this is due to the complex physicochemical environment in the tissue rather than simple reasons such as molecular size.

We attempted to solve this problem. We elucidated that the tissue has the quite similar physicochemical properties as an electrolyte gel. This finding enabled screening of superior 3D staining conditions by combining a tissue-mimicking artificial gel and computer simulation. We finally succeeded in the bottom-up design of the ideal 3D staining protocol "CUBIC-HistoVIsion (CUBIC-HV®)" by combining the screened essential conditions. CUBIC-HV can uniformly stain ~1 cm^3 3D tissue specimens, such as a whole mouse brain and a human brain block, with various antibodies and stains[18] (**Figure 3**). CUBICStars Inc. has commercialized the CUBIC-HV kits and sells them via Tokyo Chemical Industry (https://www.cubicstars.com/cubic-hv/index.html).

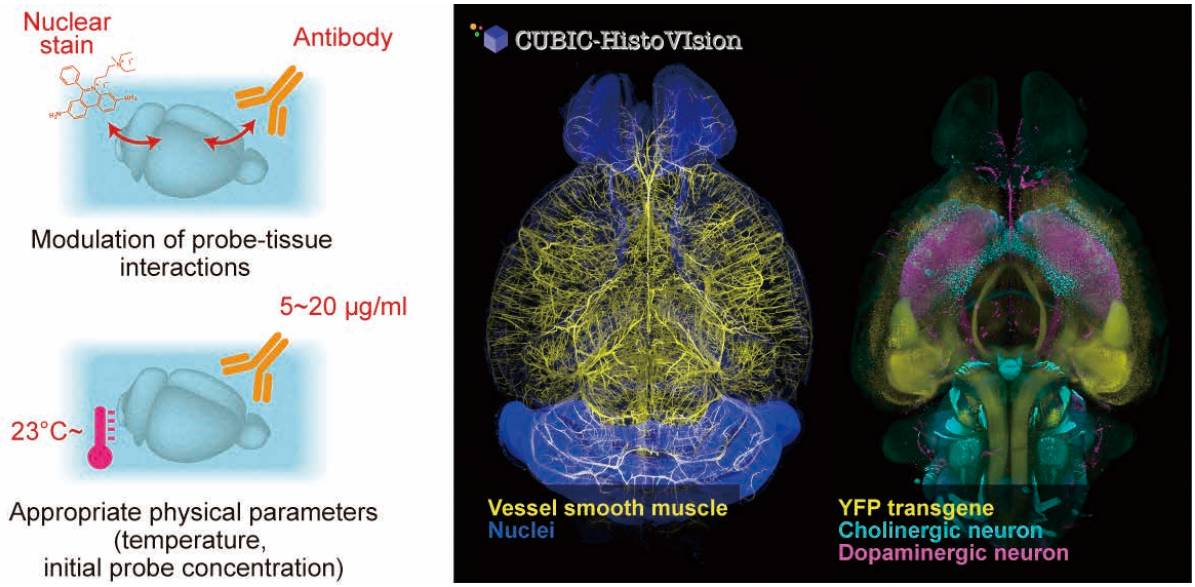

Figure 3. Development and applications of CUBIC-HistoVIsion

We screened the essential factors for improving the penetration of the staining probes to achieve uniform 3D staining. We clarified two crucial factors: (1) modulating the interaction between the staining probe and the tissue, and (2) setting appropriate parameters such as the staining temperature (room temperature or higher) and the initial concentration of the staining probes (5-20 μg/mL antibodies). We finally designed a bottom-up 3D staining and imaging protocol, "CUBIC-HistoVIsion (CUBIC-HV)," by integrating these results. CUBIC-HV made it possible to uniformly stain and visualize sizable (~1 cm^3) 3D samples, such as whole mouse brain. Figure images were adopted from reference 18 (CC-BY-4.0).

Applying an appropriate quantitative analysis is essential for extracting biological information from the acquired 3D image data. As an analysis platform for organ-scale 3D image data, we have developed "CUBIC-Atlas," a whole-brain single-cell resolution atlas consisting of all cell coordinates in the mouse brain,[8] and "CUBIC-Cloud®", a cloud-based software for whole-brain analysis and data sharing.[19] CUBICStars Inc. provides the commercialized CUBIC-Cloud software (https://cubic-cloud.com/).

Applications of tissue clearing in biomedical research

The modern tissue clearing technique was initially considered for use in neuroscience. Many studies reported applications in structural and functional analysis of the global neural circuit, such as dopamine circuits,[20] circuits in the frontal cortex,[21] a novel taste circuit,[22] and whole-brain circuits responding to drugs.[23,24] However, researchers in various fields have also adopted this technique. Cancer research is a representative area because information on 3D tissue structure is significant. Recent examples include analysis of systemic cancer metastasis,[25] detection of micrometastasis,[26] 3D pathology of prostate cancer biopsy,[27] and cancer tissue diversity analysis.[28] The technique has also been used in the clearing of spheroids and organoids.[29] Some recent protocols can even make crustaceans and insects transparent.[30,31]

Conclusions

Tissue clearing technology has rapidly spread in a wide range of biomedical research and is becoming one of the general experimental techniques in several areas. Readers who need more detailed information are encouraged to look over excellent reviews on the detailed principles, workflows, and applications.[5–7,11]

COI disclosure

The author is an employee of CUBICStars, Inc., and is a co-inventor on patents and patent applications owned or filed by RIKEN and CUBICStars, Inc., covering the CUBIC and CUBIC-HV reagents, respectively.

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Dr. Etsuo A. Susaki graduated from Kyushu University School of Medicine in 2002 and received his Ph.D. (Medicine) from the same university in 2007. His main research interests are functional states of multicellular systems and their regulatory mechanisms. In particular, he developed the CUBIC framework and realized a cell-omics approach to study multicellular systems. Recently, he has successfully developed CUBIC-HistoVIsion, an efficient 3D staining and imaging technique for organs and the whole body. He is also an expert in molecular biology, biochemistry, and genetics. He is currently using his unique research background and techniques to uncover the hidden states of multicellular systems (e.g., brain, organoid) and early processes of aging/aging-related diseases.

Related Products

Tissue-Clearing Reagent CUBIC-L [for delipidation and decoloring]	25mL	100mL	500mL	T3740
Tissue-Clearing Reagent CUBIC-R+(N) [for RI matching]	25mL	100mL	500mL	T3983
Tissue-Clearing Reagent CUBIC-R+(M) [for RI matching]		25mL	100mL	T3741
Tissue-Clearing Reagent CUBIC-B [for decalcification]		25mL	100mL	T3780
Tissue-Clearing Reagent CUBIC-HL [for highly fatty tissue and quenching autofluorescence]		25mL	100mL	T3781
Tissue-Clearing Reagent CUBIC-P [efficiently aids perfusion fixation]		25mL	100mL	T3782
Tissue-Clearing Reagent CUBIC-X1 [for tissue expansion]		25mL	100mL	T3866
Tissue-Clearing Reagent CUBIC-X2 [for RI matching while keeping the expanded size]		25mL	100mL	T3867
Mounting Solution (RI 1.520) [for CUBIC-R+]			50mL	M3294
Mounting Solution (RI 1.467) [for CUBIC-X2]			50mL	M3292
CUBIC-HV™1 3D nuclear staining kit			1KIT	C3709
CUBIC-HV™1 3D immunostaining kit (Casein separately)			1KIT	C3717