NEURAL REGENERATION RESEARCH
January 2017, Volume 12, Issue 1
www.nrronline.org

INVITED REVIEW

Three-dimensional histology: new visual approaches to morphological changes during neural regeneration

Hei Ming Lai[1], Ho Man Ng[1], Wutian Wu[1, 2, 3, 4, *]

1 School of Biomedical Sciences, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Pokfulam, Hong Kong Special Administrative Region, China

2 State Key Laboratory of Brain and Cognitive Sciences, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Pokfulam, Hong Kong Special Administrative Region, China

3 Research Center of Reproduction, Development and Growth, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Pokfulam, Hong Kong Special Administrative Region, China

4 Guangdong-Hong Kong-Macau Institute of CNS Regeneration, Jinan University, Guangzhou, Guangdong Province, China

How to cite this article: *Lai HM, Ng HM, Wu W (2017) Three-dimensional histology: new visual approaches to morphological changes during neural regeneration. Neural Regen Res 12(1):53-55.*

Abstract

Three-dimensional (3D) histology utilizes tissue clearing techniques to turn intact tissues transparent, allowing rapid interrogation of tissue architecture in three dimensions. In this article, we summarized the available tissue clearing methods and classified them according to their physicochemical principles of operation, which provided a framework for one to choose the best techniques for various research settings. Recent attempts in addressing various questions regarding the degenerating and regenerating nervous system have been promising with the use of 3D histological techniques.

Correspondence to:
Wutian Wu, M.D.,
wtwu@hku.hk.

doi: *10.4103/1673-5374.198974*

Accepted: *2016-11-10*

Key Words: *three-dimensional histology; tissue clearing; neuronal morphology; neuronal network*

Introduction

The study of neural tissue architecture and cellular morphology is important in neuroscientific research. Visualizing and understanding the three-dimensional (3D) spatial relationships of cells and molecules have been difficult using conventional histology. Serial sections of tissues are often required and this method is time- and labor-intensive, and leads to physical damage and depletion of tissue samples. Advances in optical microscopy and fluorescent probes have contributed to the emergence of tissue-clearing methods that allow intact, transparent tissues to be optically sectioned, imaged, and reconstructed in 3D *in silico*. Here, we briefly introduce the techniques that facilitate the rigorous histological evaluation of intact transparent tissues in 3D and better visualization of neuronal tracts and fibers, which could allow researchers to gain extraordinary insights into the processes of neurodegeneration and neuroregeneration.

The differences in refractive indices between different parts of the cell scatter visible light, lead to the perceived tissue opacity. Therefore, the key to transforming an opaque tissue into a transparent one is to homogenize these differences in refractive indices by immersing the tissue in an appropriate agent. The transparency of the tissue can be enhanced by prior delipidation (*e.g.*, clear lipid-exchanged acrylamide-hybridized rigid imaging/immunostaining/in situ hybridization-compatible tissue hydrogel (CLARITY) by Chung et al., 2013) or dehydration (*e.g.*, 3DISCO (3D imaging of solvent-cleared organs) by Ertürk et al., 2012), which also alters the nature of the tissue and thus its compatibility with various fluorescent labeling methods. Currently, there are no more than 20 tissue-clearing methods with multiple variants, each having its own strengths, difficulties, and issues of compatibility with fluorescent labeling methods and tissue types. Detailed description of each of these methods is beyond the scope of this brief review article; interested readers can refer to the excellent reviews published recently (Susaki et al., 2016; Treweek et al., 2016). To facilitate discussion, here we divide these tissue-clearing methods into three broad categories in terms of their operations and working principles: (1) aqueous-based refractive index homogenization (aqueous-based *n*-homogenization), (2) delipidation-facilitated *n*-homogenization, and (3) organic solvent-based *n*-homogenization. The delipidation step can be optional or partial (Lai et al., 2016), while all organic solvent-based *n*-homogenization methods require prior tissue dehydration to permit the homogeneous infiltration of hydrophobic organic solvents (**Figure 1A**). Such categorization allows the investigator to deduce the physicochemical changes made to the tissues and thus the method of choice for a particular research setting (**Table 1**).

As a general rule, aqueous-based *n*-homogenization (Susaki et al., 2016) best preserves tissue structures and fluorescence, but the lack of adequate permeabilization makes it incompatible or poorly compatible with immunohistochemistry. Moreover, the transparency of the tissues is not as good as the other two categories. In delipidation-facilitated tissue-clearing methods, the tissues are washed free of lipids in detergents with subsequent *n*-homogenization using a suitable aqueous medium (Chung et al., 2013; Susaki et al., 2016). The aqueous media and permeabilization result in good preservation of endogenous fluorescence and compatibility with immunohis-

Table 1 Selected tissue clearing methods

	Aqueous-based *n*-homogenization	Delipdation-facilitated *n*-homogenization	Organic solvent-based *n*-homogenization
Examples	ScaleS (Hama et al., 2015), Clear^{T}(Kuwajima et al., 2013)	CLARITY (Chung et al., 2013), CUBIC (Tainaka et al., 2015), SWITCH (Murray et al., 2015), FASTClear (Liu et al., 2016)	BABB (Schwarz et al., 2015), 3DISCO (Ertürk et al., 2012), uDISCO (Pan et al., 2016), FASTClear (Liu et al., 2016)
Unique advantages	Compatible with lipophilic tracers and subsequent ultrastructural studies	Best results with immunostaining, least tissue discoloration	Best for lipid-rich regions
Disadvantages	Incompatible/poorly compatible with immunostaining, comparatively poor tissue transparency	Incompatible with lipophilic tracers, ultrastructural evaluation, can be slow for lipid-rich regions, comparatively long tissue processing time	Incompatible with lipophilic tracers, ultrastructural evaluation, significant tissue discoloration and autofluorescence, can be incompatible with fluorescent proteins

Summary of selected tissue-clearing methods classified according to suggested working mechanisms, advantages, and disadvantages. Note that the list is general and exceptions exist; for example, lipophilic tracers can be made compatible with delipidation-facilitated *n*-homogenization by using fixable analogues; SWITCH, a delipidation-facilitated method, is associated with significant tissue discoloration and autofluorescence; FASTClear is a novel strategy in which tissue-clearing can be facilitated by delipidation followed by aqueous or organic solvent-based *n*-homogenization. CLARITY: Clear Lipid-exchanged, Acrylamide-hybridized Rigid Imaging/Immunostaining/In situ hybridization-compatible Tissue hYdrogel; SWITCH: System-Wide Control of Interaction Time and kinetics of Chemicals; FASTClear: Free-of-Acrylamide, SDS-based Tissue Clearing; BABB: Benzyl Alcohol-Benzyl Benzoate tissue clearing; 3DISCO: 3-Dimensional Imaging of Solvent-Cleared Organs; uDISCO: ultimate 3-Dimensional Imaging of Solvent-Cleared Organs.

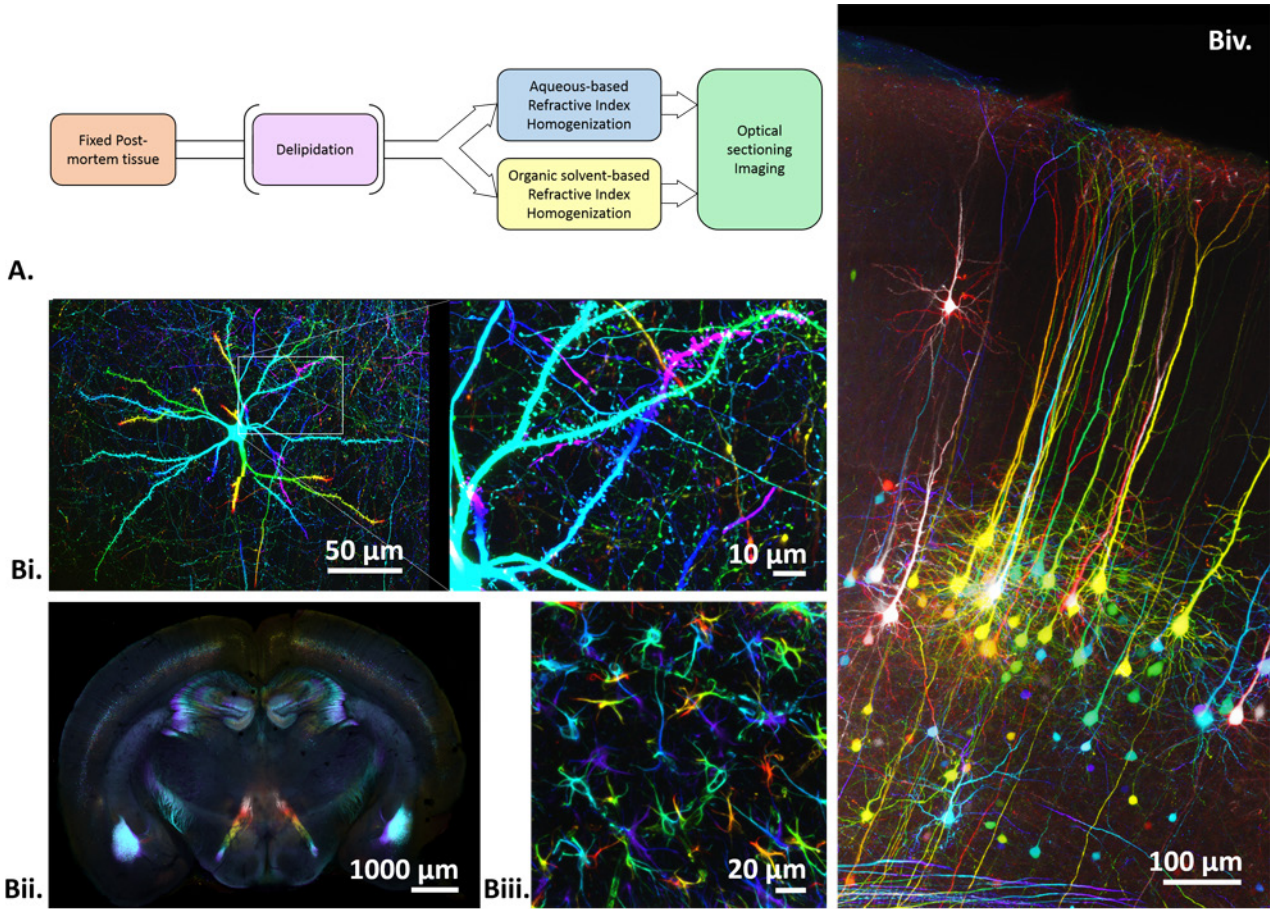

Figure 1 An introduction of 3D histology and some image examples.
(A) General workflow of 3D histology. Fixed tissue is cleared by refractive index homogenization, during which the chemical reagent used can be aqueous or organic solvent-based. The tissue transparency achieved can be enhanced by prior delipidation. Fluorescent labeling (not shown) can be performed prior to tissue harvesting, or at any time before the final refractive index homogenization step. Finally, the fluorescently-labelled, optically-cleared tissue is then imaged with optical sectioning microscopy. (B) 3D histology images generated using CLARITY and FASTClear; all are color-coded projections of Z-stacks (scale bars as labeled), where a more red-shifted color indicates the further the signal from the objective. i) A layer II neuron within the motor cortex of a Thy1-GFP mouse, tissue processed using the CLARITY method. Inset: Enlarged view showing dendritic spines on the dendritic tree of the featured neuron. ii) A tiled Z-stack of a Thy1-YFP mouse brain slice consisting of 858 images stitched together, tissue processed using the FASTClear method (Z-depth 1,100 µm). iii) GFAP staining of wild-type mouse cortical tissue with tyramide signal amplification, demonstrating astrocytic processes around a blood vessel forming the blood-brain barrier; tissue processed using the FASTClear method (Z-depth 24 µm). The shallow Z-depth was due to the limited working distance of the high-magnification objective. iv) A full layer tiled Z-stack of the motor cortex of a Thy1-GFP mouse, demonstrating sparsely-labelled layer V neurons and a layer III neuron, tissue processed using the CLARITY method (Z-depth 370 µm). CLARITY: Clear Lipid-exchanged Acrylamide-hybridized rigid imaging/immunostaining/in situ hybridization-compatible tissue; FASTClear: free-of-acrylamide, SDS-based tissue clearing; GFAP: glial fibrillary acidic protein; 3D: three-dimension.

tochemistry, respectively, but they are incompatible with most lipophilic tracers and limited by the long time required for adequate tissue delipidation. In organic solvent-based *n*-homogenization methods, the tissues are dehydrated and immersed in a hydrophobic organic solvent for homogenization (Ertürk et al., 2012). They are compatible with immunohistochemistry (Renier et al., 2014), but commonly destroy the fluorescent proteins expressed using genetic and viral tools if the pH is not well-controlled (Schwarz et al., 2015). With the differing characteristics and advantages of different methods of tissue-clearing, one size does not fit all (**Table 1**). Thus, when choosing a method of tissue-clearing for specific research questions and goals, a thorough understanding of the physicochemical basis of individual methodologies and their impact on tissues is important, as it allows the researcher to select the methodology appropriate for the desired application.

The novel perspectives brought by this added dimension in histology are obvious, especially since these techniques can be applied to previously insoluble questions. Some examples related to neural regeneration include the convenient delineation of corticospinal projection pathways (Soderbolm et al., 2015), visualization of axonal branching patterns after optic nerve crush injury (Luo et al., 2014) and peripheral nerve injury (Jung et al., 2014), and the assessment of axonal regeneration and glial responses after spinal cord injury (Ertürk et al., 2011). Our laboratory has compiled a series of 3D images of tissues processed with different optical clearing methods (**Figure 1B**) and rendered them in the form of a video that clearly demonstrates the power of this technology (**Supplementary Video 1**).

Despite the significant advances in tissue-clearing, several methodological challenges remain unresolved. First, the limited diffusion and penetration of antibodies: even with maximal tissue permeabilization by complete delipidation of tissues, the dense location of antigens at shallower depths can rapidly consume antibodies, limiting deep immunostaining. This also contributes to difficulty in the translation of these techniques to human neural tissues, which has been inherently difficult due to the substantially larger tissue volumes and variable sample quality (Lai et al., unpublished observations). Finally, with the use of ultrafast light-sheet microscopic imaging, the volume of data generated commonly lies in the gigabyte to terabyte range, requiring rigorous and robust computational infrastructures for storage and analyses (Susaki et al., 2016). Since these techniques are still in their infancy, they need to be individually optimized by users in order to obtain the best results for their own projects, emphasizing once again the importance of understanding the working principles underlying these methodologies.

The perfect combination of tissue-clearing, fluorescent labeling, and optical sectioning microscopy has led to the birth of 3D histology, providing new, exciting, and powerful visualization approaches to the study of the nervous system. Reminiscent of the invention of microtome sectioning, tissue stains, and microscopy that gave birth to histology centuries ago, we envision that the continued methodological innovations in interrogating the brain will achieve new heights in neuroscience research.

Acknowledgments: *The authors would like to thank Dr. Iain Bruce for the proofreading of the manuscript.*

Author contributions: *All authors contributed to the writing of the manuscript while HML prepared the figures and the video.*
Conflicts of interest: *None declared.*
Supplementary information: *Supplementary data associated with this article can be found, in the online version, by visiting www.nrronline.org.*

References

Chung K, Wallace J, Kim SY, Kalyanasundaram S, Andalman AS, Davidson TJ, Mirzabekov JJ, Zalocusky KA, Mattis J, Denisin AK, Pak S, Bernstein H, Ramakrishnan C, Grosenick L, Gradinaru V, Deisseroth K (2013) Structural and molecular interrogation of intact biological systems. Nature 497:332-337.

Ertürk A, Becker K, Jährling N, Mauch CP, Hojer CD, Egen JG, Hellal F, Bradke F, Sheng M, Dodt HU (2012) Three-dimensional imaging of solvent-cleared organs using 3DISCO. Nat Protoc 7:1983-1995.

Mauch CP, Hellal F, Förstner F, Keck T, Becker K, Jährling N, Steffens H, Richter M, Hübener M, Kramer E, Kirchhoff F, Dodt HU, Bradke F (2011) Three-dimensional imaging of the unsectioned adult spinal cord to assess axon regeneration and glial responses after injury. Nat Med 18:166-171.

Hama H, Hioki H, Namiki K, Hoshida T, Kuroka H, Kurokawa H, Ishidate F, Kaneka T, Akagi T, Saito T, Saido T, Miyawaki A (2015) ScaleS: an optical clearing palette for biological imaging. Nat Neurosci 18:1518-1529.

Jung Y, Ng JH, Keating CP, Senthil-Kumar P, Zhao J, Randolph MA, Winograd JM, Evans CL (2014) Comprehensive evaluation of peripheral nerve regeneration in the acute healing phase using tissue clearing and optical microscopy in a rodent model. PLoS One 9:e94054.

Kuwajima T, Sitko AA, Bhansali P, Jurgens C, Guido W, Mason C (2013) ClearT: a detergent- and solvent-free clearing method for neuronal and non-neuronal tissue. Development 140:1364-1368.

Lai HM, Liu AK, Ng WL, DeFelice J, Lee WS, Li H, Li W, Ng HM, Chang RC, Lin B, Wu W, Gentleman SM (2016) Rationalisation and validation of an acrylamide-free procedure in three-dimensional histological imaging. PLoS One 11:e0158628.

Liu AK, Lai HM, Chang RC, Gentleman SM (2016) Free-of-Acrylamide SDS-based Tissue Clearing (FASTClear): A novel protocol of tissue clearing for three-dimensional visualisation of human brain tissues. Neuropathol Appl Neurobiol doi: 10.1111/nan.12361.

Luo X, Yungher B, Park KK (2014) Application of tissue clearing and light sheet fluorescence microscopy to assess optic nerve regeneration in unsectioned tissue. Methods Mol Biol 1162:209-217.

Murray E, Cho J, Goodwin D, Ku T, Swaney J, Kim S, Choi H, Park Y, Park J, Hubbert A, McCue M, Vassallo S, Bakh N, Frosch MP, Wedeen VJ, Seung S, Chung K (2015) Simple, scalable proteomic imaging for high-dimensional profiling of intact systems. Cell 163:1500-1514.

Pan C, Cai R, Quacquarelli FP, Ghasemigharagoz A, Lourbopoulos A, Matryba P, Plesnila N, Dichgans M, Hellal F, Ertürk A (2016) Shrinkage-mediated imaging of entire organs and organisms using uDISCO. Nat Methods 13:859-867.

Renier N, Wu Z, Simon DJ, Yang J, Ariel P, Tessier-Lavigne M (2014) iDISCO: a simple, rapid method to immunolabel large tissue samples for volume imaging. Cell 159:896-910.

Schwarz MK, Scherbath A, Sprengel R, Engelhardt J, Theer P, Giese G (2015) Fluorescent protein-stabilization and high-resolution imaging of cleared, intact mouse brains. PLoS One 10:e0124650.

Soderbolm C, Lee D, Dawood A, Carballosa M, Santamaria JA, Benavides FD, Jergova S, Grumbles RM, Thomas CK, Park KK, Guest JD, Lemmon VP, Lee JK, Tsoulfas P (2015) 3D imaging of axons in transparent spinal cords from rodents and nonhuman primates. eNeuro 2.

Susaki EA, Ueda HR (2016) Whole-body and whole-organ clearing and imaging techniques with single-cell resolution: toward organisms-level systems biology in mammals. Cell Chem Biol 23:137-157.

Tainaka K, Kubota SI, Suyama TQ, Susaki EA, Perrin D, Ukai-Tadenuma M, Ukai H, Ueda HR (2015) Whole-body imaging with single-cell resolution by tissue decolorization. Cell 159:911-924

Treweek JB, Gradinaru V (2016) Extracting structural and functional features of widely distributed biological circuits with single cell resolution via tissue clearing and delivery vectors. Curr Opin Biotechnol 40:193-207.