HHS Public Access

Author manuscript

J Neurosci Methods. Author manuscript; available in PMC 2023 November 25.

Published in final edited form as:

J Neurosci Methods. ; 334: 108594. doi:10.1016/j.jneumeth.2020.108594.

Clearing Techniques for Visualizing the Nervous System in Development, Injury, and Disease

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Abstract

Modern clearing techniques enable high resolution visualization and 3D reconstruction of cell populations and their structural details throughout large biological samples, including intact organs and even entire organisms. In the past decade, these methods have become more tractable and are now being utilized to provide unforeseen insights into the complexities of the nervous system. While several iterations of optical clearing techniques have been developed, some are more suitable for specific applications than others depending on the type of specimen under study. Here we review findings from select studies utilizing clearing methods to visualize the developing, injured, and diseased nervous system within numerous model systems and species. We note trends and imbalances in the types of research questions being addressed with clearing methods across these fields in neuroscience. In addition, we discuss restrictions in applying optical clearing methods for postmortem tissue from humans and large animals and emphasize the lack in continuity between studies of these species. We aim for this review to serve as a key outline of available tissue clearing methods used successfully to address issues across neuronal development, injury/repair, and aging/disease.

Keywords

Tissue clearing; Nervous system; Development; Disease; Large animals; Humans

Introduction

The primary goal of clearing tissue is to render biological specimens transparent for deep three- dimensional visualization of labeled cells of interest. Traditional methods were problematic in that clearing reagents caused damage to cellular morphological features (Steinke and Wolff, 2001; Alnuami et al., 2008), and utilization was limited to thin

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sections (Hofman and Taylor, 2013). In recent years, the resurgence of optical clearing methods have aimed to preserve tissue integrity and fluorophore (i.e., fluorescent markers) stability following treatment. Other approaches in the vast number of newly developed clearing techniques improved by reducing the number of chemical treatment steps which dramatically accelerates tissue processing for quantification and validation. Moreover, these newer techniques are enhancing the capabilities for analysis of thicker samples (~>500 μm) and archival human brain tissues, enabling spatial analysis of neuronal microstructures with unbiased characterization (Lai et al. 2018). Discussed in this review are the advancements as well as limitations for current methodology to explore large animals and human nervous system complexities. The functional utilization and application of optical clearing for large volume histological fixation and maintenance of tissue integrity while reducing light scattering has been conceptually described over numerous reviews (Silvestri et al., 2016; Ariel, 2017; Richardson and Lichtman, 2017). Here, we examine the bidirectionality of optical clearing methods used to study brain development, injury/repair, aging/disease states in human and large animal samples.

In particular, tissue clearing methods have been efficient in mapping normal neuronal connectivity and studying brain injury and neuropathology in Alzheimer's disease (AD), an age-related neurodegenerative disease characterized by progressive dementia. Nonetheless, profound advancements in clearing methods have aided in identifying novel cellular connections and β-amyloid plaque distribution, a main pathological identifier in AD (Liebmann et al, 2016). In contrast, the application of optical clearing in neurogenesis, peripheral nerve injury, and other pathological disorders besides AD remain less explored, despite the potential of clearing techniques to provide fast subsequent reconstruction of key cell players. This review brings light to the disparities between the utilization of clearing methods within the neuroscience field, and provides a resource for researchers looking to apply clearing methods to future CNS studies.

Clearing Overview

Optical clearing of large tissues was first described over a hundred years ago; however, this approach was limited in utility until the advent of key technologies such as confocal, two-photon, and light sheet microscopy. The end of the 20th century marked a massive resurgence and evolution in tissue clearing approaches and today many groups are using these techniques to visualize whole cell populations, circuitry and connectivity across entire, intact nervous systems. To date, several clearing methods have been developed and each vary in cost, suitability for certain structures, and time-consumption (Richardson and Lichtman, 2015; Tainaka et al., 2016; Mano et al., 2018). Biological tissues, such as the brain and spinal cord, are composed of a diverse number of cell types which results in tissue inhomogeneity in terms of how visible light scatters. In order to minimize light scattering, tissue clearing techniques aim to balance the refractive index throughout samples, resulting in tissue homogeneity and transparency. This refractive index matching is typically achieved in one of three ways: (1) aqueous-based matching through immersion (e.g. – SeeDB), (2) solvent-based removal of lipids following dehydration (e.g. – BABB), and (3) gel embedding to enhance tissue integrity followed by delipidation (e.g. – CLARITY). Numerous derivatives of these approaches and their original papers have been extensively

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reviewed and are summarized in Table 1 (Dodt et al., 2007; Hama et al., 2011; Becker et al., 2012; Erturk et al., 2012; Chung et al., 2013; Fujimoto and Imai, 2013; Kuwajima et al., 2013; Renier et al., 2014; Tomer et al., 2014; Aoyagi et al., 2015; Hama et al., 2015; Liu et al., 2017; Vigouroux et al., 2017).

When selecting a clearing method to visualize the nervous system, there are several key considerations one should make: tissue size, antibody compatibility, lipid preservation, tissue integrity (shrinkage vs. expansion), suitability for RNA labeling, chemical probe compatibility, length of fluorescence stability, multiplexing, optics and computational tools required for analyses. Such considerations have been reviewed in detail (Seo et al., 2016; Susaki and Ueda, 2016; Aswendt et al., 2017; Lai et al., 2017; Zhu et al., 2017); here we focus on how optical clearing approaches have been recently employed to better study development, injury and diseases of the nervous system in numerous model organisms and postmortem human tissue.

Development

With recent improvements of various optical clearing techniques, key developmental stages can now be observed with advanced three-dimensional (3D) imaging. Prior to these advances, probing the spatial relationships between neurons traversing long distances and their targets was difficult. Several groups are now utilizing these techniques to anatomically map individual cell populations, cytoarchitecture and structural features, microcircuitry, and connectivity throughout the entire nervous system. Along with this approach many unexpected discoveries have been made in model organisms during pre- and postnatal stages of development.

Building an intact and functional nervous system involves many critical stages including stem cell proliferation, progenitor migration, cell maturation, and functional integration. Optical clearing methods provide a practical means to visualize these processes throughout development without loss of spatial information. For example, the effects of amniotic contaminants and thyroid signaling on neuronal cell fate were assessed in the entire hindbrain of tadpoles (Fini et al., 2017). More recently, researchers performed whole-mount labeling and subsequent clearing of mouse embryos to demonstrate the role of netrin 1 in the confinement of precerebellar neurons to the CNS at the CNS/PNS border (Moreno-Bravo et al., 2018). In addition, another group was able to identify precise morphological changes in migrating neuroblasts, within and surrounding the rostral migratory stream, during mouse postnatal development; they also shed light on additional migratory pathways (Aoyagi et al., 2018).

Currently, one of the fundamental challenges of imaging the entire mammalian brain with adequate resolution is the complex organization of cellular networks. Over the years, the nervous system has been viewed as a ‘multiplex network’ consisting of different cell types and morphologies forming elaborate connections that are most appreciable when mapped in three dimensions (Amunts et al., 2013; Murakami et al., 2018: Zhu et al., 2019). To provide comprehensive insight into long-range synaptic connections governing neural circuits, researchers have employed the use of dual immmuno-labeling and three-

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dimensional imaging to visualize neuronal projections and termination patterns involved with somatosensory perception and locomotion (Liang et al., 2015; Kardamakis et al., 2016). Furthermore, incorporation of optical clearing technology into brain mapping studies, have opened doors to understanding the organization and microarchitecture within the PNS as well as neuronal profiling throughout development. Liang et al., have provided novel insight into pain perception by visualizing the collateralization and termination patterns of fiber systems within the spinal cord (Liang et al., 2015, 2016). Kardamakis and Fürth's groups have utilized clearing techniques and computational tools to access multisensory integration and monosynaptic connectivity within an intact brain. Notably, innovations to tissue-clearing techniques have provided further understanding into neuronal connectivity throughout development while preserving tissue ultrastructure.

A second challenge relates to the sophisticated software and computational power necessary to process, analyze, and store massive three-dimensional datasets generated from cleared tissues. Many strides have been made to overcome this hurdle through the development of novel algorithms to perform precise readout descriptions and 'automated' analyses of data sets from large biological specimens. For example, the manual segmentation of neural structures in specific anatomical locations acquired from a 3D image using 1mm thick slice brain tissue has been evaluated using novel software tools such as ManSegTool (Magliaro et al., 2017). In addition, Renier et al., created "ClearMap", an open-source software that automatically analyzes evidence of neuronal activity (with c-fos expression) at cellular levels and generates registered brain anatomical atlases (Renier et al., 2016). Utilization of such cellular atlases could be a valuable step in identifying and mapping new neuron types (i.e., cells in general), circuity (i.e., large-scale connectomes) (Lo and Chiang, 2016), and brain activity in responses to drug treatments.

In recent years, modifications to classical tissue-clearing methods have provided useful anatomical maps and cell population profiles illuminating a clearer picture of intact, healthy nervous systems and a stronger understanding of the circuitry underlying proper cognitive function. Although these methods are suitable to study many facets of development, they have been primarily utilized to reveal how neurons form local and distal connections within the brain (Table 2). Future studies aimed at applying these methods to understand other aspects of development (e.g. – neurogenesis, axonal refinement, cell fate, synaptic pruning) will accelerate our understanding into the dynamic cellular mechanisms underlying CNS and PNS development and will better inform studies on how to repair an injured nervous system.

Injury and Repair

While there are many animal models of CNS and PNS injury, recent studies have implemented optical clearing techniques largely in the study of spinal cord, peripheral nerve, TBI, and ischemic injury. Recent advances in light sheet fluorescence microscopy and optical clearing have provided in-depth access to 3D information in whole-mount tissue, such as nerve fibers, axonal branches, and target reinnervation points. The ability to track individual axons from sites of innervation to the corresponding cell bodies may guide therapeutic development through understanding the full regenerative capacity following injury (Bray et al., 2017; Ertürk et al., 2012; Jung et al., 2014).

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Interestingly, optimization of tissue clearing protocols integrated with neuronal labeling has cultivated ‘novel’ segmentation platforms and promoted the re-evaluation of axonal regeneration. For example, using CLARITY, ‘novel’ bilateral neuronal connectivity from dendrites to the dorsal columns of the spinal cord were observed in a study of the regulation of calcium binding proteins following peripheral nerve injury (Zhang et al. 2014). Use of vDISCO - a modified immunolabeling method using “nanoboosters” which enhances fluorescent signals – allowed effective reconstruction and imaging of peripheral axonal degradation within a mouse’s torso following traumatic brain injury. Furthermore, vDISCO technology revealed immune infiltration by monocytes and macrophages in lymphatic vessels, muscles, and at the site of trauma following spinal cord injury (SCI) (Cai et al. 2018). Limitations of clearing techniques (i.e., complete immunofluorescence labeling and accurate imaging processing/analysis) motivated the development of “StereoMate”, a multimodal platform/framework with various protocols for clearing, immunofluorescence labeling, and imaging for robust data reconstruction and analysis (West and Bennett et al. 2019). This platform revealed profound loss of neurons in the dorsal root ganglia (DRG) following peripheral nerve injury and unique heterogeneity within DRG nuclei (i.e., trimodal distribution) which, hitherto, had not been shown- contrary to results seen in 2D analysis (i.e., bimodal distribution).

Notably, visualization into an intact spinal cord has proven to be very toilsome due to the abundance of lipids and myelin encasing the gray matter compared to the brain with a surface covered in gray matter. While the ability to fully clear a spinal cord to tract-trace axonal projections seems like a daunting task, Ertürk et al., reported increased regenerative axonal trajectories (i.e., axonal sprouts) in conditioned – injured peripheral axons prior to injury of central axons - as well as unconditioned axons; most of the sprouting occurring through conditioned axons (i.e., 20% more than unconditioned) within and through the lesion site using 3DISCO, following conditioned sciatic nerve injury (Ertürk et al. 2012a; Ertürk et al. 2012b). Beyond the complexities of nerve degeneration and regeneration, researchers have utilized clearing methodologies to understand retinal vascular development and remodeling following ischemic injury (i.e., hypoxia, optic nerve injury, etc.) (Luo et al. 2014, Bray et al. 2017, Singh et al. 2017) as well as ‘novel’ visualization into microvascular architecture after ischemic brain injury (i.e., stroke) (Lugo-Hernandez et al. 2017). Thus, studies applying clearing methods are already providing important insights into injury response and repair programs; as these tools gain more traction in other injury paradigms, we will have a deeper understanding and clues into how to promote recovery from trauma to the nervous system.

Aging and Disease

Although aging is considered a natural biological occurrence, alterations in normal cellular processes including but not limited to telomere attrition, loss of proteostasis, mitochondrial dysfunction, cellular senescence, and stem cell exhaustion, initiate and increase the risk in developing neurological diseases. Current approaches in neurodegenerative research are often aimed at studying neuropathological features in early disease states within intact clinical tissue samples (Ando et al., 2014; Gitler et al., 2017; Hussain et al., 2018). Nonetheless, a major limitation associated with studying neuronal abnormalities within

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pathological specimens is the utilization of thin brain tissue sections, which restricts the ability of visualizing spatial interactions of individual cell types and their connections. Recently, there have been a number of animal studies addressing this problem by adopting clearing techniques to provide further insight into key pathological signatures such as Aβ plaque formation, tau pathology, vascular network remodeling, and viral invasion into the CNS.

The ability to examine thick tissue samples in high resolution, provides researchers opportunities to uncover molecular mechanisms within biological systems that may underlie diverse neurological outcomes. Compared with other diseases of the adult nervous system, optical clearing has been extensively used for investigations of Alzheimer's disease (AD) pathology (Table 3). In particular Hama et al., took advantage of their original technique (i.e., ScaleS), and created AbScale- for deep immunolabeling of Aβ plaques. Using imaging and quantitative analysis, a majority of the Aβ plaque distribution was found specifically in the cortex, and lesser in other brain regions of an AD mouse model (Hama et al., 2015). Furthermore, modernization of clearing techniques has enabled characterization of molecular triggers for disease formation/ progression such as calcium deposits in degrading skeletal muscles (i.e., triceps brachii, quadriceps femoris, and spinalis pars lumborum) within Duchene muscular dystrophy mice models using CUBIC (Bozycki et al., 2018), as well as pathogen invasion into the zebrafish CNS with CLARITY (Passoni et al., 2017). Thus, clearing methodologies could reveal underlying molecular events in other neurological diseases for future studies.

Studies of other neurological diseases remain largely untethered by the advent of clearing technology. Collectively, researchers should continue to use tissue clearing innovations for in-depth analysis of human pathological developments in postmortem tissue generated from animal models of aging and neurodegeneration following a battery of behavioral and neurological assessments; such studies will likely provide unprecedented understandings into disease progression to identify new pathological signatures and potential targets for treatment.

Humans and Large Animals

Optical clearing of human tissue is hampered by lack of fluorescent reporter genes and lengthy postmortem intervals which affect protein stability. In addition, access to human postmortem tissue is limited, and it is difficult to acquire neurotypical specimens from early developmental stages. An advantage to conventional cryosectioning of these rare specimens is the number of sections available to address numerous questions; however, multiplexing has been demonstrated with optically-cleared human tissue affording the opportunity to evaluate several markers within the same thick specimen (Phillips et al., 2016; Hsueh et al., 2017). Overall, there have been few studies utilizing clearing on human tissues in development and disease.

Belle et al. took advantage of two facets of a solvent based clearing technique (3DISCO) to study human peripheral nerve development during the first trimester: tissue shrinkage to a manageable size for microscopy and compatibility with whole-mount immunolabeling.

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3D reconstructions of sensory and motor nerves revealed notable differences in nerve organization between the left and right hands as well as signatures of adult-like patterning of nerve coverage during embryonic-fetal development. Using the same approach, a 3D atlas of gonadotropin-releasing hormone neurons, involved in reproduction and fertility through innervation of the hypothalamus, was generated in humans during the first trimester (Casoni et al., 2016). Tracking these neurons throughout the entire fetus revealed migration to extrahypothalamic regions, raising the question of whether they have additional functions unrelated to fertility. Recently, the spatiotemporal expression pattern of a receptor (Robo3) involved with commissural axonal crossing was assessed using whole-mount labeling and optical clearing in the hindbrain and spinal cord of several species including human fetuses (Friocourt et al., 2019). With this approach, the researchers were not only able to make important comparisons between amniotes but also documented migratory patterns of Robo3+pontine neurons and expression in the human fetal ganglionic eminences which give rise to interneurons.

A handful of neurological disorders have been evaluated with tissue clearing methods in human tissues including cerebellar ataxia, Alzheimer’s and Parkinson’s disease. Because the spatial distribution of β-amyloid plaques varies widely in AD, a 3D approach to visualize their distribution and morphologies is ideal. Liebmann et al., evaluated Aβ plaques in postmortem human tissues from AD patients within and around the hippocampus using iDISCO. Compared to a mouse model of AD, 3D amyloid patterns were found to be larger and more complex, and morphologically diverse between individual humans. Another group performed optical sectioning following ScaleS clearing of elderly human AD samples to quantitatively determine the spatial relationship between microglia and two classes of amyloid plaques: diffuse and cored. ScaleS, is a sorbitol-based clearing method that preserves fluorescence signals, has minimal tissue shrinkage, and a turnover time ranging between weeks to months depending on tissue thickness. 3D volume rendering of double labeled specimens showed that microglia are near diffuse and not cored plaques, and the presence of cored plaques was typically associated with an absence of diffuse plaques which potentially supports the idea that cored plaques are derived from diffuse plaques (Hama et al., 2015).

Tissue clearing methods have also allowed 3D visualization into the spatial relationship between midbrain fibers and Lewy pathologies (Liu et al., 2016). Axonal processes and Lewy body-like inclusions were visualized in the cortex, nucleus basalis, and midbrain of postmortem tissues from a human Parkinson’s disease patient using CLARITY (Liu et al., 2016). The cerebellar circuitry of mitochondrial diseased patients was recently evaluated on quadruple labeled tissue and passive CLARITY (Phillips et al., 2016). Not only were respiratory chain deficiencies identified by assessing complex I subunits, but this group was able to remove antibodies from labeled tissue with a detergent and re-stain for new markers following clearing. Advances in clearing technologies enable 3D visualization and evaluation of human specimens which have already led to new insights into developmental and degenerative processes that would have likely gone unnoticed with more traditional histological approaches.

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Surprisingly few studies utilizing clearing methods on large animal brains have been reported. Many large mammals share similarities to their human counterparts and represent ideal intermediate model organisms to guide studies on human brain development, injury and diseases. Although scalability has been demonstrated by clearing an entire adult pig and marmoset brain using SHANEL and SWITCH methods (Murray et al., 2015; Zhao et al., 2019), using thick sections to gain deeper understandings of the CNS landscape has been the favorable approach.

Recently, solvent-based clearing approaches were used on thick sections to demonstrate the presence of migratory streams filled with newborn neurons oriented toward the prefrontal cortex of early postnatal ferrets and piglets (Morton et al., 2017; Ellis et al., 2018); a feature previously thought to be unique to human infants. Evidence of an additional migratory stream of young interneurons was also discovered in the ferret, offering clues on where to look in future human studies (Ellis et al., 2018). A class of neurons involved with fertility regulation were recently visualized in an intact sheep hypothalamus using a solvent-based clearing technique, allowing anatomical mapping and sexually dimorphic comparisons in hypothalamic nuclei throughout the rostral-caudal axis (Moore et al., 2018). Optical clearing has also been utilized to study long range connections in a nonhuman primate; by injecting a fluorescent tracer into the motor cortex of a macaque, researchers were able to track and visualize corticospinal tract axons in the optically cleared spinal cord 10 weeks later (Soderblom et al., 2015).

Future Perspectives

Clearing methodologies have revolutionized our exploration of spatial cellular, subcellular, and structural details throughout the nervous system increasing our understanding of neurodevelopment, injury, and diseases. Within a few years, several discoveries related to key biological events such as generation of newborn neurons, cell migration, and mature circuitry wiring the brain have been made owing to recent, synergistic advances in labeling methods, clearing technologies, and optics/microscopy. While these methods have been primarily utilized in smaller organisms, their scalability to larger tissues derived from higher order mammals including humans has been demonstrated. Nevertheless, the limitations imposed by applying these techniques to humans (e.g., long postmortem intervals, need to probe with antibodies, size of an intact adult brain) still remains a challenge for current technologies. However, much progress has been made and it will be of great value to make comparisons with macrostructural information gained from noninvasive neuroimaging modalities such as MRI. In addition, utilization of large animal models resembling their human counterparts along with these techniques may foster translation to human studies. With future improvements in chemical probes, deep tissue penetration, optics, and computational platforms, interrogating the complexities of the whole human brain may be possible.

Despite the utility of modern clearing techniques to visualize cells within the nervous system before birth to adulthood, there has been seemingly more efforts to utilize these applications to understand neuronal connectivity than endeavors to map other cells critical for normal development (e.g. – oligodendrocytes, astrocytes, and microglia). Fewer studies have

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employed these powerful techniques to evaluate brain injury which would offer a clearer view of how the brain remodels and insight into new strategies for repair. Likewise, while significant advancements have been made in understanding Alzheimer's disease etiology, we highlight the potential for clearing techniques to fill current gaps in knowledge in other neurodegenerative diseases. As applications of optical clearing technologies continue to evolve, we look forward to a greater collective understanding of normal neural development and its response during physiological or pathological conditions as well as after injuries, with the hope of generating more effective treatments and improve human health.

Acknowledgements

This work was supported by the US National Institutes of Health (R15NS108183 to P.D.M.), the IMSD National Institute for General Medical Sciences Fellowship (R25GM072767 to D.D.L.P.), and departmental start-up funds to P.D.M.

Glossary

Aβ	amyloid beta
ACT-PRESTO	active clarity technique-pressure related efficient and stable transfer of macromolecules into organs
AD	Alzheimer's Disease
APP	amyloid beta precursor protein
BABB	benzyl aclcohol/benzyl benzoate
BDA	Biotinylated dextran amine
C_e3D	clearing-enhanced 3D
CLARITY	Clear lipid-exchanged Acrylamide-hybridized Rigid Imaging/immunostaining/in situ hybridization-compatible Tissue hYdrogel
CNS	central nervous system
CSF-c	cerebrospinal fluid-contacting cells
CUBIC	Clear, Unobstructed Brain/body imaging Cocktails and Computational analysis
CUBIC-L	Clear, Unobstructed Brain/body imaging Cocktails and Computational analysis- delipidation
CUBIC-R	Clear, Unobstructed Brain/body imaging Cocktails and Computational analysis- RI matching
DA	dopamine
DRG	dorsal root ganglion

FASTClear	Free of Acrylamide SDS-based Tissue Clearing
FDISCO	fluorescence-preserving DISCO
iDISCO	immunolabeling-enabled three-dimensional imaging of solvent-cleared organs
iDISCO+	immunolabelling-enabled DISCO plus
iExM	iterative expansion microscopy
KNDy	endogenous opioid dynorphin A
ManSegTool	Manual Segmentation Tool
NECAB1	Neuronal calcium (Ca^{2+})-binding protein 1
MAP	magnified analysis of the proteome
NECAB1	Neuronal calcium (Ca^{2+})-binding protein 1
NKB	neurokinin B
PACT	passive clarity technique
PARS	perfusion-assisted agent release in situ
PEGASOS	polyethylene glycol-associated solvent system
PNS	peripheral nervous system
Robo3	Roundabout Guidance Receptor 3
RGC	retinal ganglion cells
RI	refractive index
ScaleS	sorbitol- based optical clearing method
SCI	spinal cord injury
SCGN	Secretagogin
SCM	simplified CLARITY method
sDISCO	stabilized DISCO
SeeDB	See Deep Brain
SHANEL	Small-micelle- mediated Human organ Efficient clearing and Labeling
SHIELD	stabilization under harsh conditions via intramolecular epoxide linkages to prevent degradation

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SWITCH	System-Wide control of Interaction Time and kinetics of CHemicals
TBI	traumatic brain injury
TDE	2,2'-thiodiethanol
TH	tyrosine hydroxylase
TH1	tyrosine hydroxylase gene 1
TH2	tyrosine hydroxylase gene 2
uDISCO	ultimate DISCO
vDISCO	nanobody(V_HH)-boosted 3D imaging of solvent-cleared organs
3D	three-dimensional
3DISCO	3D imaging of solvent-cleared organs
5-HT	Serotonin

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Highlights

- One-stop resource for available clearing and staining methods in CNS research
- Tissue clearing methods provide new insights into many facets of the nervous system
- Trends are emerging in the types of neuroscience questions being addressed
- Advancements and limitations of current protocols for human & large animal studies

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Table 1.

Summary of current optical clearing techniques.

		Clearing Time	Immunostaining/ Fluorescent Protein[#]	Lipid Preservation	Size Change	Final RI	Citation
Solvent-based	BABB	Days	I	No	Shrinkage	1.55	Dodt et al., 2007
	FluoClearBABB	Days, Weeks	F	No	Shrinkage	1.56	Schwarz et al., 2015
	3DISCO	Hours, Days	I (limited)/ F (couple of days)	No	Shrinkage	1.55	Ertürk et al., 2012a; Ertürk et al., 2012 (b)
	iDISCO	Hours, Days	I/ F (couple of days)	No	Shrinkage	1.56	Renier et al., 2014
	iDISCO plus	Weeks	I	No	Shrinkage[M]	1.56	Renier et al., 2016
	uDISCO	Hours, Days	I/F	No	Up to 65 % Shrinkage	1.57	Pan et al., 2016
	vDISCO	Weeks, Months	I/F	No	Shrinkage	1.55	Cai et al., 2018
	FDISCO	Days	I/F	No	Shrinkage	1.56	Qi et al., 2019
	sDISCO	Days, Months	F	No	Shrinkage	1.55; 1.56	Hahn et al., 2019
	sPEGASOS	Days, Weeks	I/F	No	Shrinkage	1.54	Jing et al., 2018
Simple Immersion	Sucrose	Days	I/F	No	Shrinkage	1.44	Tsai et al., 2009
	FocusClear	Hours, Days	I/F	Yes	No Change	1.47	Chiang et al., 2002
	ClearT	Hours, Days	I	Yes	No Change	1.44	Kuwajima et al., 2013
	ClearT2	Hours, Days	I/F	Yes	No Change	1.44	Kuwajima et al., 2013
	SeeDB	Days	F	Yes	No Change	1.48	Ke et al., 2013
	SeeDB2 G/S	Days	F	Yes	Shrinkage[M]	G-1.46; S-1.52	Ke et al., 2016
	FRUIT	Days	F	Yes	Expansion[M]	1.48	Hou et al., 2015
	TDE	Days, Weeks	I/F	No	No Change	1.42	Costantini et al., 2015; Aoyagi et al., 2015; Staudt et al., 2007
	FASTClear	Weeks	I/F	No	Shrinkage[M]	1.42-1.56	Liu et al., 2017
	Ce3D	Weeks	I/F	No	Shrinkage[M]	1.49	Li et al., 2017
Hyperhydration	ScaleA2	Weeks	I (limited)/ F	No	Expansion	1.38	Hama et al., 2011
	ScaleU2	Months	I (limited)/ F	No	No Change	1.38	Hama et al., 2011
	ScaleS	Weeks	I/F	No	Shrinkage[M]	1.47	Hama et al., 2015
	CUBIC	Days	I/ F	No	Expansion	1.38; 1.48	Susaki et al., 2014

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		Clearing Time	Immunostaining/ Fluorescent Protein#	Lipid Preservation	Size Change	Final RI	Citation
	CUBIC-L CUBIC-R	Days, Weeks	I/F	No	Expansion ^{M}	1.44-1.52	Tainaka et al., 2018; Kubota et al., 2017
	Whole-Body CUBIC	Days	I/ F	No	Expansion	1.38	Tainaka et al., 2014
	ScaleCUBIC (1,2)	Days, Weeks	F	No	Expansion	~1.49	Susaki et al., 2014 (a); Susaki et al., 2015 (b)
	UbasM	Days, Weeks	I/F	No	Expansion ^{M}	1.45-1.48	Chen et al., 2017
	CUBIC-X	Days, Weeks	F	No	Expansion	1.47	Murakami et al., 2018
Hydrogel Embedding	CLARITY	Days	I/F	No	Expansion ^{M}	1.45	Chung et al., 2013
	Bone CLARITY	Days, Weeks	F	No	Shrinkage ^{M}	1.47	Greenbaum et al., 2017
	PACT	Days, Weeks	I/F	No	Expansion ^{M}	1.38-1.48	Yang et al., 2014
	PARS	Days	I/F	No	No Change	1.38-1.49	Yang et al., 2014
	ACT-PRESTO	Hours, Days	I/F	No	Expansion	1.47	Lee et al., 2016
	SWITCH	Days, Weeks	I	No	Shrinkage ^{M}	1.47	Murray et al., 2015
	SCM	Hours, Weeks	I/F	No	Expansion	1.47	Sung et al., 2016
	Stochastic electrotransport	Days	I/F	No	Expansion ^{T}	1.47	Kim et al., 2015
	SHIELD	Days, Weeks	I/F (limited)	No	Expansion	1.46	Park et al., 2019
	iExM†	Days	I	No	Expansion up to ~20x	1.33	Chang et al., 2017a
	MAP†	Days	I/F	No	Expansion up to 4-5x	1.33	Ku et al., 2016

RI, refractive index; I, immunostaining; F, fluorescent protein.

#Indicates whether immunolabeling and/or fluorescent protein emission was demonstrated in the original publication.

†Hydrogel embedding followed by hyperhydration.

^{M}Minimal

^{T}Transient.

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Table 2.

Summary of optical clearing techniques used in neurodevelopment studies.

		Specimen	Species	Age	Technique	Cell Type/ Structure/ Marker	Citation
Development	Neurogenesis	Whole Brain	Zebrafish	3. 5. 7DPF/ 6-12M	BABB	Proliferating stem cells, glia, GFP-reporter	Lindsey et al, 2018
		Whole Brain	Xenopus/ Tg Xenopus	Stages NF45 (1 week old) & NF46/47	CLARITY	Oligodendrocytes, neurons, nuclei, mitotic cells	Fini et al, 2017
	Proliferation	Brain	Xenopus/ Zebrafish/ Chicken	E15/P0/4W/ Adult (1-3Y)/ Adult (3M-2Y)	CLARITY PACT	TH Immunoreactive cells, nuclei, 5-HT+ CSF-c cells, DA+ cells, TH1+ cells, TH2+ cells	Xavier et al, 2017
	Connectivity/ Circuitry/ Tracing/ Mapping	Whole brain Brain	Mouse/Tg Mouse	2-3M	CLARITY	Membrane-localized proteins, dendritic spines, synaptic puncta, neurons, axonal fibers	Chung et al, 2013
		Brain Spinal cord	Mouse	12–14W	CLARITY	Raphe nuclei, reticular nuclei, BDA fibers, raphespinal fibers, serotonergic fibers	Liang et al, 2015
		Whole brain Whole spinal cord	Tg Zebrafish/ Tg Mouse	Larva	CLARITY	Neurons	Tomer et al, 2015
		Brain	Mouse	P70	tB-BABB	Subcortical nuclei, neurons, dendrites, glia, axons	Schwarz et al, 2015
		Brain	Mouse/Tg Mouse	Adult/P1-3/ ~8-12W	iDISCO+ClearMap	Axon projections, neurons, c-Fos+ cells- immediate early genes	Renier et al, 2016
		Brain Spinal cord	Tg Mouse/Rat	Adult	CLARITY	Nuclear DNA, astrocytes, and DiI labeled cells	Jensen and Berg et al, 2016
		Spinal cord	Mouse	-	CLARITY CUBIC	Serotonergic fibers	Liang et al, 2016
		Whole brain	River lamprey	Adult	CLARITY	Neurons, intemeurons, neurobiotin-injection, afferent and efferent projections	Kardamakis et al, 2016
		Brain	Tg Mouse	E0/ E15.5/P0/P4	iDISCO+3DISCO	Axons, ß-galactosidase in nuclei, trigemino-thalamic (TT) tract trajectory, c-Fos+ cells	Renier et al, 2017
		Brain	Mouse	6-8W	SWITCH	Neurofilaments, blood vessels	Ren et al, 2017
		Brain	Tg Mouse	P42–49/ P70-80	ScaleS	Astrocytes	Chai et al, 2017

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		Specimen	Species	Age	Technique	Cell Type/ Structure/ Marker	Citation
		Brain	Mouse/Rat/ Marmoset	6W/3Y	CUBIC	Cell bodies, dendrites, neurons, microvasculature	Watson et al, 2017
		Brain	Tg Mouse	Adult	CLARITY2	Purkinje neurons, dendrites, cell soma, neurites	Magliaro et al, 2017
		Whole brain	Mouse/Tg Mouse	8-13W	CLARITY Whole Brain Openbrainmap	d3/d5 dendrites, cell bodies, neurons, intemeurons	Furth et al, 2018
		Whole mouse paw	Tg Mouse	E14.5/P0.5	CLARITY	Fgfr1 expression/GFP/ cell nuclei	Collette et al. 2017
		Whole Brain	Mouse/Tg Mouse	3-6M	CLARITY	Myelinated WM tracts	Chang et al, 2017
		Brain Pancreas Whole Mouse	Mouse/ TgMouse/ Human	91–117DPC/E15/ P2-42/2–8Y	CLARITY	Neural projections, neurons, vasculature, pancreatic islets cells	Hsueh et al, 2017
		Brain	Mouse	E15/P21/P70	SeeDB2G BABB 3DISCO	cortical neurons, mitral and tufted cells, axons, dendrites	Sakaguchi et al, 2018
		Whole fruit fly	Tg Fruit Fly	Pupae/Adult 4–5 days	FlyClear	Dorsal cluster neurons, medulla columnar neurons	Pende et al, 2018
		Brain	Mouse/Tg Mouse	8-12W	uDISCO	Parvalbumin and somatostatin intemeurons, dopaminergic neurons, TH+ neurons	Lin et al, 2018
		Whole Brain Brain	Tg Mouse	1W/3W/8W/10W/11W/4M/ 6M	CUBIC X ScaleCUBIC CUBIC-Atlas	Cell nuclei and spines	Murakami et al, 2018
		Brain	Tg Mouse	P5-80	SeeDB2	Microglia, purkinje cells, climbing fibers, liposomes, synapses	Nakayama et al, 2018
		Whole Mouse Whole Brain	Tg Mouse	Adult	uDISCO BABB 3DISCO vDISCO	Neurons, neurites, meningeal lymphatic vessels, microglia, axonal projections	Cai et al, 2018*
		Brain	Tg Mouse	P21	SeeDB2	Mossy fibers terminals, thorny excrescences, dendritic spines, pyramidal cells	Weng et al, 2018
		Whole Brain Brain	Rat/Sheep	Adult	iDISCO	TH neurons, nuclei, fibers, cell bodies, kisspeptin and NKB cells, KNDy cells, GnRH fbers	Moore et al, 2018
		Whole Brain	Zebra finches	Adult	CUBIC iDISCO+	Vasculature, fiber tracts, nuclei	Rocha et al, 2019

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		Specimen	Species	Age	Technique	Cell Type/ Structure/ Marker	Citation
		Brain	Mouse/Tg Mouse	9W	FOCM	Neurons, neuron bundles, dendrites, synaptic boutons	Zhu et al, 2019
	Vascular Network	Brain Whole Spinal cord	Tg Mouse/Rat	Adult	PACT PARS	Endothelial cells, cell nuclei, glia, blood vessel, neurons	Yang et al, 2014
		Retinal flat mount	Rats	P7/P14	CLARITY	Vasculature, vertical sprouts, endothelial cells	Singh et al, 2017 *
		Whole Brain	Mouse	3M	3DISCO	Vessels, capillaries, vascular wall	Todorov et al, 2019
	Migration	Brain	Piglet	1W	iDISCO	Neuroblasts, migrating neuroblasts	Morton et al, 2017
		Brain	Mouse	P11/15/16/29–31/55–57/ Adult (3M)	TDE	Neuroblasts , mature neuronal cell nuclei, blood vessels, astrocytes	Aoyagi et al, 2018
		Brain	Ferret	P20/40/65/90	iDISCO+	Neuroblasts, migrating neuroblasts, SCGN+ cells, cell nuclei, myelin	Ellis et al, 2019

DPF, days post fertilization; M, months of age; Tg, transgenic or reporter; NF, nieuwkoop and Faber stage; E, embryonic day; P, postnatal day; W, weeks of age; Y, years of age; DPC, days post conception.

*denotes publications that also use an injury model.

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Table 3.

Summary of optical clearing techniques used in injury and diseases of the nervous system studies.

		Specimen	Species	Age	Technique	Cell Type/Structure/ Marker	Citation
Injury	**Nerve**	Sciatic nerve	Rat/Tg Rat	Adult	THF	Individual axons	Jung et al, 2014
		Whole DRG Spinal cord	Tg Mouse	Adult	BABB	DRG neurons, neuronal nuclei, small C fibres	West and Bennett, 2019
	Spinal Cord	Spinal cord	Tg Mouse	2–18M	THF	Axonal boutons, neurons, microglia, astrocytes	Ertürk et al, 2012
		Spinal cord	Mouse/Tg Mouse	1-2D	CLARITY	DRGs, motor neurons, NECAB1+ neurons, dendrites	Zhang et al, 2014
		Spinal cord	Tg Mouse/Rat/ Macaque	6-9W/5Y	3DISCO THF BABB	Axon collaterals and fibers, fibroblasts, astrocytes, motor and sensory neurons	Soderblom et al, 2015
	Retina	Whole Tissue	Tg Mouse	Adult	THF BABB	RGC axons	Luo et al, 2014
		Whole Tissue	Tg Mouse	6-8W	iDISCO	RGC axons	Bray et al, 2017
	Brain	Whole Brain	Mouse	10–12W	3DISCO	Blood vessels, capillaries, endothelial cells	Lugo-Hemandez et al. 2017
		Brain	Tg Mouse	Adult	CLARITY	oligodendrocytes, microglia, astrocytes, neuronal nucleolus and nuclear envelope	Gaire et al, 2018
		Dura mater	Human	3D-36W. 2Y	Glycerol & Mannitol	Dura, blood vesssels	Cheshire et al., 2015
		Brain	Rats	P90	SeeDB	Embryonic stem cells, cell bodies, neurons	Nudi et al.. 2015
		Whole & Section Brain	Tg Mouse	~3M	THF & DBE	Dendritic spines, immune cells (microglia, cerebral lymphocytes), astrocytes	Ertürk et al., 2016
		Brain	Tg Mouse	6W	PACT	Glial cells- astrocytes, microglia	Merkel et al.. 2017
		Brain	Tg Mouse	2-4M	THF & BABB	Endothelial cells, blood vessels	Assis-Nascimento et al., 2016
		Whole Brain	Mouse	8-10W	CLARITY	Peptides, commissural fibers	Mann et al., 2016
		Whole Brain	Tg Mouse	8-10W	CLARITY	Axons, Node of Ranvier, flanking paranodes, cell nuclei, cell membranes	Marion et al., 2018
	Ischemic model (Cerebral artery	Whole Brain	Tg Mouse	10W	CLARITY	Vessel endothelium, tight junction protien (Claudin-5	Zhang et al., 2018
	Hypoxic Injury	Brain	Rats	7D	CLARITY	Neurofilaments	Lee et al., 2017
Disease	**Alzheimer's**	Brain	Mouse/Tg Mouse	8-19W/ 9-24M	ScaleS AbScale ScaleSQ	Aβ plaques, neuronal nuclei, neurons, synapse regions, blood vessels, dendrites, axon terminals, neurites, microglia	Hama et al, 2015
		Brain hemispheres Brain	Tg Mouse/ Human	4.4-37M	iDISCO ClearMap	Aβ plaques, microglial aggregates, axon filaments,	Liebmann et al, 2016

		Specimen	Species	Age	Technique	Cell Type/Structure/ Marker	Citation
						blood vessels, cell nuclei, tau, neurofibrillary tangles	
		Brain hemispheres	Tg Mouse	8-34M	iDISCO+	Somatodendritic tau, neurons	Fu et al, 2016
		Brain	Tg Mouse	4-80W/15 M	UbasM CUBIC uDISCO ScaleS SeeDB	Aβ plaques, dendritic spines; motor, sensory, and central neurons	Chen et al, 2017
		Brain hemisphere	Tg Mouse	1-6M	CUBIC Scale S4	Aβ aggregates, neurons, glia	Tanaka et al, 2018
		Brain	Mouse/Tg Mouse	4-6W/25 M	CUBIC	Aβ plaques, neurons, neurites, dendritic spines, blood vessels	Vints et al, 2019
		Brain	Tg Mouse	90 ± 5D/6, 9M	iDISCO uDISCO	Hyperphosphorylated tau, protein aggregates	Detrez et al, 2019
		Brain	Mouse/Tg Mouse	6M	CLARITY	Aβ plaques, microglia, astrocytes, vascular endothelium, GFAP positive cells	Martorell et al, 2019
	PD	Brain	Mouse/Rat/ Human	12W	CLARITY	Lewy body-like inclusions, neurofilament, neuronal soma, monoaminergic neurones and fibers, axonal processes, microglia	Liu et al, 2016
	DMD	Whole Body	Mouse/Tg Rats	8W	CUBIC	Calcium deposits, nuclei, intestinal villi and folia	Bozycki et al, 2018
	Viral	Whole Brain	Zebra Fish	4-7DPF	CLARITY	Microvasculature, endothelial cells	Passoni et al, 2017
	Glioma	Whole Brain	Tg Zebra Fish	1-14M	CLARITY	GFP malformations	Mayrhofer et al, 2017
	MtD	Brain	Mouse/ Human	12M	CLARITY	Mitochondrial proteins, porin, neurofilament H, myelin, calbindin and parvalbumin interneurons, purkinje cell bodies, axons, mitochondria, vascular network	Phillips et al, 2016

PD, Parkinson's disease; DMD, Duchenne muscular dystrophy; MtD, Mitochondrial disease; Tg, transgenic or reporter; DRG, dorsal root ganglia; M, months of age; D, days of age; W, weeks of age; Y, years of age.

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