CLARITY for mapping the nervous system

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With potential relevance for brain-mapping work, hydrogel-based structures can now be built from within biological tissue to allow subsequent removal of lipids without mechanical disassembly of the tissue. This process creates a tissue-hydrogel hybrid that is physically stable, that preserves fine structure, proteins and nucleic acids, and that is permeable to both visible-spectrum photons and exogenous macromolecules. Here we highlight relevant challenges and opportunities of this approach, especially with regard to integration with complementary methodologies for brain-mapping studies.

Mammalian brains are staggeringly complex in terms of both scale and diversity; many billions of neurons are present, among them likely at least hundreds of genetically distinct cell types, with each type of cell represented by many distinct projection patterns. CLARITY[1] is a newly developed technology that can be used to transform intact biological tissue into a hybrid form in which tissue components are removed and replaced with exogenous elements for increased accessibility and functionality. CLARITY has the potential to facilitate rapid extraction of anatomical-projection information important for many fields of neuroscience research[2,3]; such information can be collected along with molecular-phenotype information at the resolution of single cells. Alone or in combination with other methods[4,5], such an approach could contribute to the study of function and dysfunction in this complex system.

In general, obtaining system-wide detailed information from neural tissue is a formidable challenge (to say nothing of subsequent data curation and analysis). In the mammalian central nervous system, seamlessly intertwined neural processes leave little extracellular space, creating barriers to macromolecule diffusion for *in situ* hybridization, antibody staining or other forms of molecular phenotyping deeper than the first few cellular layers of intact tissue[6]. The high density of irregularly arranged lipid interfaces that characterizes this tissue likewise creates an effective scattering barrier to photon penetration for optical interrogation of mammalian brains[7], unlike the *Caenorhabditis elegans* (worm) or larval *Danio rerio* (zebrafish) nervous systems, which are more accessible owing in part to smaller size and less myelination. Single-photon microscopy can provide optical transmission of information from only about 50 micrometers below the mammalian brain surface, and even well-optimized two-photon microscopy cannot be used to image deeper than about 800 micrometers, far short of enabling visualization of full projection patterns and global arrangement of cell populations in the intact brain[8].

Over the past few decades, a great deal of technological innovation has been stimulated by these challenges[8–21]. First, newer automated methods for mechanical sectioning of tissue have overcome some of the drawbacks of traditional sectioning methods that were laborious, expensive and damaged the tissue. Serial block-face mechanical[9–13] or optical-ablative[14] methods, in combination with imaging readouts such as two-photon tomography[14,15], electron microscopy[16] or array tomography[17], have been used to map macroscopic to nanoscopic brain structures (see Review[18] in this issue). In some cases, molecular labeling is built into these processes, and ongoing work includes approaches for addressing additional challenges such as generation of contrast in tissue before sectioning[19,20]. Tools for automated analysis, efficient reconstruction and error-free alignment also continue to be developed[21] as well as for registration with activity information, and indeed detailed wiring information linked to activity has been obtained from well-defined volumes[22,23].

Second, optical clearing methods have been developed that involve immersion of the specimen in medium that matches the refractive index of the tissue, thereby reducing light scattering and extending

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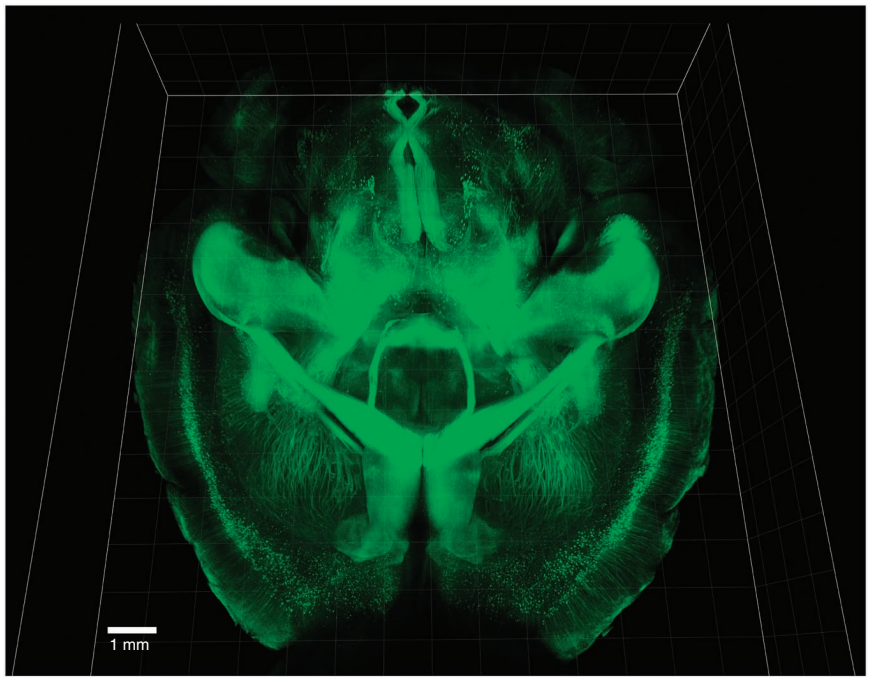

Figure 1 | Imaging of nervous system projections in the intact mouse brain with CLARITY. Adult *Thy1-EYFP* H line mice (4 months old) were perfused transcardially followed by hybridization of biomolecule-bound monomers into a hydrogel mesh and lipid removal as described[1]. The clarified mouse brain was imaged from the dorsal region to the ventral region (3.4 mm to the midpoint) using a 10× water-immersion objective. Image adapted from ref. 1.

the range of optical imaging[24–26]. For example, benzyl alcohol–benzyl benzoate is an organic solvent that effectively renders biological specimens transparent but reduces the stability of fluorescent protein signals[24,25]; in Sca*l*e, another clearing method, an aqueous solution is used to preserve fluorescence signals, but the rate and the extent of clearing remain limiting[26]. Notably all current tissue-clearing methods leave the densely packed lipid bilayers intact and therefore still face challenges with regard to penetration by visible-spectrum light and molecules, making these methods largely incompatible with whole-tissue molecular phenotyping.

The CLARITY approach[1] helps address ongoing challenges by enabling molecular and optical interrogation of large assembled biological systems, such as the entire adult mouse brain. Light-microscopy (**Fig. 1**) and biochemical-phenotyping (**Fig. 2**) techniques can be used to rapidly access the entire intact clarified mouse brain with fine structural resolution and molecular detail (to the level of spines, synapses, proteins, single-amino-acid neurotransmitters and nucleic acids) while in the same preparation maintaining global structural information including brain-wide macroscopic connectivity. To clarify tissue (**Fig. 3**), lipid bilayers are replaced with a more rigid and porous hydrogel-based infrastructure (in a process conceptually akin to petrification or fossilization, except that not only structure but also native biomolecules such as proteins and nucleic acids are preserved). This outcome is achieved by first infusing small organic hydrogel-monomer molecules into the intact brain along with cross-linkers and thermally triggered polymerization initiators; subsequent temperature elevation triggers formation, from within the brain, of a hydrogel meshwork covalently linked to native proteins, small molecules and nucleic acids but not to lipids, which lack the necessary reactive groups (**Fig. 3a**). Subsequent whole-brain electrophoresis in the presence of ionic detergents actively removes the lipids (**Fig. 3b**), resulting in a transparent brain-hydrogel hybrid that both preserves, and makes accessible, structural and molecular information for visualization and analysis.

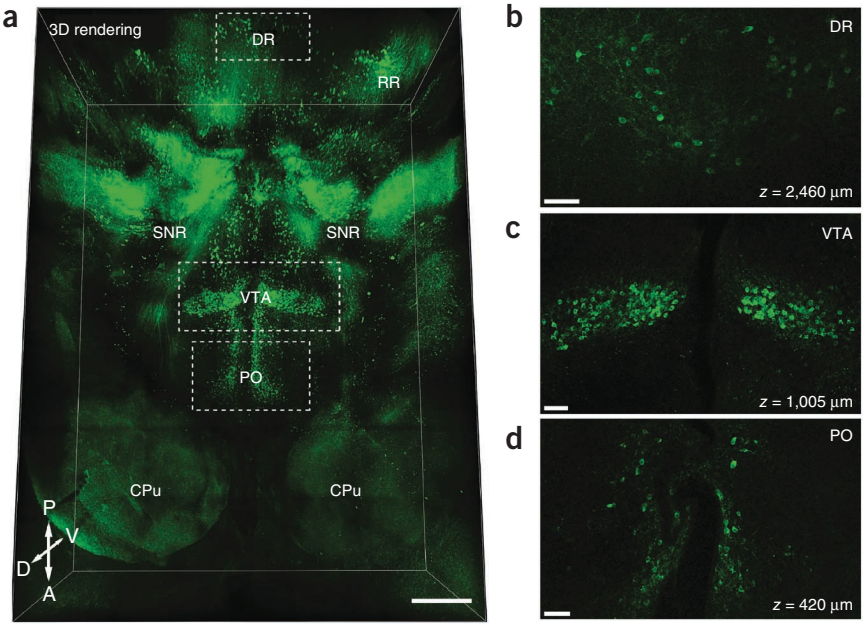

Figure 2 | Intact mouse brain molecular phenotyping and imaging with CLARITY. (**a**) Three-dimensional (3D) visualization of immunohistology data, showing tyrosine hydroxylase (TH)-positive neurons and fibers in the mouse brain. The intact clarified brain was stained for 6 weeks as described[1], with primary antibody for 2 weeks, followed by a 1-week wash, then stained with secondary antibody for 2 weeks followed by a 1-week wash and imaged 2,500 µm from ventral side using the 10× water-immersion objective. D, V, A and P indicate dorsal, ventral, anterior and posterior, respectively. (**b**–**d**) Optical sections at different depths, corresponding respectively to the upper, middle and lower dashed box regions in **a**. Note that TH-positive neurons are well-labeled and clearly visible even at a depth of 2,460 µm in the intact brain. CPu, caudate putamen; PO, preoptic nucleus; VTA, ventral tegmental area; SNR, substantia nigra; RR, retrorubral nucleus; DR, dorsal raphe. Scale bars, 700 µm (**a**) and 100 µm (**b**–**d**). Image adapted from ref. 1.

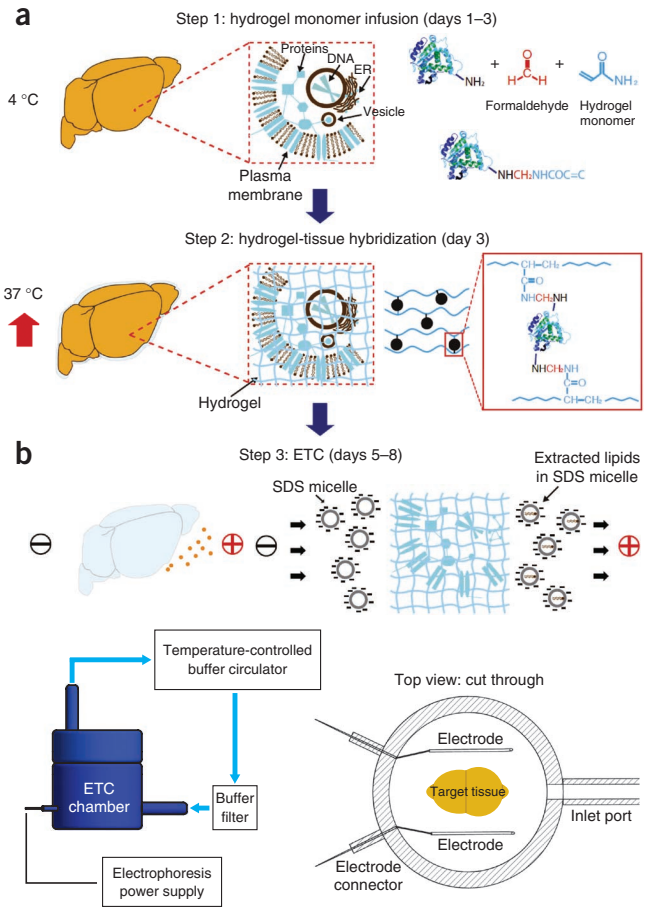

Figure 3 | CLARITY technology and instrumentation. (**a**) Tissue is cross-linked with formaldehyde in the presence of infused hydrogel monomers. Thermally triggered polymerization then results in a hydrogel-tissue hybrid which physically supports tissue structure and chemically incorporates native biomolecules into the hydrogel mesh. (**b**) In electrophoretic tissue clearing (ETC), an electric field is applied across the hybrid immersed in an ionic detergent solution to actively transport ionic micelles into the hybrid and extract membrane lipids out of the tissue, leaving structures and cross-linked biomolecules in place and available for imaging and molecular phenotyping[1]. The ETC setup consists of the custom ETC chamber, a temperature-controlled buffer circulator (RE415, Lauda), a buffer filter (McMaster) and a power supply (Bio-Rad). The sample is electrophoresed by applying 20–60 volts to the electrodes. Buffer solution is circulated through the chamber to maintain temperature and the composition of the buffer solution constant throughout the clearing process. The cut-through view (bottom right) shows placement of the hydrogel-embedded tissue in the sample holder (Cell Strainer, BD Biosciences) located in the middle of the chamber between the two electrodes. The end of each electrode exposed outside the chamber is connected to a power supply. Image adapted from ref. 1.

Here we address opportunities and challenges for CLARITY methods in brain mapping, in combination with other emerging genetic and imaging technologies.

Imaging methods for CLARITY

CLARITY is compatible with most fluorescence microscopy techniques. Conventional laser-scanning approaches, such as confocal and multiphoton microscopies, are well-suited to image samples prepared using CLARITY because the excitation and emission wavelengths of light involved can penetrate deep into the transparent tissue. Objective working distance can be limiting in this setting, although already single-photon imaging of the intact clarified adult mouse brain with a 3.6-millimeter working distance objective has been achieved without noticeable degradation in resolution (**Fig. 1**)[1]. Even greater depth of imaging, for larger brains, tissues or organisms, is possible with longer working distance and high-numerical-aperture objectives (such as the 5–8-millimeter working distance versions available from several manufacturers), but it will be important for optics to be refined and for objectives to be developed that are matched to the refractive index of clarified tissue to minimize aberration and maintain both high resolution and long working distance.

Single-photon laser-scanning confocal imaging is in many respects appropriate for CLARITY, given the broad range of suitable fluorescent labels available for multicolor confocal interrogation of clarified tissue. But confocal microscopy does expose the entire depth of tissue to excitation light and therefore induces substantial photobleaching of fluorescent molecules, a particularly acute problem in the setting of slow, high-resolution whole-brain imaging. Nonlinear imaging techniques such as two-photon microscopy address this out-of-focus photobleaching issue, but both single and two-photon imaging methods suffer from low image acquisition rate. With these scanning methods, imaging a mouse brain may take days at single-cell resolution and months at single-neurite resolution—a key practical issue when dealing with the larger samples that are characteristic of CLARITY approaches.

For imaging large samples at high resolution and at high data-acquisition rates, we suggest that selective-plane illumination microscopy (SPIM) may be a method of choice. In this technique, the sample is illuminated with a thin sheet of light from the side and fluorescence emission is collected along an axis perpendicular to the plane of illumination by wide-field acquisition[27–32]. This unique illumination and imaging modality substantially reduces both photobleaching and imaging time (for imaging whole mouse brain, closer to hours at single-cell resolution, and days at single-neurite resolution). CLARITY will naturally work well with SPIM; for example, the high and uniform transparency of clarified tissue will minimize light-plane broadening that causes degradation in resolution toward the center of the tissue in SPIM and therefore enable uniform high-resolution imaging of large samples. Other rapidly developing imaging technologies, such as structured illumination and Bessel-beam illumination[32], will enhance resolution and may potentially allow imaging in large and intact tissue specimens even beyond the diffraction limit. The CLARITY-SPIM combination of high resolution, independence from mechanical sectioning and reconstruction, and fast data-acquisition rate may become particularly useful for distinguishing and tracing neural projections with high throughput and accuracy in the course of generating maps of brain connectivity.

When indicated, after global maps of intact systems are generated, individual portions of tissue such as the downstream target of an imaged projection may be extracted for analyses such as electron microscopy that require sectioning. CLARITY is compatible with subsequent electron microscopy and preserves some ultrastructural features such as postsynaptic densities[1], although because of the absence of lipid, conventional electron microscopy staining does not currently provide enough contrast to identify all

relevant ultrastructures and boundaries, pointing to the need for optimization of the electron microscopy preparation and staining process. Alternatively, when combined with array tomography and/or super-resolution imaging in neural applications[33,34], clarified tissue may provide for nanoscopic molecular characterization of synaptic connections defined by paired presynaptic and postsynaptic proteins, so that the synaptic wiring logic of incoming projections to a brain region can be established in detail. Of course, no single imaging modality that may be used with CLARITY provides all potentially important information on functional connectivity. For example, conventional light microscopy may not readily provide definitive identification of synaptic contacts, whereas electron microscopy analysis does not typically reveal rich molecular information on detected synapses relevant to functional properties, nor would an absence of synaptic junctions between cells identified by any method demonstrate absence of direct cellular communication, which can occur via nonlocal and volume-transmission influences, as in the case of neuromodulators or extrasynaptic neurotransmitter action. Consistent with possible integrative strategies, clarified tissue appears compatible with diverse imaging readout modalities that complement each other; moreover, the multiround molecular phenotyping capacity of CLARITY (see below) may be suitable for providing enriched detail on the composition and relationships of subcellular structures such as synapses[34].

Integrating circuit maps with molecular information

A unique feature of CLARITY is its potential for intact-tissue molecular phenotyping studies. The hydrogel-tissue hybridization preserves endogenous biomolecules ranging from neurotransmitters to proteins and nucleic acids[1]; soluble proteins and cell-membrane proteins alike are secured by chemical tethering to the hydrogel mesh. Moreover, removal of the lipid membrane makes this retained molecular information accessible via passive diffusion of macromolecular probes into the tissue, and the enhanced structural integrity of clarified tissue allows multiple rounds of antibody staining, elution or destaining and restaining that are not typically feasible with conventionally fixed tissue[1,35]. Immunostaining of 1-millimeter-thick clarified tissue blocks takes days, and immunostaining of entire intact adult mouse brains is possible on practical timescales of several weeks (**Fig. 2**). This process could be accelerated by increasing probe diffusion rate with electrophoresis or other methods. The fact that CLARITY supports multiple (at least three) rounds of molecular phenotyping may be of value to define cell types and to link form and function in brain-mapping studies, allowing the integration of rich local and global morphological details (for example, type of synapse, shape of cell body, and information about dendritic arborization and axonal projections) with genetic fingerprints.

In addition, the combination of CLARITY with genetic methods for identification of synapses or for labeling specific circuits is also of interest. First, GFP reconstitution across synaptic partners (GRASP) has emerged as a light microscopy–based synapse-detection technique[36]. In this method, two nonfluorescent split-GFP fragments can be virally expressed in the synaptic membrane of two separate neuronal populations; these two fragments reconstitute fluorescent GFP only across a synaptic cleft so that the location of synapses between the two populations can be visualized[36,37]. This approach was validated with electron microscopy and was used to map synaptic connectivity in the nematode[36] and the fruit fly[38]; most recently, applicability to mammalian systems was demonstrated: mammalian GRASP (mGRASP) revealed labeling of both excitatory and inhibitory synapses[39]. Second, in recent years many mouse lines and viral targeting techniques have been developed for intersectional labeling of spatially, genetically, synaptically and functionally defined neuronal circuit elements. For instance, an engineered rabies virus encoding EGFP can be injected into a particular brain region to label neurons that are presynaptic to the injection site[40,41]. More recently, activity-dependent expression of channelrhodopsin (ChR2) conjugated with EYFP was used to label and drive a subset of synaptically recruited neurons associated with fear memory[42], and a technique has been developed and validated that allows permanent marking (by filling a cell with fluorescent protein labels) of neurons across the mouse brain that were active during a relatively restricted time window (on a time scale of hours)[43]. These versatile targeting approaches, in combination with CLARITY, could provide a high-throughput approach for globally mapping synaptically connected and synaptically activated populations across the brain that could complement the use of electron microscopy in some settings.

Although new genetic strategies such as these are quite powerful, for studying and mapping the human brain, more conventional antibody-based (nongenetic) molecular phenotyping alone is particularly important. Lipophilic dyes (for example, 1,1′-dioctadecyl-3,3,3′3′-tetramethylindocarbocyanine perchlorate) can be injected into postmortem human brain to effectively label local projections through passive diffusion[44]; however, in fixed tissue without active axonal transport, diffusion rates (and therefore tracing distance) are substantially limited and may not be ideally suited to mapping long-range connectivity. With CLARITY, molecular markers have already been used to identify individual structures and projections (cell bodies and fibers) in human tissue on the millimeter length scale[1]; although this approach has not yet been tested for tracking long-range projections in the human brain, neurofilament protein staining reliably highlights a major subset of axonal fibers, and because continuity of labeled projections is preserved in the intact tissue, fibers can be traced with diminished risk of alignment and/or reconstruction error. Cell type–specific markers (for example, parvalbumin and tyrosine hydroxylase) can also be used to label both cell bodies and projections in human brain tissue[1]. CLARITY may in this way help unlock a rich source of clinically relevant information, providing a means to interrogate and make accessible brain-bank samples that are unique or rare, for the purpose of understanding disease mechanisms as well as the native structure, organization and complexity of the human brain.

Limitations, challenges and opportunities

CLARITY is currently in the early stages of development; innovation will be needed over the coming years. First, we have noted that tissues can expand after electrophoretic tissue clearing and return to the original size after refractive-index matching[1]; although macroscopic observation indicates that the changes in volume are largely isotropic and reversible, quantitative monitoring at microscopic and nanoscopic resolution will be required to confirm that loss in structural connectivity or occurrence of

other tissue artifacts is minimal. Second, although adherence to the described protocol[1] will minimize tissue damage, the precise extent to which CLARITY under best current practice secures specific molecular information represented in proteins, nucleic acids and small molecules must be further explored. Only ~8% of total protein content is lost in CLARITY[1], substantially lower than with other methods over the same timescale (for example, ~24% loss with conventional fixative (paraformaldehyde) and detergent (0.1% Triton X-100) histological solution compositions[1]). However, the nature of the remaining loss should be investigated, and we note that the current chemistry of CLARITY (by design) does not preserve lipids or other molecules lacking functional groups required for chemical tethering to the hydrogel mesh, such as phosphatidylinositol 4,5-bisphosphate, or PIP2. Additional chemical-treatment strategies may need to be explored for specialized experimental questions.

It also remains to be determined how accessible different classes of biological information from clarified tissue may be, in clinical or animal settings, for high-content quantitative data-extraction pipelines. Mapping this landscape will be of value for developing strategies to maximize information extraction after global (brain-wide) projection mapping has been completed, and may be carried out in the context of normal function, disease states and treatment regimens (in mapping effects of brain-stimulation treatments or in drug screening, for example). Another open question is how long clarified tissue may be maintained or stored for such analysis, assessment, imaging or remodeling in subsequent rounds of CLARITY processing.

CLARITY can be seen as a prototype for a general approach for building new structures and installing new functions from within biological systems—an approach that may find other instantiations as the technology is developed for introducing components or monomers into biological tissue that are then triggered subsequently to form a polymer, gel, mesh, network or assembled structure with desired physical parameters (for example, stiffness, transparency, pore size, conductivity and permeability) or active properties (for interfacing, catalysis or functionalization). Relevant exogenous components may include proteins, oligonucleotides, stains, chemicals or even small mechanical, electronic or optical components. These introduced components could be designed for either constitutive or inducible functionality—in the latter case, such that they can be activated by external elements including heat, mechanical force, redox changes, electromagnetic triggers such as light, and other accelerators, thereby enabling temporally precise initiation of the structural and functional tissue transformation. The capacity to trigger functionality could help enable versions of this general approach (not involving lipid removal) that may be compatible with ongoing vital functions of the tissue.

On the brain-mapping front, as pioneering technologies such as array tomography and serial block face scanning electron microscopy have already proven, turning large data sets into useful and tractable deliverables still poses an immense challenge. Relevant computational approaches for registration in three dimensions will need to be developed, and particularly in the setting of variable, quenching and/or bleaching fluorescent signals, automated image acquisition, segmentation and tracing algorithms will be helpful. We note that molecular landmarks are useful for navigating three-dimensional data, and here the intact-tissue molecular phenotyping capability of CLARITY becomes particularly important. As in conventional histology, broadly staining markers (which are dense yet distinct) can be useful as landmarks to navigate and identify brain regions of interest[45]. In this regard, DAPI staining is compatible with CLARITY[1] and appears particularly useful as a Nissl-like registration method.

Registration of CLARITY data with other data is both a challenge and an opportunity. For example, in an animal-subject brain in which a particular set of neurons becomes known (via imaging of activity [46–49] and/or via optogenetic control[4]) to be involved in a specific behavioral function or dysfunction, to then clarify the same preparation and obtain brain-wide wiring and molecular information on those same cells (and their connection partners) would be of substantial value. This approach could be used to address fundamental questions in both basic and clinical or preclinical neuroscience but will require the development of efficient workflows for the registration of the different types of experimental data. CLARITY may also help to conceptually link future high-resolution activity maps[50] with structural or functional macroscale maps (for example, http://www.neuroscienceblueprint.nih.gov/connectome/), providing an anatomical foundation that could help researchers decipher the meaning of brain-activity patterns linked to health and disease[4,50]. Beyond neuroscience, CLARITY is currently being explored for the evaluation, diagnosis and prognosis of pathological states including cancer, infection, autoimmune disease and other clinical conditions as well as for the study of normal tissue, organ and organism function, including development and relationships of cells and tissues. Resources that may help enable the general user to establish the methodology are available online (http://clarityresourcecenter.org/).

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The authors declare competing financial interests: details are available in the online version of the paper.

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