

## Hydrogel-Tissue Chemistry: Principles and Applications

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### Abstract

Over the past five years, a rapidly developing experimental approach has enabled high-resolution and high-content information retrieval from intact multicellular animal (metazoan) systems. New chemical and physical forms are created in the hydrogel-tissue chemistry process, and the retention and retrieval of crucial phenotypic information regarding constituent cells and molecules (and their joint interrelationships) are thereby enabled. For example, rich data sets defining both single-cell-resolution gene expression and single-cell-resolution activity during behavior can now be collected while still preserving information on three-dimensional positioning and/or brain-wide wiring of those very same neurons—even within vertebrate brains. This new approach and its variants, as applied to neuroscience, are beginning to illuminate the fundamental cellular and chemical representations of sensation, cognition, and action. More generally, reimagining metazoans as metareactants—or positionally defined three-dimensional graphs of constituent chemicals made available for ongoing functionalization, transformation, and readout—is stimulating innovation across biology and medicine.

### Keywords

CLARITY; hydrogels; metareactant; HTC; hydrogel-tissue; clearing

## INTRODUCTION

In the study of complex biological systems, a powerful experimental approach is that of analysis or disassembly (removing components, such as a particular type of cell or complex

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### DISCLOSURE STATEMENT

All protocols, software, and other information regarding these methods is freely available from the authors and online, and disseminated via free hands-on training courses ([clarityresource-center.org](http://clarityresource-center.org) and [clover.caltech.edu](http://clover.caltech.edu)). V.G. and K.D. have disclosed intellectual property regarding HTC methods to Caltech and Stanford, some of which has been licensed to ClearLight Diagnostics, which is exploring applications for cancer diagnostics, and with which there are consulting arrangements and equity; V.G. and K.D. each also have grant support from the US federal government (National Institutes of Health and National Science Foundation) to further develop, apply, and disseminate these methods.

of molecules, from the native context for further study). For example, the current revolution in cancer treatment was in part enabled by reductionist molecular and cellular-level analysis of isolated cancer cells and of specific immune-system cells that play a role in suppressing tumor growth. The success of this analytical paradigm has, in part, extended to neuroscience as well; studies of isolated neurons and axons have facilitated elucidation of the fundamental logic of single-neuron information processing.

However, for systems like the intact vertebrate brain (composed of  $10^7$ – $10^{11}$  interconnected neurons and characterized by crucial emergent properties), studying constituent components in isolation can provide little insight into many of the most significant mysteries. Alternatively, converting the brain—or more broadly the entire metazoan (multicellular animal) organism—into an assembly of reactants anchored onto a new and versatile three-dimensional (3D) coordinate system has recently emerged as a complementary strategy (23, 24). Coupling individual subsets of chemically defined biomolecules to functional groups, covalently anchoring or entangling these in turn within a polymer lattice, and then working with this structure (effectively a 3D assembly of spatially tagged molecular reactants) (23, 24) has already opened the door to a diverse array of novel approaches and discoveries in biology.

The technique builds in part from (among several other foundations in science and engineering) the chemistry of hydrogels, which are 3D polymeric networks of connected hydrophilic components. Gels and polymers have a long history of use in biology, including for providing physical support of tissues during sectioning and imaging, as well as for a number of important clinical applications in regenerative medicine and tissue engineering. But in the basic science of hydrogel-tissue chemistry (23, 24), specific classes of native biomolecules in tissue are immobilized or covalently anchored (for example, through individualized interface molecules to gel monomer molecules) and precisely timed polymerization causing tissue-gel hybrid formation is triggered within all the cells across the tissue in an ordered and controlled process (Figure 1) to ultimately create an optically and chemically accessible biomolecular matrix. Indeed, when the biomolecules of interest are thereby transferred to the polymer lattice, a robust new composite hydrogel-tissue material results (23, 24), which becomes the substrate for future chemical and optical interrogation that can be probed and manipulated in new ways. This approach has been diversified (Figure 2) to address needs and opportunities in organisms and tissues across biology (including in cancer diagnostics, bacterial and HIV infection of mammalian tissues, developmental biology, parkinsonism, Alzheimer's disease, multiple sclerosis, autism, drug abuse, and fear/anxiety disorders). Here, we review the fundamentals of this approach, the rapidly expanding scope of discoveries that have resulted, and emerging directions and opportunities for the future.

## DEVELOPMENT OF METHODS

Biomolecule functionalization and multistep linkage to a versatile tissue-hydrogel scaffold (Figure 2) within the cells of vertebrates (mouse, fish, and human) (15, 16, 23) were described in an initial version called CLARITY; this method was optimized for application to the vertebrate nervous system (15, 16, 23). The hydrogel-tissue hybrid brains were

transparent (i.e., clarified) and thus permissive of intact whole-organ imaging at high resolution (16). It was noted that the resulting hydrogel-tissue hybrid “expanded” upon lipid removal in aqueous solution but “did not cause net tissue deformation...[R]emaining secured in place were fine structural details” (16, p. 334) since the expansion could be reversed with a solution change. Other diverse strategies for reducing opacity of intact tissue had been explored for years (though with varying degrees of efficacy and versatility) (Figure 3), but transparency was not the only experimental leverage achieved with the hydrogel-tissue chemistry (HTC) approach; for example, the new hybrids were designed to be macromolecule permeant—enabling multiple rounds of molecular interrogation of preserved biomolecules (proteins and nucleic acids) that had been anchored into the new physical structure (16, 23, 125).

Single-photon confocal microscopy was initially used to image many-millimeter-thick blocks of the resulting clarified and fluorescently labeled human brain tissue, zebrafish central nervous systems, and whole adult mouse brain hemispheres (16). Diverse lines of work eventually emerged from this publication (23); as was noted therein, “infused elements need not be exclusively hydrogel monomers or acrylamide-based, and the properties of infused elements may be adjusted for varying degrees of clarity, rigidity, macromolecule-permeability or other functionality” (16, pp. 336–37). Also in 2013, a broad diversity of additional compositions, including those with acrylates or alginates, was described (25), and indeed variations and innovations on the theme rapidly emerged (Figure 4) (reviewed in 23, 53).

Also introduced was an electrophoretic tissue clearing (ETC) technique to accelerate lipid removal (16); lipid removal promotes tissue transparency and macromolecular interrogation, and this process can be carried out nondestructively after hydrogel-tissue hybrid formation (Figure 1). ETC employs electric field-forced clearance of lipid-containing ionic-detergent sodium dodecyl sulfate (SDS) micelles (Figure 1). Although helpful, ETC is not absolutely necessary to remove lipids, and the following year an ETC-independent approach was reported—passive CLARITY. This variant was initially described by Zhang et al. (147) and was found to be effective for adult central nervous systems and spinal cords. Passive CLARITY was soon thereafter reported to apply also to brain slices (104), and when combined with CLARITY-optimized light-sheet microscopy (COLM) this variant enabled imaging of entire adult mouse brains at subcellular resolution within several hours (131). At the same time, another CLARITY variant (PACT) was described (142), presenting modifications to the CLARITY reagents to passively achieve fast clearing of thick samples. After overnight tissue fixation in 4% paraformaldehyde (PFA), tissues were embedded in a 4% acrylamide hydrogel solution without the 4% PFA and 0.05% bisacrylamide of the original hydrogel formulation to minimize cross-linking (133, 142). In addition, a relatively inexpensive refractive index-matching solution, termed RIMS, was introduced (142).

The data of both Yang et al. (142) and Tomer et al. (131) in 2014 showed a moderate degree of tissue expansion associated with the HTC process, as had been described by Chung et al. (16) and indeed also as had been seen with earlier tissue clearing approaches (Figure 5). Although this effect had not been amplified to explore potential advantages, over the next two years, several HTC papers {11 [expansion microscopy (ExM) in 2015], 131 [expansion

passive CLARITY technique (ePACT) in 2015], and 62 [magnified analysis of the proteome (MAP) in 2016]} soon enabled much-enhanced swelling of HTC hybrids to improve resolution of densely packed features. In a method unique for preserving endogenous fluorescence, ePACT (133) uses collagenase to enhance the magnitude of the size change. Two of the other versions, ExM (11, 30) and MAP (64), also embed tissue in a similar hydrogel network (reviewed in 53). In these formulations, which prescribe inclusion of acrylates (R2 in Figure 2) alongside acrylamide to enhance swelling (Figures 2 and 4), proteolysis can be carried out to facilitate this process but is not required. MAP additionally allows reversible expansion of the tissue-hydrogel hybrid (Figure 5) and super-resolution imaging of subcellular structures using high concentrations of acrylamide (30% acrylamide with 10% acrylate) to promote protein attachment to the hydrogel and prevent intra- and inter-protein cross-linking (64).

A large number of subsequent HTC studies put forward additional enhancements, including modifications of the ETC process and device (5, 59, 71, 72, 117, 121), of the hydrogel monomer and cross-linker levels (5, 32, 63, 131, 133, 142) and of other parameters while maintaining the basic hydrogel-tissue chemistry (18, 20, 22, 32, 63, 80, 84, 108, 122, 140, 142, 143, 145, 149). In addition to the acrylamide and/or acrylate-based PFA-coupled hydrogels noted above (PACT/ePACT, ExM, MAP), other gelation mechanisms have also been described. The SWITCH approach uses pH changes to synchronize formation of a glutaraldehyde-crosslinked matrix within tissue before CLARITY-type lipid removal via SDS, resulting in a heat- and chemical-resistant tissue-hydrogel hybrid that facilitates multiple rounds of labeling, elution, and relabeling (94, 106). Also described in the study that introduced PACT was a strategy termed PARS (perfusion-assisted agent release in situ) for whole-body clearing and labeling using perfusion through the vasculature to deliver hydrogel, clearing, labeling, and imaging reagents (133, 142). PACT and other passive CLARITY-based HTC methods were further adapted to tissues otherwise difficult or impossible to image intact, from the rigid and opaque bone [PACT-deCAL (133, 140) and Bone CLARITY (44)] to the soft and friable clinical samples and embryos (27, 51, 148).

In addition to small-molecule dyes, cellular stains, and protein labels (e.g., lectin) that can directly target proteins, DNA, and other biomolecules, tissues cleared using HTC can be stained using fluorescently tagged whole antibodies as well as smaller antibody formulations such as FAB (fragment antigen-binding antibody) fragments (15, 16, 131, 133). Nanobodies were effective in staining PACT-cleared tissues (142); at 10% the size of full antibodies and stable over a variety of pH and temperature conditions, nanobodies are particularly appealing for labeling cleared thick tissues (133). The ETC process was accelerated using an approach called stochastic electrotransport (59), and an electrophoretically driven approach transported antibodies across a few millimeters of cleared tissue in less than an hour, approximately 800 times faster than via passive diffusion (75). PRESTO (pressure-related efficient and stable transfer of macromolecules into organs) conferred increased antibody penetration depth and speed, particularly in cleared peripheral organs, by application of either centrifugal force or convection flow using a syringe pump during sample incubation in an antibody solution (71).

To broaden the types of macromolecular information obtained, recent studies have developed methods for visualizing lipids and RNA in HTC samples. Following earlier work that demonstrated the detection of endogenous mRNA in CLARITY specimens via standard in situ hybridization protocols (16), Yang et al. (142) showed that PACT hydrogels supported the use of single-molecule fluorescence in situ hybridization (smFISH) to detect individual mRNA transcripts at depth. In optimizing retention of RNA for labeling in cleared hydrogel-tissue hybrids, a carbodiimide compound [1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC)] was discovered to be useful for specifically linking RNA nucleotides directly to the tissue hydrogel (125) (Figure 2), and application of the hairpin chain reaction (HCR) amplification system facilitated multiplexed RNA labeling in these EDC-CLARITY samples that could be at least 3 mm thick. A 1% acrylamide hydrogel exhibited improved RNA labeling (for both total RNA and specifically mRNA) when compared to CLARITY samples (with 4% acrylamide) (125). Multiplexed single-molecule HCR was also demonstrated as an effective in situ hybridization technique in HTC brain slices embedded and cleared with PACT or ExM (12, 27, 115). Other methods led to improved visualization of fluorescent nanoparticles (polyethylene glycol-coated quantum dots) (116, 117), creation of nonfluorescent (dark) reaction products (horseradish peroxidase colorimetric labeling) (122), and development of lipophilic dyes that were altered to be aldehyde fixable to proteins to mark membranes even after HTC lipid removal (52).

## HYDROGEL-TISSUE CHEMISTRY-BASED DISCOVERY IN NEUROSCIENCE AND THROUGHOUT THE ORGANISM

HTC methods have proven powerful for neuroscience; only a few examples of resulting discoveries are collected here to illustrate current capabilities and opportunities. First, a large number of studies have used the HTC approach to identify local and global wiring patterns of targeted neurons, beginning with the demonstration that a specific class of spinal cord neuron (NECAB expressing) exhibits midline crossing (147), and subsequently with the mapping of infection distribution for viral vectors microinjected into the lateral amygdala (LA) to analyze the neural mechanism of cocaine-cue memory engram formation in mice (50). Similarly, in a study analyzing the morphology of raphe-spinal fibers in the spinal cord, passive CLARITY provided visualization of a unique branching pattern of serotonergic fibers along the rostrocaudal axis as they extended toward the lateral motor neuron column (77, 78). Using rabies virus-based circuit mapping, passive CLARITY and COLM provided unbiased global mapping of all the neurons in the brain that project to dopamine neurons in the substantia nigra pars compacta, which in turn project to dorsolateral versus dorsomedial striatum (73). Likewise, rabies virus-based methods were used to trace monosynaptic inputs to projection-defined dopamine neurons via whole-brain CLARITY (in this case also with ETC and light-sheet imaging) (90). Anterograde tracing followed by CLARITY (using both ETC and passive clearing) provided visualization of synaptic targets of GABAergic projections from the medial septum (136). And in a study analyzing top-down control of anxiety and fear, passive CLARITY was used to track and map a distinct novel projection from ventromedial prefrontal cortex to basomedial amygdala (1). Integrating passive CLARITY with light-sheet microscopy and behavior, researchers implemented multiple-

animal whole-brain activity mapping protocols for HTC alongside a strategy termed CAPTURE (143) for quantifying numbers and projections of behaviorally activated neurons.

PACT was used to study the distribution and morphology of astroglia in thick tissue sections (92) and the 3D distribution of multiple genetically defined neuron types in mouse brains (103). Passive CLARITY on sections of medial prefrontal cortex (mPFC) established the presence of nonoverlapping corticotropin-releasing factor and corticotropin-releasing factor receptor-1 circuits relevant to acute stress (138) and was used to map brain-wide viral expression in mice inoculated with western equine encephalitis virus in the foot pad (101). The distribution of microglia within the subventricular zone (a neurogenic region of the adult central nervous system) was mapped using passive CLARITY (38), and in the periventricular zone of the cerebellum, passive CLARITY was employed to analyze the organization of astrocytes during development (43). Passive CLARITY was used to show increased dendritic complexity in hippocampal pyramidal neurons of transgenic mice that exhibit enhanced learning (114) and to observe the localization of cells expressing neuromedin B, a bombesin-like neuropeptide that influences sighing behavior, around the facial nucleus, including the retrotrapezoid nucleus (a control center for breathing) (76). In transgenic mice using the nicotinic acetylcholine receptor  $\alpha 2$  subunit (*Chrna2*) locus to mark deep-layer V Martinotti cells, passive CLARITY was used to verify labeling, specificity, and morphology of the targeted cells (47). For examining somatostatin-expressing interneurons in the dentate gyrus, CLARITY allowed demonstration of the axonal projections of a specific subset to the medial septum (146). Subcellular localization of a specific transcription factor, ESRRA, was analyzed using CLARITY (1% acrylamide with ETC) in brain sections (200  $\mu\text{m}$ ) to help elucidate the protein's role in cell signaling (111). Using viral vector tracing to label mPFC-projecting neurons in the basolateral amygdala (BLA), CLARITY provided visualization of the target specificity of those neurons, which aided in investigation of their role in manipulating fear associations (60). To analyze neuronal organization in the hypothalamus, whole-brain mapping of tyrosine hydroxylase (TH)-positive neurons and projections was performed with CLARITY followed by immunostaining and COLM (109).

In addition to enabling these basic discoveries, HTC work has also stimulated technical and engineering advances. Passive CLARITY of electrolytically lesioned slices was used to correct electrode placement for fast-scan cyclic voltammetry (120) and to identify locations of implanted optical fibers (89). Following penetrating brain injury, passive CLARITY permitted brain-wide visualization of specific peptide accumulation in studies exploring targeted delivery of diagnostic and therapeutic compounds (86). And more broadly, body-wide biodistribution studies looking at chemicals or biologicals were found to benefit from HTC; for example, Treweek and coworkers (134) and Deverman et al. (28) demonstrated that whole-body PARS (142) could facilitate the generation of transduction maps of systemically delivered genes by adeno-associated viruses, which in turn facilitated characterization and discovery of new viral variants for targeting the central and peripheral nervous systems (8). HTC-based clearing has also technically enabled quadruple immunofluorescent staining as well as multiple rounds of labeling to reveal a variety of richly defined subcellular domains and molecule types in single human cerebellar sections (102).

Several studies have combined magnetic resonance imaging (MRI) with CLARITY. In probing the contribution of myelination to measurables from diffusion tensor imaging, passive CLARITY revealed that myelination correlates strongly with fractional anisotropy but only partially with radial diffusivity (9). The differential contributions of lipids and proteins to MRI contrast were analyzed using passive CLARITY to remove lipids and preserve proteins: Cleared tissues showed minimal contrast, increased relaxation times, and diffusion rates similar to free water, and lipids were thus demonstrated to be the dominant source of MRI contrast in brain tissue (74). In experimental autoimmune encephalomyelitis (a mouse model of multiple sclerosis), a direct relationship was defined between gray matter atrophy visualized using MRI and the number of axonal end bulbs in spinal cord visualized using passive CLARITY (118). This type of ground-truth work on clinical biomarkers is of immense and rapidly increasing value, particularly given the epidemiology of neurodegenerative diseases.

Disease model work in general has progressed rapidly with HTC. In a mouse model for Parkinson's disease, passive CLARITY revealed fragmented nigrostriatal axons (97). In addition to related studies in rat models (80, 119), direct human-disease HTC applications have also advanced rapidly. The effectiveness of CLARITY on postmortem human brain tissue was demonstrated using 500- $\mu\text{m}$  thick tissue blocks from clinical autism samples that had been stored in formalin for over six years, revealing 3D morphologies not readily accessible using traditional sectioning (16). Similarly, passive CLARITY has been used to examine the 3D architecture of amyloid and tau aggregates in 500- $\mu\text{m}$  thick banked tissue from Alzheimer's disease patients (3), and passive CLARITY has been used on 3-mm thick blocks of fresh or formalin-fixed tissue from Parkinson's disease patients to reveal Lewy body inclusions nearly 1 mm deep in the tissue (80).

## NONNEURAL TISSUES

Although originally conceived for studying the brain (23, 24), the HTC approach can be extended to a wide variety of other organs and tissue types, including spinal cord, lung, heart, intestine, spleen, kidney, muscle, testis, pancreas, liver, skin, and bone (32, 44, 71, 72, 100, 140, 142). Its usefulness for imaging infection was demonstrated using PACT in mice infected with fluorescent *Mycobacterium tuberculosis*, which enabled visualization of 3D spatial distribution of bacteria throughout intact lungs (20). A modified PACT, MiPACT (for microbial identification after PACT) was designed to label bacterial rRNA (via HCR) for analysis of spatial organization and metabolic activity of bacteria in amorphous sputum samples from cystic fibrosis patients (27). Also in lung, localization of nestin-expressing cells was observed throughout the vasculature (not the airway system) of tissue cleared via PACT, which motivated and guided investigation of the role of these cells in development of pulmonary hypertension (110). In a mouse model of lung adenocarcinoma, applying CLARITY to whole-lung tumors (clearing with two days of ETC) provided a comprehensive demonstration of significant differences in the cellular density and morphology of tumor cells with and without depletion of regulatory T cells (54). In pancreatic tissue, an evaluation of p53 loss of heterozygosity in tumor progression was enabled by HTC (95).

In liver, 3D positioning within the portal system (relative to the canals of Hering) was demonstrated using passive CLARITY for periportal hepatocytes, which undergo proliferation following injury (37). After application of passive CLARITY to rat kidneys, superresolution-STED microscopy revealed 3D positioning information at the nanometer scale (137). HTC on mouse and human gut tissue was achieved using passive CLARITY and immunostaining to visualize structures in the enteric nervous system, vasculature, smooth muscle layers, and epithelium, while also demonstrating compatibility with classical pathological stains such as hematoxylin-eosin and Heidenhain's Azan (96). Early systemic viral spread of human immunodeficiency virus 1 (HIV-1) in humanized mice was analyzed from gut-associated lymphoid tissues using PACT (58), and HTC (with ETC) was found useful for studying even dense and fibrous mouse hind-limb skeletal muscle tissue (91). In virgin and lactating mouse mammary glands, epithelial and tumor cells were made visible using PACT (82), whereas with passive CLARITY on intact mouse ovaries, the architecture and growth of ovarian follicles and their relationship to vasculature was analyzed throughout the mouse reproductive life (35, 83). Embryonic and neoplastic tissue analysis has been similarly optimized (48, 88, 132), and fast clearing was achieved by HTC in liver tissue (69) as well as in the growth plates of distal limbs (17).

In hatched chickens, adult *Xenopus*, and adult zebrafish, the comparative organization of HTC-stabilized cerebrospinal fluid-contacting cells revealed similarities pointing to a common bony vertebrate ancestor (141). Legs from chicken embryos were analyzed using passive CLARITY to reveal embryonic development of hallux positioning in the avian grasping foot (6). Passive CLARITY was also applied to the mouse nasal septum to visualize the morphology of horizontal basal cells in the olfactory epithelium following lesion of the olfactory bulb (112). The effect of subcutaneous injection of poly(methacrylic acid-co-methyl methacrylate) beads on vascularization was observed using passive CLARITY in mouse skin tissue (79). A dual-illumination-side light-sheet microscope optimized for imaging cardiac tissue over 1 cm<sup>3</sup> in volume, combined with HTC, enabled researchers to measure ventricular dimensions, track the lineage of cardiac cells, and view the spatial distribution of cardiac-specific proteins within intact hearts (29). CLARITY also has been employed in intact mouse hearts as well as human heart tissue up to several millimeters thick (42, 62).

Host-pathogen interactions were studied using passive CLARITY and PACT to comprehensively examine morphology of necrotic granulomas from adult zebrafish infected with *Mycobacterium marinum* (19, 20). PACT and CUBIC (123) were found well suited for imaging the intact zebrafish testis at cellular resolution (39). Passive CLARITY was applied to transgenic *Xenopus* tadpoles to locate and quantify thyroid hormone signaling disruption by contaminants introduced during brain development (36). Applying passive CLARITY to the intact liver of lamprey undergoing metamorphosis provided visualization of the process of biliary degeneration, a process that occurs in human infants with biliary atresia via a mechanism that is still unknown (14), and passive CLARITY/COLM imaging in the lamprey was used to visualize the spatial organization of neuronal inputs and outputs in the optic tectum with the Neurobiotin tracer (55).

Addressing challenges beyond soft tissue, Bone CLARITY (44) was developed and applied along with a CLARITY-optimized light-sheet microscope to quantify marrow cells from cleared adult intact mouse bones, revealing differences in fluorescent stem cell count and distribution after bone-forming agent administration (44). HTC approaches have been applied to multicellular plants as well via plant-enzyme-assisted (PEA)-CLARITY, an adaptation to perform optical clearing and antibody interrogation on plant tissues. Using cell wall-degrading enzymes to increase permeability and starch-hydrolyzing enzymes to improve transparency following passive clearing, PEA-CLARITY enabled visualization of fluorescent signals from expressed proteins as well as antibody staining in whole, intact tobacco and *Arabidopsis* leaves (98). The PEA-CLARITY protocol was later applied to study the 3D architecture of the *Medicago truncatula* root nodules (128).

## OUTLOOK

The proven application domain of HTC in biology and medicine is rapidly expanding and has already resulted in numerous basic science discoveries and opportunities for clinical medicine (e.g., 24, 51, 143). However, the novelty of the preparation and its resulting data streams have created challenges. Here, we consider the current rate-limiting steps as well as opportunities for the future.

Early on, one of the clearest applications of the HTC approach was enabling high-resolution optical access to large intact tissues, organs, and organisms. Although this major goal was achieved, collecting high-resolution volumetric image data from large samples created new issues. For example, the transparency of the hydrogel-tissue hybrid allowed confocal or two-photon imaging over large volumes, but these slow point-scanning techniques led to bottlenecks in image acquisition (e.g., the collection of high-resolution structural data sets for an adult mouse brain required several days of imaging). Data collection on this timescale is associated with problems ranging from photobleaching to simple microscope overoccupancy, but rapid development of advanced light-sheet imaging, which offers orders-of-magnitude improvement in speed (29, 41, 44, 107, 115, 130, 131, 143), addressed this acquisition problem. Subsequent HTC-focused work included stochastic electrotransport (59); super-resolution-STED microscopy (137); adaptive optics (105); HTC sample handling chambers (44, 92, 93, 135); custom ETC and staining chambers (59, 71); and microfluidic chip-based embedding, clearing, and labeling (13).

The initial expansion found associated with HTC methods (16, 131, 142) was counteracted with size-normalization/contraction strategies during the refractive index-matching step to allow high-resolution objectives with limited working distance to access more of the brain (16). This strategy also had the effect of reducing the data set size, an important consideration for tractability. However, these considerations have become progressively less important with the advent of new hardware, including customized long-working-distance and high-resolution CLARITY objectives (87, 131) as well as distributed computing strategies.

Many studies have employed automated analysis pipelines for manipulating large CLARITY data sets; commercial 3D rendering software programs, such as Imaris or Arivis, can

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automate manually intensive data processing steps such as cell counting. Automation becomes even more valuable when analyzing thicker tissue sections or whole organs (44, 92, 143), but the utility of automated analysis extends beyond the domain of cell body recognition and counting. To quantify neural projection patterns, an automated method has been developed to compute 3D structure tensors from CLARITY images, and input of the tensors into diffusion tractography software yielded reconstruction of calculated streamlines mapped onto fibers from the CLARITY images (143). With this approach, connectivity between a seed region and specific downstream targets could be visualized and quantitatively evaluated by counting streamlines (143). In addition, alignment of autofluorescence images from multiple sample organs can be used to create a common reference space. When autofluorescence is combined with segmentation algorithms for automated cell detection, a transformation of the acquired signal from each sample onto this reference space can be used to compare the regional distribution of labeled cells across brain samples and allow registration to public atlases, such as the Allen Brain Institute's Mouse Reference Atlas (90, 107, 143). Automatic annotation of CLARITY brain images (67) has been enabled by registering CLARITY brain images to the Allen atlas using a method called Mask-LDDMM. TeraFly is a free, open-source software tool designed specifically for 3D integrated visualization and annotation of massive, terabyte-sized image data sets like those acquired using the COLM system (7), and a manual segmentation tool (ManSegTool) for segmenting 3D neuronal data sets was demonstrated to enable neuroscientists to extract neurons from cerebellum slices cleared and imaged using passive CLARITY (85). For automatic annotation and standardization of brainwide data sets, WholeBrain is a free, open-source software that provides connectivity and activity-based mapping and quantification of multidimensional data, using a scale-invariant anatomical mouse brain atlas, which allows comparison of results across experiments and imaging platforms (40). Concurrently, an interactive Web-based framework, Openbrainmap (<http://openbrainmap.org>), was developed for data visualization and sharing between laboratories (40).

Tissue clarification is only one of many application domains of HTC methods, although it is arguably the most developed. Beyond tissue transparency, two studies have applied the hydrogel tissue-embedding step of CLARITY to stabilize mouse embryos or adult mouse brain tissue for micro-computed tomography (micro-CT) imaging using contrast agents that typically shrink tissue (2, 139). CLARITY was also used to reveal the 3D structure of patterned microtissues (129). And in stem cell-derived organoids, passive CLARITY followed by immunostaining was used to model and explore effects of cocaine exposure on the human fetal brain (70).

A final emerging domain of substantial interest, and an initial motivation for HTC (26), is the development of hydrogel-tissue hybrids with diverse types of functionalization, which would enable experiments extending far beyond static structural and molecular analysis. For example, creation of active constructs based on polymers with electrically conductive properties could allow new forms of interrogation of biological systems, and diverse additional forms of HTC and variants are in the process of emerging. Rooted in fundamental chemistry, the broad concept of envisioning (and remaking) metazoan animals and tissues as metareactants—that is, positionally intact and chemically versatile scaffolds of molecular

reactants—may continue to open up new and unanticipated domains of investigation and discovery across diverse fields of biology.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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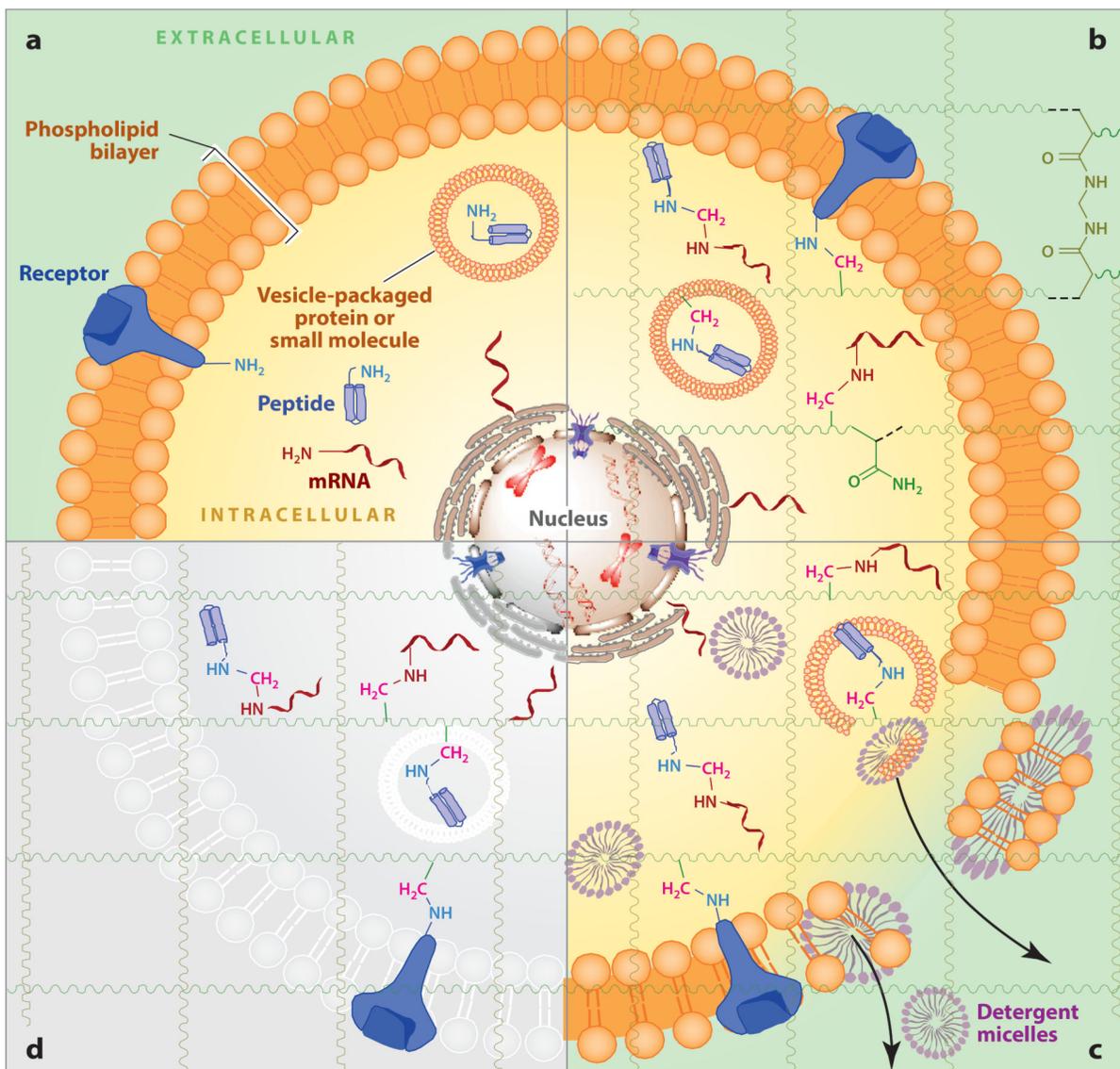
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**NH<sub>2</sub>** Amino group of nucleophilic small-molecule, peptide N-terminus, or amino acid side chain, e.g., lysine's ε-amine

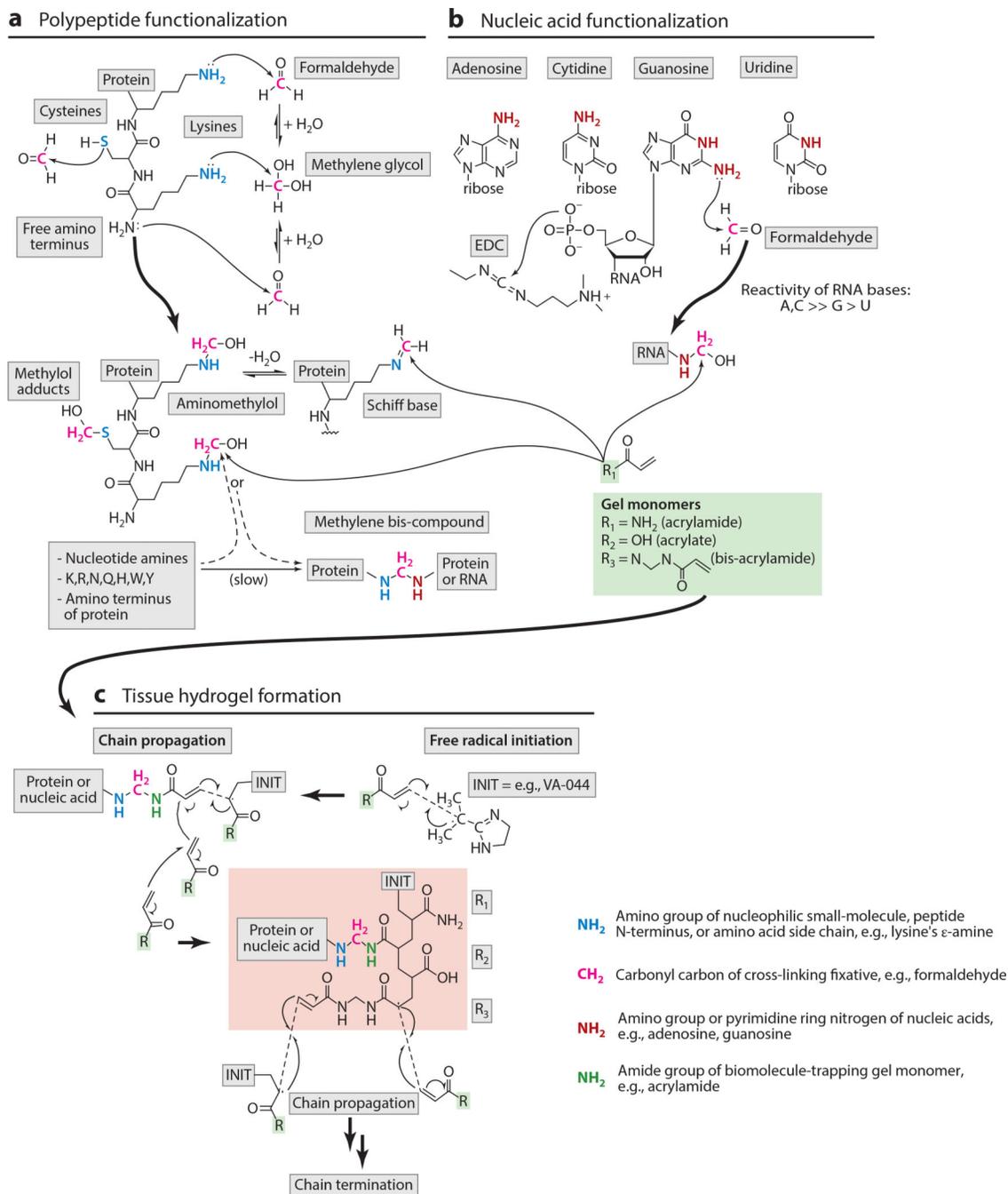
**CH<sub>2</sub>** Carbonyl carbon of cross-linking fixative, e.g., formaldehyde

**NH<sub>2</sub>** Amino group or pyrimidine ring nitrogen of nucleic acids, e.g., adenosine, guanosine

**NH<sub>2</sub>** Amide group of biomolecule-trapping gel monomer, e.g., acrylamide

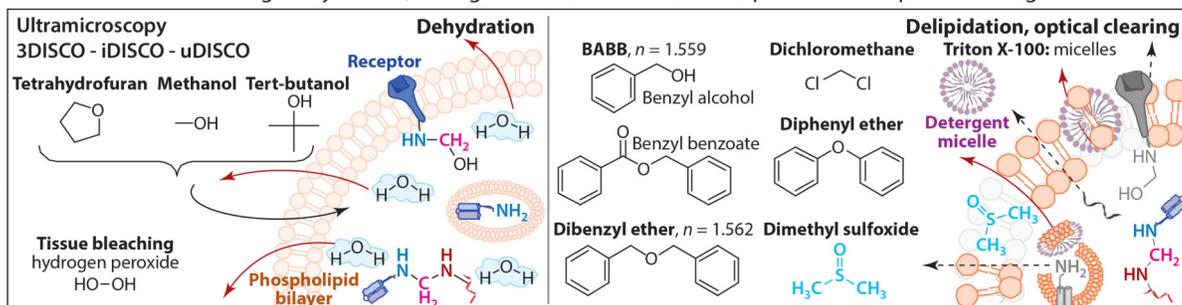
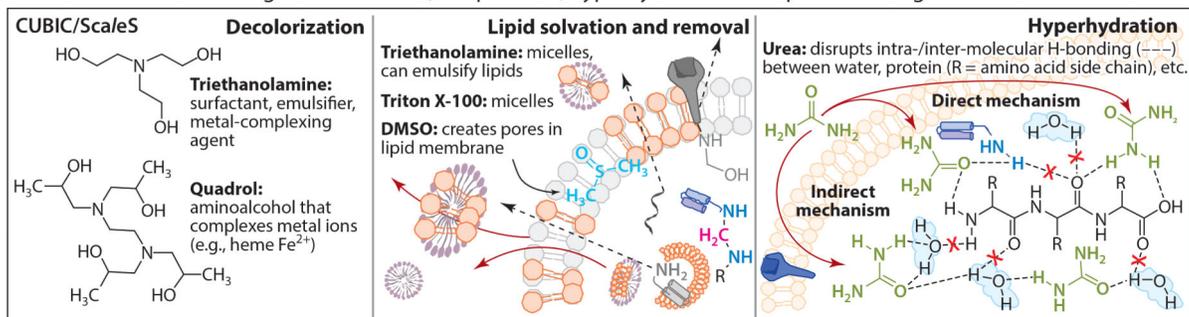
**Figure 1.** Hydrogel-tissue chemistry (HTC) steps toward tissue functionalization. The example shown (initial HTC formulation) is termed CLARITY (16). The main steps for transformation of the tissue, as shown for (a) the diagrammed cell prior to the HTC process, include (b) hydrogel-monomer infusion followed by cross-linking to native macromolecules and then gelation (*dashed black lines* denote newly formed C-C bonds to the hydrogel lattice, which in turn is shown as *wavy green lines*). Details of the chemistry are shown in Figure 2. For all figures, the color code for tracking source of C and N atoms is as follows: blue N(H)=protein-derived amine moiety; magenta C(H)=formaldehyde-derived carbon moiety; red N(H)=nucleic acid-derived amine moiety; and dark green=amide of exogenous gel

monomer (e.g., acrylamide). (c) The delipidation process is shown after the anchoring of proteins directly to the new hydrogel lattice; nucleic acid anchoring is chiefly indirect via protein bridges, anchoring via EDC (1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide) for linking the 5-phosphate group to surrounding amines (125) (Figure 2), or polymer entanglement. Stringent delipidation with detergent can then proceed without the risk of extensive biomolecule loss or structural disruption. (d) Optical clearing, refractive index matching, and high-resolution volumetric imaging (through delipidized tissue) can now occur for reading out molecules replotted onto the new tissue-hydrogel coordinate system shown in green (23).



**Figure 2.** Example functionalization chemistry. Most current hydrogel-tissue chemistry (HTC) protocols include a preliminary biomolecule fixation step, such as aldehyde-based cross-linking of (a) proteins, peptides, and small-molecule amines and/or (b) nucleic acids, including targeted coupling of nucleic acids to the matrix via EDC (16, 125). (c) Biological macromolecule retention is next enhanced via creation and conjugation to (for example) an acrylamide-bisacrylamide gel matrix. Note that direct aromatic amine coupling of the RNA with aldehyde shown is expected to be a minor reaction compared to coupling reactions with

protein aminomethylol moieties and compared to noncovalent caging of extensively crosslinked and protein-bound RNA in the hydrogel matrix. Depicted here are certain reactions as designed, but as Feldman pointed out 45 years ago, “The use of nucleic acid reactions with formaldehyde has outstripped our knowledge of their mode of action” (34, p. 2), and the same could be said of many modern tissue-based chemistries. A fundamental theme, however, is a gel monomer (green box, in this case showing three well-defined demonstrated R-moiety variants with the R1 acrylamide common to many current formulations) and the resulting tissue-hydrogel scaffold (here peach box, showing a representative HTC structure) into which the biological system is transformed; this provides the new coordinate system for replotting and jointly working with functionalized biomolecules stably in 3D space. Abbreviations: EDC, 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide; INIT, free radical initiator.

**a** Other forms of clearing: dehydration, detergent- and solvent-based delipidation and optical clearing**b** Other forms of clearing: decolorization, delipidation, hyperhydration and optical clearing

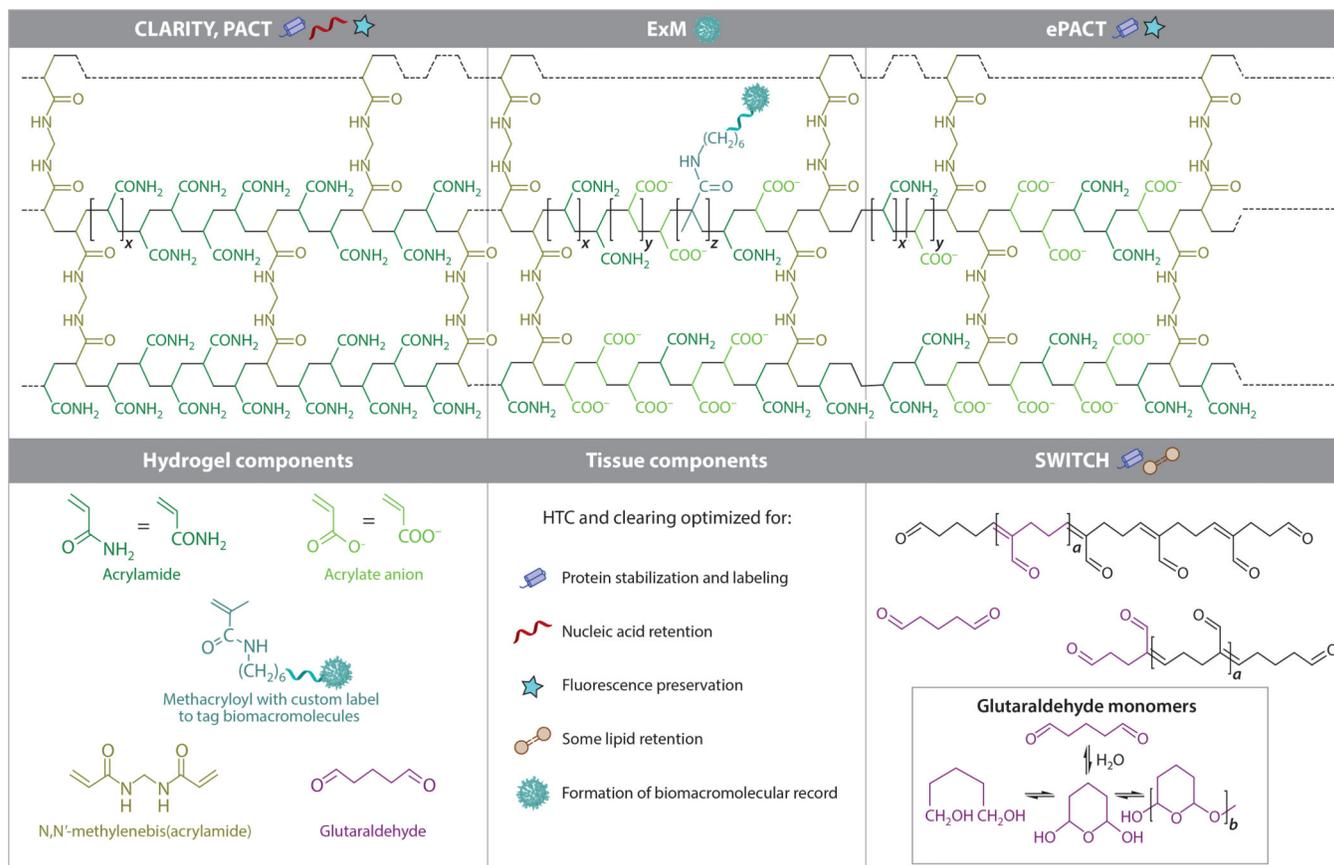
$\text{NH}_2$  Amino group of nucleophilic small-molecule, peptide N-terminus, or amino acid side chain, e.g., lysine's  $\epsilon$ -amine

$\text{NH}_2$  Amino group or pyrimidine ring nitrogen of nucleic acids, e.g., adenosine, guanosine

$\text{CH}_2$  Carbonyl carbon of cross-linking fixative, e.g., formaldehyde

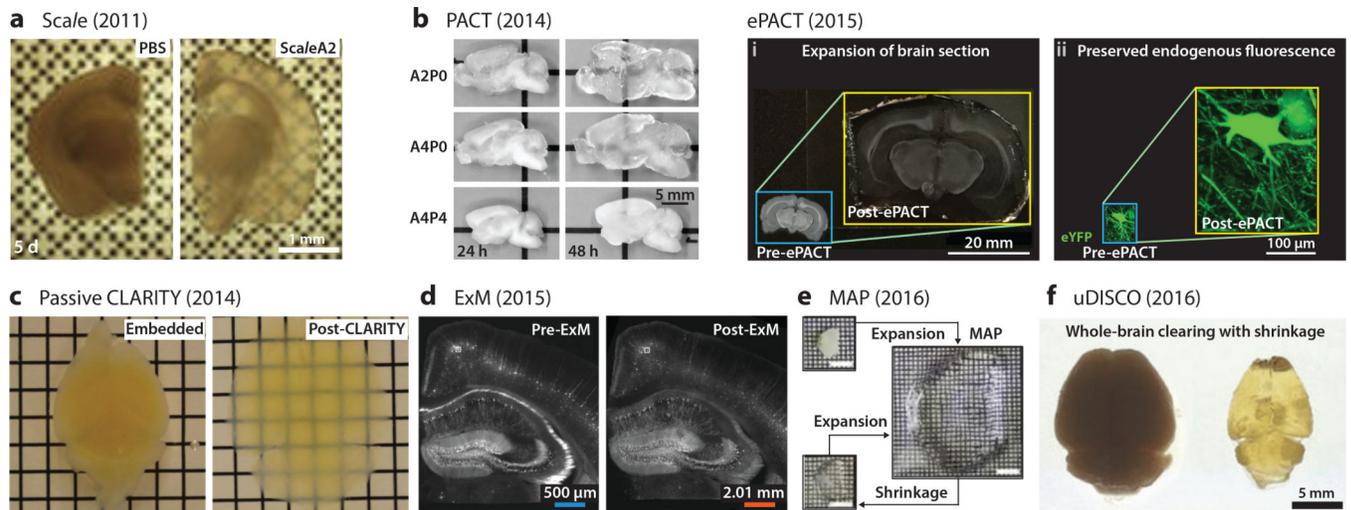
**Figure 3.**

Non-hydrogel approaches for optical access to tissue. Beyond the hydrogel-tissue chemistry (HTC) concept, distinct transparency methods have been reported on the basis of various combinations of organic solvent-based dehydration and delipidation, or of hyperhydration-based optical clearing after less stringent permeabilization and delipidation steps. Unlike HTC constructs, these are all generally limited to optical imaging as the next and final step, rather than specifically enabling additional chemistry. The color code for tracking source of C and N atoms is as follows: blue N(H)=protein-derived amine moiety, magenta C(H)=formaldehyde-derived carbon moiety, red N(H)=nucleic acid-derived amine moiety. (a) Organic solvent-based clearing (dehydration, lipid removal, and refractive index matching) methods include BABB/ultramicroscopy (31), 3DISCO (33), iDISCO (107), FluoClearBABB (113), uDISCO (99), RetroDISCO (150), CRISTAL (57), and ethanol/ethyl cinnamate (61). (b) Aqueous-based clearing (refractive index matching, with optional hyperhydration and lipid removal) methods include: Scale and ScaleS (45, 46), SeeDB (56), CUBIC (65, 77, 123, 124, 126), 2,2'-thiodiethanol (TDE) (4, 18), FRUIT (49), ClearSee (66), acrylamide-free CLARITY (68, 81), sorbitol/sucrose/ fructose (144), and single-cell optical clearing (21). Abbreviations: 3DISCO, 3-dimensional imaging of solvent-cleared organs; BABB, benzylalcohol/benzyl benzoate; CRISTAL, curing resin-infiltrated sample for transparent analysis with light; CUBIC, clear, unobstructed brain imaging cocktails and computational analysis; DMSO, dimethylsulfoxide; iDISCO, immunolabeling-enabled 3-DISCO; SeeDB, See Deep Brain; uDISCO, ultimate DISCO.

**Figure 4.**

Hydrogel-tissue hybrid backbone concepts. Hydrogel-tissue chemistry (HTC) structures involve integration of native biomolecules as part of the hydrogel framework as shown in Figures 1 and 2; for clarity on HTC subtypes, shown here are only the designs for exogenous chemical-derived backbones, while a fuller perspective with details on integration of native biomolecules appears as Supplemental Figure 1. HTC backbone formulations (a selected subset shown) allow customizable biological macromolecule anchoring and functionalization within a variety of frameworks. Molecular design of the initial backbone (*top left*; 16) and some of the subsequent early variants (*top middle, top right, bottom right*) are shown; color-coded backbone constituents are shown at lower left, and symbols for design properties of different methods are shown at bottom middle. The chemical backbone of the hydrogel built within the cells (*top left*) interacts with tissue elements through two principal routes: (i) covalent integration of amine-containing and otherwise functionalized proteins, nucleic acids, and small biomolecules; and (ii) noncovalent trapping of bulky moieties, such as extensively cross-linked protein networks within the hydrogel matrix. As with the initial HTC design, subsequent HTC variants are similarly capable of preserving molecular information during tissue processing through physically securing tissue macromolecules within the hydrogel or through recording their cellular location using custom labels that can withstand processing steps. Among the notable formulations, ExM (11), ePACT (133), and MAP (64) incorporate acrylate alongside acrylamide (as R2 of the R1, R2, and R3 moieties shown in Figure 2) into the basic HTC formulation to further

enhance expansion. Bisacrylamide was not described in the initial PACT paper but can be included in the PACT formulation. SWITCH (94) gelation forgoes paraformaldehyde/acrylamide in favor of glutaraldehyde, a dialdehyde fixative that confers more robust protein cross-linking and some fixation of amine-containing phospholipids. EDC-CLARITY (125) provides a dedicated covalent-linkage pathway for more robust mRNA cross-linking to the tissue-hydrogel matrix. Other properties of interest seen with different variants include increased rigidity (seen with SWITCH) or conversely increased size-flexibility [as seen with PACT (142), ExM (11), ePACT (133), and with other methods not shown, including proExM (127), ExFISH (12), iExM (10), and MAP (64); Supplemental Figure 1]. Tissue components are critical constituents of the HTC backbone, as shown in more detail in Supplemental Figure 1; for example, in the top-row HTC methods, native proteins (with multiple reactive amines) can support cross-linking as well as bisacrylamide (R3 moiety of Figure 2) does. Moreover, native amines play a crucial role in catalyzing glutaraldehyde polymerization in the bottom right SWITCH method, in which low pH is used to initially slow down polymerization as glutaraldehyde is exposed to tissue amines until gel formation is desired. Abbreviations: EDC, 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide; ePACT, expansion passive CLARITY technique; ExFISH, expansion fluorescent in situ hybridization; ExM, expansion microscopy; iExM, iterative expansion microscopy; MAP, magnified analysis of the proteome; PACT, passive CLARITY technique; proExM, protein-retention expansion microscopy; SWITCH, system-wide control of interaction time and kinetics of chemicals.



**Figure 5.**

Examples of topology-preserving size changes. Size-adjustable tissue matrices can facilitate visualization of tissue architecture at the cellular or molecular level. The degree of intrinsic tissue-hydrogel expansion may be enhanced via the inclusion of yet more absorbent hydrogel monomers in the hydrogel-tissue chemistry (HTC) gel formulation. Expansion was seen with (a) the non-HTC method Sca/e in 2011 (46), (b) two HTC-based passive CLARITY techniques that preserve endogenous fluorescence during expansion: PACT in 2014 (142) and expansion-PACT (ePACT) in 2015 (133), and several additional HTC methods: (c) passive-CLARITY in 2014 (131), (d) expansion microscopy (ExM) in 2015 (11), and (e) magnified analysis of the proteome (MAP) in 2016 (64). By contrast, (f) the non-HTC organic solvent-based method uDISCO shrinks tissue (99). In (b) PACT/ePACT, mild enzymatic digestion followed by delipidation and hydration of the tissue-hydrogel matrix allows fourfold expansion with the preservation of biomolecular content and native fluorescence via expansion inversely proportional to the degree of hydrogel cross-linking; matrices of 2% acrylamide and no paraformaldehyde (A2P0) exhibited greatest expansion over delipidation (133). HTC methods have progressively achieved greater expansion of the tissue-hydrogel matrix [magnified analysis of the proteome (MAP): 5-fold expansion; iterative expansion microscopy (iExM): 20-fold expansion] via modified HTC, stringent temperature degradation of the tissue matrix, and/or alternate enzymatic digestion steps. However, increased hydrogel-tissue hybrid expansion for visualization tends to come at the cost of lost biomolecular content and reduced capability for ongoing functionalization, as well as reduced practicality for large-volume imaging. Images adapted as follows: (a) figure 1 of fiamma et al. (46); (b) figure 1 of Yang et al. (142), supplemental figure 4 of Treweek et al. (133) and figure 2 of Treweek & Gradinaru (134); (c) supplemental figure 1 of Tomer et al. (131); (d) figure 3 of Chen et al. (11); (e) figure 1 of Ku et al. (64); (f) figure 1 of Pan et al. (99). Additional abbreviation: eYFP, enhanced yellow fluorescent protein.