Extracting structural and functional features of widely distributed biological circuits with single cell resolution via tissue clearing and delivery vectors
Jennifer Brooke Treweek and Viviana Gradinaru

The scientific community has learned a great deal from imaging small and naturally transparent organisms such as nematodes and zebrafish. The consequences of genetic mutations on their organ development and survival can be visualized easily and with high-throughput at the organism-wide scale. In contrast, three-dimensional information is less accessible in mammalian subjects because the heterogeneity of light-scattering tissue elements renders their organs opaque. Likewise, genetically labeling desired circuits across mammalian bodies is prohibitively slow and costly via the transgenic route. Emerging breakthroughs in viral vector engineering, genome editing tools, and tissue clearing can render larger opaque organisms genetically tractable and transparent for whole-organ-cell phenotyping, tract tracing and imaging at depth.

Address
Division of Biology and Biological Engineering, California Institute of Technology, Pasadena, CA, USA

Corresponding author: Gradinaru, Viviana (viviana@caltech.edu)

Introduction — The case for a hermeneutic approach to biological investigation
From slime mold to the rhesus macaque, countless species have contributed to our current understanding of the biological processes that grant life. The optimum animal model for a line of research is often determined by a particular anatomical feature that makes the organism uniquely suitable for experimentation. For example, although the giant squid may seem an unusual choice to further understanding of mammalian neural circuits, the sheer size and slow conduction velocity of its axons enabled scientists to study neuronal firing with the rudimentary electrophysiological techniques available during the early 20th century [1], giving rise to the field of modern cellular neuroscience. By examining individual aspects of a diverse range of organisms in great detail, scientists have been able to amass a set of unifying principles for the field of neural sciences [2]. The route to this understanding parallels the hermeneutic circle, a classic concept in theology and logic [3]. In hermeneutics, the process of interpretation follows a spiraling path in which one first studies the overall body, then examines its composite parts, and lastly revisits the concept of the whole body as a sum of the parts. Similarly, in neuroscience, observation of a particular sensory or motor system in an organism leads to investigation of the cellular underpinnings of the related circuits, which are then placed in the larger context of the central and peripheral nervous systems.

Applying this approach to investigations of molecular and cellular physiology in health and disease can be both technologically challenging and time-consuming in mammalian subjects. Mammalian tissues can be easily photographed at the macroscopic level, and then the organs and tissues can be dissected and thinly sliced for microscopic analysis. However, the process of aligning these two different perspectives to reconstruct a whole-organism map with subcellular resolution remains nontrivial [4]. Without a clear methodology for integrating microscale and macroscale views, it is difficult to apply newly-discovered molecular mechanisms to systems-level questions and to recognize how systems-level findings may in turn inform novel hypotheses on molecular processes.

Two recent technical advances can bridge the divide between cellular and systems-level studies (Table 1). First, improved viral-vector-based strategies can deliver cargo, such as fluorescent labels, efficiently and with cell specificity over entire organs or the whole body (Figure 1). This enables tracing of, for example, wide-coverage brain networks or peripheral nerves ([5**,6,7,8,9–13,14**], reviews: [15–20]). Second, optimized tissue-clearing methodologies (Tables 2 and 3, with key terminology defined in Box 1) can map intact local and long-range circuits [21*,22**,23,24,25,26–31,32**,33,34,35**,36,37,38**,39,40**,41**]. The former merges two powerful biological techniques: the use of genetically encoded tools for studying cellular function and connectivity, and the development of viral vectors as a vehicle for delivering these tools into cells [10,11,13,19,42–52,53**,54*]. The latter illustrates how the century-old technique [55,56] of tissue clearing may gain renewed importance when it is refined to
incorporate current advances in microscopy [27,29**, 57–61], genetically encoded fluorescent labeling tools [7,8,14**, 62**, 63, 64*, 65, 66], protein affinity tags [67–71], and tissue-binding size-adjustable polymeric scaffolding [22**, 29**, 32**, 37, 41**, 72**] (for review: [73]). This brief review will highlight recent work on generating adeno-associated viruses (AAVs) with unique properties via specialized viral-vector screening methods [53**, 74–80] (Figure 1, Box 1), and on modern tissue-clearing methodologies that preserve fluorescence and support high-resolution imaging at depth (Figure 2, Table 2, and Box 1; the following protocols generally achieve both goals: [21**, 22**, 23, 24, 25**, 28–31, 32**, 35**, 36, 37, 39**, 40**, 41**, 81–83]).

### Table 1

**Anticipated biomedical applications of modern clearing techniques**

<table>
<thead>
<tr>
<th>Application areas</th>
<th>Cleared tissue and complementary technologies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assessing biodistribution of chemicals or biologicals; screening compound libraries [53**, 91, 145–151]</td>
<td>Whole-body clearing a of rodents (embryo through aged adult; see Figure 1)</td>
</tr>
</tbody>
</table>
| Labeling and imaging through dense, complex tissues [153–160]; mapping discrete cellular niches (e.g., stem cells, tumors) [161–167] | Specialized clearing of bone (PACT-deCAL [41**]), BABB [27,108], tissue biopsies a and excised organs的整体清除方法在显微镜下[27,29**,57–61]，通过基因编码的荧光标记工具[7,8,14**，62**,63,64*,65,66]，蛋白质亲和标签[67–71]，以及组织绑定的大小可调的聚合物骨架[22**,29**，32**，37,41**，72**](对回顾: [73])。本文将概述近年来在腺相关病毒(AAVs)中开发具有独特性质的方法，特别是针对病毒载体筛选方法[53**，74–80](图1，盒1)，以及现代组织清除技术，这些方法可以在一定程度上保留荧光并支持高分辨率成像深度(图2，表2，以及盒1；以下的协议通常可以同时实现这两个目标: [21**，22**，23，24，25**，28–31，32**，35**，36，37，39**，40**，41**，81–83])。

**Scientific motivation for broad coverage gene delivery and imaging of whole intact tissues in mammals by tissue clearing**

Although viral vectors are commonly used for delivering genetically encoded cargo to mammalian cells in vivo, therefore avoiding slow and costly transgenic means, few are capable of both safe and efficient transduction of specific cellular targets. Fewer still are capable of broad coverage across all cellular connectivity under study. For example, AAVs are widely used, especially in nondividing cells, due to their safety [78,84–86]; however, the handful of serotypes available cannot efficiently and specifically target many populations of interest. Past and ongoing efforts on engineering viral vectors with desired properties [79,87,88], including cell-type and/or organ specificity [74–89], will greatly benefit research in tissue mapping, gene therapy, and genome editing [90]. To contribute to and complement these efforts we have recently developed an in vivo Cre-REcombination-based AAV Targeted Evolution (CREATE) selection platform for identifying AAVs that more efficiently transduce genetically defined cell populations (Figure 1a) [53**]. We used CREATE to identify variants from a systemically delivered AAV capsid library that cross the blood-brain barrier and transduce neurons and astrocytes brain-wide. Using this method, we identified one variant, AAV-PHP.B, that achieves 40-fold to 90-fold more efficient brain-wide transduction than the current standard, AAV9 (Figure 1d) [91]. AAV-PHP.B transduces most neuronal types and glia throughout the brain, which supports its use to deliver multicolor labels to genetically defined circuits for

---

**Note**: Novel AAV capsid: AAV-PHP.B was named in honor of Caltech Professor Paul H. Patterson (1943–2014).
Current viral labeling and tissue clearing techniques

Treweek and Gradinaru

Figure 1

(a) In vivo selection for viral capsids with desirable properties

1 - create capsid library
2 - deliver library in vivo to select for capsid sequences that target region/cells of interest
3 - recover capsid sequences from cells of interest
4a - repeat selection with recovered sequences

Ex vivo selection via whole-body clearing

(b) Before Clearing - After Clearing
1 - lungs
2 - heart
3 - liver
4 - kidney
5 - pancreas
6 - ovaries
7 - intestine
8 - brain

4b - characterize tropism of individual capsids using PARS

3 - recover capsid sequences from cells of interest

Concept for an in vivo selection technology for panning large-scale libraries to identify compounds or biologicals with optimized physiological properties. Within this framework, whole-body tissue clearing can facilitate biodistribution mapping. For example, to engineer viral vectors for more effective transgene delivery, one strategy involves exposing live cells or whole-organisms to AAV capsid libraries and then identifying...
mapping distributed networks, such as those recruited by deep brain stimulation [92–97]. Furthermore, engineered vectors that label discrete cell populations could be put to immediate use, for example in elucidating points of contact between major somatic sensory nerves and the CNS, or in mapping the autonomic motor branch of the PNS to better understand metabolic and endocrine disorders.

Despite these and other labeling technologies, it remains difficult to create maps for phenotypically distinct fine axons that run in bundles throughout the brain when the traditional method involves sectioning the tissue into paper-thin slices, imaging each slice, and recovering the 3D perspective with imaging software: it is slow, tedious, costly, and error prone. Over the past decade there has been a surge in methods for increasing the transparency of thick mammalian tissue samples and whole organs so that they may be examined intact (Table 2). Here, the scientific value of fluorescence-preserving tissue clearing for vector engineering is also apparent: the ability to process major organs simultaneously and without sectioning (Figure 1b and c) will greatly facilitate transduction mapping of systemically delivered genes (Figure 1d), including small-molecule tags and fluorescent labels.

**Whole-organ and whole-body tissue-clearing methodologies**

The original century-old tissue-clearing techniques [55,56] enable deep imaging into tissue without physical sectioning, but the harsh organic solvents damage cellular architecture and are incompatible with modern immunolabeling and fluorescence microscopy tools. Thus, the application of modern labeling technologies to *ex vivo* mapping studies requires new developments to render tissues transparent while also stabilizing critical macromolecules and preserving endogenous fluorescence (see Table 2 for a comprehensive list of major clearing protocols of the last decade). In particular, the CLARITY technique provides a method to further stabilize samples by anchoring tissue components in place using an interpolating hydrogel scaffold [22**]. This transparent, tissue-binding hydrogel mesh secures proteins and nucleic acids into place without causing epitable masking and allows scientists to visualize intact organs at the subcellular [22**,29**,32**,41**,98**] and even single transcript scale [32**]. Recently, a method to clear whole adult rodents and organs emerged from the realization that hydrogel monomers as well as clearing detergents and immunolabeling reagents could all be infused throughout the intact post-mortem organism using the intrinsic circulatory system (vasculature) (Figures 1b–c and 2a–b) [32**,41**]. PARS (Perfusion-assisted Agent Release in Situ) achieves both delipidation and labeling rapidly in the intact post-mortem organism via perfusion. The PARS approach, which has also proven compatible with a variety of tissue-clearing reagents [257,31,39], can prepare transparent whole organisms for imaging at depth and aligns with a paradigm shift in biomedical research. Namely, efforts to profile the two-dimensional molecular content of samples have been superseded by more comprehensive inquiries into the relationship between an organ’s volumetric composition and its resulting biological function [99].

Across most disciplines and within a variety of laboratory settings, it has become increasingly relevant to engage in the fine-scale phenotyping of whole specimens, whether of intact samples, such as tissue biopsies, excised organs and cultured organs in a dish [100,101], or of whole organisms. Thus, tissue clearing methods must be simple, economical and adaptable to a variety of applications to be adopted across scientific fields. Toward this goal, we developed a set of hydrogel-embedding and delipidation protocols that can be used to rapidly clear excised organs individually or all organs simultaneously within the intact body without compromising cellular architecture or endogenous fluorescence (for experimental timeline and details, see Tables 2 and 3; for troubleshooting advice, see: http://www.nature.com/nprot/journal/v10/n11/fig_tab/nprot.2015.122_T5.html) [32**,41**]. PACT (PAssive CLARITY Technique) and PARS entail hybridizing the tissue sample to polymers in order to anchor proteins and nucleic acids during detergent-mediated lipid extraction and to preserve gross tissue architecture during all tissue-processing steps (Figures 1b–c and 2a–b) [32**,41**]. While many clearing protocols are successful at removing lipids through detergent treatment alone, we have found that the porous structure of the tissue–hydrogel matrix, particularly when coupled to the driving force of detergent perfusion, facilitates rapid diffusion of solubilized lipids out of the tissue and the subsequent penetration of immunolabels into the remaining tissue–hydrogel matrix. Furthermore, the utility of tissue–hydrogel hybridization and detergent perfusion extends...
<table>
<thead>
<tr>
<th>Motivation</th>
<th>Method Variations</th>
<th>Pretreatment</th>
<th>Clearing reagents</th>
<th>Size change</th>
<th>Fluorescence</th>
<th>IHC</th>
</tr>
</thead>
<tbody>
<tr>
<td>To achieve transparency rapidly using organic solvents of high RI (precursors: Spalteholz [53], BABB [27])</td>
<td>3DISCO [28,38*,105*], iDISCO [39,113]</td>
<td>4% SDS ETC delipidation</td>
<td>Dichloromethane</td>
<td>Shrinkage</td>
<td>YES (1–4 d)</td>
<td>Limited</td>
</tr>
<tr>
<td></td>
<td>uDISCO</td>
<td>4% SDS ETC delipidation</td>
<td>Dichloromethane</td>
<td>Shrinkage</td>
<td>YES (1–4 d)</td>
<td>YES</td>
</tr>
<tr>
<td>To obtain a fluorescence-compatible solvent alternative via hyper-hydration of the delipidized sample</td>
<td>Sca/eA2-U2 [23]</td>
<td>n/a</td>
<td>4 M urea, 10–30% glycerol hyperhydration</td>
<td>Expansion</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td></td>
<td>Sca/eS (AbScale, ChemScale, ScaleSQ [40**])</td>
<td>n/a</td>
<td>2.7–9.1 M urea, 20–40% sorbitol, ≤5.0% Triton X-100, DMSO</td>
<td>NO</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td></td>
<td>CUBIC [25*], with decolorization [35*,39]</td>
<td>n/a</td>
<td>25% urea–50% sucrose aminoalcohol decolorization</td>
<td>Reversible expansion</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>To stabilize sample structure, macromolecular content, and fluorescent labeling using size adjustable, tissue-binding hydrogels</td>
<td>CLARITY [22**,37,62*,112]</td>
<td>4% SDS ETC delipidation</td>
<td>Tissue-hydrogel</td>
<td>YES</td>
<td>YES*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CLARITY variations [29,30,98**,111,112]</td>
<td>4% SDS ETC</td>
<td>4% SDS ETC</td>
<td>Reversible expansion</td>
<td>YES</td>
<td>YES*</td>
</tr>
<tr>
<td></td>
<td>Stochastic Electrotransport [81]</td>
<td>4% SDS diffusion</td>
<td>4% SDS diffusion</td>
<td>YES</td>
<td>YES</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PARS [32**,41**]</td>
<td>4% SDS stochastic ETC</td>
<td>4% SDS stochastic ETC</td>
<td>YES</td>
<td>YES</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PACT [32**,41**]</td>
<td>8% SDS perfusion</td>
<td>Tissue-hydrogel</td>
<td>Minimal expansion</td>
<td>YES</td>
<td>YES*</td>
</tr>
<tr>
<td></td>
<td>PACT-deCAL</td>
<td>8% SDS diffusion</td>
<td>Tissue-hydrogel</td>
<td>Controllable expansion</td>
<td>NO</td>
<td>YES</td>
</tr>
<tr>
<td></td>
<td>ExM [72**]</td>
<td>8% SDS diffusion</td>
<td>Superabsorbent hydrogel</td>
<td>NO</td>
<td>YES</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ePACT [41**]</td>
<td>EDTA/EGTA decalcification</td>
<td>Glutaraldehyde-tissue gel</td>
<td>4–5× expansion</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td></td>
<td>SWITCH [206]</td>
<td>Enzymatic digestion</td>
<td>± clearing</td>
<td>NO</td>
<td>YES</td>
<td></td>
</tr>
<tr>
<td>To preserve body-brain connections and accelerate adult whole-organ preparation via clearing in situ</td>
<td>PARS</td>
<td>8% SDS perfusion</td>
<td>Tissue-hydrogel</td>
<td>Minimal expansion</td>
<td>YES</td>
<td>YES*</td>
</tr>
<tr>
<td></td>
<td>Perfusion CUBIC [39]</td>
<td>CUBIC reagent perfusion</td>
<td>n/a</td>
<td>YES</td>
<td>YES</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Perfusion FRUIT [31]</td>
<td>FRUIT reagent perfusion</td>
<td>n/a</td>
<td>n/d</td>
<td>NO</td>
<td></td>
</tr>
</tbody>
</table>

* Sample preparation aside from standard fixation and brief post-fixation (e.g., transcardial perfusion with 4% PFA).
* Chemically/mechanically removing tissue macromolecular components (e.g. lipids, heme) to improve light probe penetration and reduce light scattering.
* Fluorescence preservation varies with fluorophore and user technique. A binary classification: YES denotes overall preservation of endogenous fluorescence, with any signal dimming mild; NO denotes major or complete endogenous fluorescence quenching due to reagent or procedural incompatibility with common fluorophores; if rapid signal decay, timeline for imaging in *’. ExM [72**] does not preserve endogenous fluorescence — however, updated protocols do (unpublished, personal communication with Prof. Boyden). Also of note, moderate loss of fluorescence in CUBIC, PACT, and SeeDB tissues, and major quenching in 3DISCO tissues has been reported elsewhere [40**].
* YES, NO: compatible, incompatible with IHC and fluorescent labeling in thick samples and with multiple fluorophores; Limited: IHC possible with small-molecule stains and some antibodies, restrictions in immunofluorescence and/or deep antibody penetration; in perfusion-clearing, YES denotes perfusion-delivery of IHC reagents.
* Personal communication with Dr. Erturk: μDISCO allows preservation of endogenous fluorescence in fine processes for several weeks to months.
* Abbreviations: n/p = not published, n/d = not determined, n/a = not applicable; see Box 1 for complete list.
* mRNA preservation and detection also demonstrated in these clearing methods (PACT [32**], CLARITY [98**]).
beyond their capacity to facilitate rodent organ and whole-body clearing and immunolabeling [32**,41**]. For example, hydrogel embedding effectively stabilizes amorphous or fragile samples [41**], such as sputum (unpublished results), for processing and secures microorganisms to sites of infection. This latter property has proven valuable in studying bacterial colonization (personal communication with Prof. Dianne Newman). Although originally demonstrated in whole rodents, perfusion-based methods may also render large, excised samples such as primate and pig organs transparent via the reirculation of PARS reagents through catheterized organ vasculature [102], an undertaking which would be prohibitively slow via passive immersion-based clearing. Finally, after initial lipid extraction and/or solvation steps (Table 2, chemical clearing), most tissue-clearing protocols employ a refractive-index homogenization step to minimize differences in light deflection by the heterogeneous tissue biomolecules (Table 3, optical clearing). This is most commonly accomplished via immersing chemically cleared samples in a solution of matched refractive index, such as RIMS (Figure 2a) [32**,41**]; polyol and concentrated sugar or sugar alcohol solutions such as glycerol [22**,29**], sorbitol [32**,40**], sucrose [25**,35**,39] and fructose [21*,103]; organic solvents such as BABB [26,27,82,104] and dibenzyl ether [26,28,36,38**,105]; and others (see Table 3).

For example, tissue clearing allows pathologists to map tumor cells in whole human biopsies and postmortem samples [28,32**,33]. Likewise, the ability to conduct fast whole-body clearing, as granted by the perfusive force in the PARS methodology, opens new avenues for exploring small-molecule biodistribution, examining viral-vector tropism (Figure 1), and tracing peripheral nerve networks to their target organs (Table 1) [31,32**,39**,41**]. For difficult-to-clear bone samples, PACT with decalcification (PACT-deCAL [41**]), SeeDB [21*,106], 3DISCO [28], Murray’s clear (1:2 Benzyl Alcohol: Benzyl Benzoate; BABB [27,104]) and other decalcification methods [107] can facilitate mapping the three-dimensional architecture of skeletal tissue and stem cell niches [108]. Regardless of the clearing protocol used, all carry trade-offs in terms of the degree of optical transparency achieved, the maintenance of endogenous fluorescence, the preservation of cellular integrity, and the permeability of cleared tissue to macromolecule labels. Although clearing with organic solvents or with electrophoresis may deliver more rapid and effective clearing than passive methods involving immersion in aqueous solutions, these harsher methods may also hinder fluorescent imaging, prove incompatible with immunolabeling, or risk tissue damage (see Table 2). In selecting a specific protocol or merging aspects of multiple protocols [109], researchers must consider their project objectives (e.g., imaging sparse epitopes, whole-body tract tracing) and experimental constraints (e.g., integration with smFISH or electron microscopy studies on cleared samples). Thus, this comprehensive list of

---

Table 3

<table>
<thead>
<tr>
<th>Sample mounting for enhanced optical clarity.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method (^a)</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>Organic solvents</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Amides</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Polyol and concentrated</td>
</tr>
<tr>
<td>sugar or sugar alcohol solutions</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Aqueous contrast media</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Aqueous mounting media</td>
</tr>
</tbody>
</table>

\(^a\) Optical clarity, or reduced light scattering through tissue, may be enhanced via homogenizing the refractive indices throughout heterogeneous tissues and at all material interfaces between the sample and objective lens.

\(^b\) Y/N denotes that rapid signal decay may necessitate prompt imaging upon sample mounting.

\(^c\) Net size change depends on tissue type and degree of tissue-hydrogel cross-linking [22**,29**,32**,41**].
available protocols (Tables 2 and 3) will serve most experimental needs (Table 1).

**Toward structure–function mapping with tissue clearing**

For tissue-clearing methods to reach their full potential, several major challenges must first be tackled. First, **imaging**: large tissue volumes require specialized microscopy; second, **data analysis**: meaningful data must be extracted from terabyte data sets; third, **access to functional information**: markers of activity must be preserved during tissue processing.

Regarding the extraction of functional information, a record of neuronal activity can be encoded via transcriptional or biochemical changes. For example, immediately-gene activation (e.g., through *Targeted Recombination in Active Populations (TRAP)* [110–113]), Ca²⁺ influx [62*,63], and voltage spikes [114*] can all be detected by genetically encoded fluorescing sensors (for reviews, see [115–118]). An exciting possibility for resolving neuronal activation across longer timescales is to pair this *in vivo* activity sensing with *ex vivo* analysis of previously active cell circuits using thick-tissue clearing. Specifically, genetically encoded stable fluorescent markers can permanently tag living cells that respond to time-restricted stimuli so that their chemical identity and connectivity can be probed post-mortem. One such marker, CaMPARI (calcium-modulated photoactivatable ratiometric integrator of neuronal activity), grants persistent quantitative detection of any neuronal activity that occurs during subsecond application of the photoconversion light [64*]. One can envision the combined use of CaMPARI with subsequent tissue-clearing methods that preserve endogenous fluorescence (such as Scale/S [40*] clearing and RIMS incubation [32*–41*]) to map the activity of intact biological networks in response to behaviorally relevant stimuli.
As we learn more about the transcriptional correlates of neuronal activity, quantitative and multiplexed RNA detection in intact tissue could also serve the role of extracting functional proxies from deceased tissue. Combinatorial labeling (barcoding) via single-molecule fluorescence in situ hybridization (smFISH) [119,120] allows for simultaneous detection of mRNA transcripts for multiple genes within individual cells [121,122**]. Importantly, both HCR-based transcriptional analysis [123–125] and smFISH [32**,121,122**] have been validated in thick sections, wherein tissue clearing and the controlled swelling of tissue-hydrogels can improve single-transcript resolution through reducing background and physically separating single-molecule labels. Further enlargement of the optical space within a cell, either for fluorescencely barcoding multiple transcripts or for examining single-cell morphology, may be achieved through recent protocols (e.g., ExM [72**], ePACT [41**]) that expand tissue 4-fold or more with the possibility to retain endogenous fluorescence (Figure 2c and d for ePACT; [41**]). Combined, these evolving technologies raise the possibility of single-cell transcriptomics with preserved spatial information. By applying high-resolution microscopy [58–60,126] to the detection of mRNA-binding probes (e.g., single-molecule hybridization chain reaction (smHCR) probes with high signal-to-noise [123–125]) in cleared and, if needed, expanded tissue, scientists will be able to achieve more robust single-molecule RNA detection.

Figure 2

Clearing techniques that enable high-resolution, volumetric imaging of tissue architecture and cellular morphology. Whole-body hydrogel embedding and detergent-based clearing via the PARS-CLARITY method [22**,32**,41**] preserve gross tissue structure (a) and fine neuronal processes (b) alike, while the purposeful expansion of these tissue-hydrogel hybrids via water absorption (c) allows the visualization of subcellular detail via either native fluorescence [41**] (d), or probes for protein and nucleic acid detection [32**]. ePACT permits the clearing and 4-fold expansion of 100 μm thick coronal brain sections with preservation of tissue shape, cellular morphology and native fluorescence. Source: (a) and (b) adapted from [32**], and (c) and (d) adapted from [41**].
and hence quantitative data for transcriptional profiling of intact circuits across organs [121,122**].

**Outlook**

Studies in naturally transparent organisms have recently progressed to real-time monitoring of neuronal activity during controlled behavior via light-gated and light-emitting tools [114*,127]. Although the protocols for tissue stabilization and lipid removal described above can produce samples with sufficient transparency for intact tissue imaging and rapid tissue phenotyping, these methods are limited to ex vivo use. Transparent or not, deceased tissue can offer only a static picture of neuronal connections. Even with a connectome in hand as a road-map for cellular networks, we would still be far from understanding the brain. For example, neuropeptides and hormones can act at a distance disregarding explicit wiring [128–130], parallel pathways within a network can result in degeneracy in circuit function [131], and apparent structural connectivity (e.g., as elucidated via GRASP [132]) does not imply active synaptic connectivity [133]. A crucial next step will involve registering the three-dimensional information obtained through tissue-clearing with either ex vivo or in vivo cellular activity mapping. Compatible with cleared tissue imaging, methods such as TRAP [110–113] and smFISH [32**,121] enable the permanent tagging of recently active cells in thick tissues. This snapshot only captures a single time point, however. What remains to be developed is a method for time-stamping signaling events across bulk cell populations such that the time-varying metabolic information from a single-cell’s lifetime can be retrieved and cross-correlated to the metabolic records of all neighboring cells. To this end, single-cell transcriptomics [134,135] and ‘molecular ticker tapes’ (i.e., an engineered DNA polymerase mis-incorporates nucleotides into a DNA ‘ticker tape’ based on spikes in ion concentration [136–138]) represent two areas of promise.

A second approach under development aims to bring the CLARITY concept to living tissue. Namely, instead of altering tissue to reduce light-scattering, scientists are recruiting the power of ultrasound focusing at depth to deliver and collect light noninvasively from living tissue. Methods such as Time-Reversal Ultrasound-Encoded (TRUE) focusing aim to correct the light-wavefront in scattering tissue [141] and currently enable focusing at depth within ex vivo tissue [139,142]. Because of its high sensitivity to motion, challenges remain in using TRUE for noninvasive deep-tissue imaging and light delivery in vivo [140]. One possibility is to combine advances in optical imaging, such as TRUE, with the application of gentle tissue clearing reagents in vivo to decrease autofluorescent background and homogenize the refractive index [143,144].

Bringing ‘clarity’ to living tissue, when combined with developments in labeling, imaging, and computation, will enable mapping of anatomical and functional connectivity and will illuminate the workings of intact circuits with high temporal precision. Although whole-body imaging is still a nascent technology, analysis of the resulting volumetric datasets will convey a level of scientific understanding that cannot be replicated in a two-dimensional context. Akin to previous work in the nematode and zebrafish, large-scale tissue clearing represents a first step toward a hermeneutic approach to mammalian biology.

**Conflict of interest**

California Institute of Technology filed intellectual property for some of the technologies described with authors as inventors.

**Acknowledgements**

We thank members of the Long Cai, Viviana Gradinaru, Dianne Newman, and Changhui Yang groups at Caltech for useful discussions. Dr. Gradinaru is a Heritage Principal Investigator supported by the Heritage Medical Research Institute and a Pew Scholar in the Biomedical Sciences, supported by the Pew Charitable Trusts. This work was funded by grants to VG: the NIH Director’s New Innovator IDP, 5D10OD017582-01, PECASE, and NIH/NIA 1R01AG047664-01; as well as by funding support from the Beckman Institute for the Resource Center on CLARITY, Optogenetics, and Vector Engineering for technology development and broad dissemination (http://www.beckmaninstitute.caltech.edu/clover.shtml).

**References and recommended reading**

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest

A ‘GPS’ for the brain. From the lab that brought brainbow comes a technique for mapping the location of axonal branches of many individual neurons simultaneously and at the resolution of individual axons.


The INTRSECT (INTronic Recombinase Sites Enabling Combinatorial Targeting) strategy grants the conditional labelling of discrete neuron populations and circuits using viral vectors with specialized trafficking properties and taking advantage of available recombinase tools. Importantly, INTRSECT can replicate numerous Boolean logical operations within the same subject, allowing researchers to control multiple genetically and/or connectivity-defined pathways.


21. Ke M-T, Fujimoto S, Imai T: SeeDB: a simple and morphology-preserving optical clearing agent for neuronal circuit reconstruction. Nat Neurosci 2013, 16:1154-1161. A passive clearing method based on immersion in aqueous fructose solutions of high refractive index, SeeDB minimizes tissue deformation and is faster than comparable methods (e.g., ScaleA2), although complete tissue transparency for deep imaging can be difficult to achieve, and the ability to immobilize thick tissues is limited.


An extension of ScaleA2 that provides faster clearing via partial lipid extraction and hyperhydration of remaining tissue components.


This Nature Protocol describes the design and build of a CLARITY-optimized light-sheet microscope (COLM) to rapidly image thick tissue volumes, as well as detailed protocols for electrophoresis-based CLARITY and passive CLARITY — the later is gentle on the tissue while preserving the key advantages of efficient clearing and labeling, with preserved endogenous fluorescence.


32. Yang B, Treweek JB, Kulkarni RP, Deverman BE, Chen CK, ** Lubec B, Shah S, Cai L, Gradinaru V: Single-cell phenotyping within transparent tissue through whole-body clearing. Cell 2014, 158:945-958. First study to realize whole body clearing and labeling in adult rodents via perfusion through vasculature (PARS) and to show RNA detection in cleared tissue and with hydrogel formulations that can cause significant (~<2x) tissue expansion (PACT) with retained endogenous fluorescence.


First clearing method to work towards decolorizing tissue via eluting a major chromophore — heme — during clearing.


An overview of how to apply the 3DISCO method, which employs dehydration, delipidation and refractive index matching with organic
solvents (THF and BABE), to rapidly clear and image heavily myelinated whole-organs. Although native and immunofluorescence fades quickly, this method permits the clear visualization of fine-scale processes (spines) and nerve networks in the CNS and PNS.


A comprehensive description of the CUBIC (original CUBIC and CUBIC with decolorization), including updates to the reagent list and step-by-step protocols for CUBIC tissue clearing variations and subsequent CUBIC informatics.


This study describes multiple notable improvements to the previously reported ScaleA2 method — a clearing protocol that, while gentle on tissues, was slow, immunolabeling-limited, and resulted in incomplete transparency in large samples. ScaleS addresses many of these original limitations with case-specific methods: for rapid clearing, immunostaining and clearing, large-organ clearing, etc.


Detailed, step-wise instructions for: (1) conducting tissue clearing and labeling based on the PACT, PARS, and RIMS methodologies from Yang et al.; (2) imaging large, cleared tissues with an affordable, custom-built light-sheet microscope; and (3) handling and analyzing large image data files in commercial and open-source software packages. In addition, this Nature Protocols article describes novel developments to the clearing techniques, including designated procedures for PACT-based expansion-clearing (ePACT), for PACT-based bone-clearing via a decalcification step, and for the integration of PACT with standard histological methods for quenching autofluorescence.


Introduces a novel selection platform for identifying adeno-associated viruses (AAVs) that can be delivered systematically and more efficiently transduce genetically defined cell populations. The methodology (CRE-ATE) eliminates the need for lengthy selection steps traditionally used in panned capsid libraries. As proof-of-principle of the CREATE platform, the isolation of an engineered AAV variant, PHP.B, capable of crossing the blood-brain barrier and conferring efficient CNS transduction is described.


A depiction of how the monosynaptic retrograde spread of glycoprotein (G)-deleted rabies virus can achieve improved cell-type specificity for circuit tracing through EnvA pseudotyping, crossing Cre driver lines with Cre-dependent TATA-expressing mice, and cell-specifically delivering the G transgene using Cre-dependent AAV.


A paper reporting on the generation of ultrasensitive genetically encoded calcium sensors, GCaMP6 variants with slow, medium, or fast kinetics, that can be used to accurately follow neuronal activity long-term in vivo. Variants displayed similar baseline fluorescence as previous GCaMP sensors, but markedly improved dynamic range. The sensitivity of GCaMP6 allows for the detection of single action potentials as well as activity in individual synaptic compartments (e.g., transients in dendrites).


Report of a neuronal activity sensor-CaMPARI, based on Ca2+-binding proteins calmodulin (CaM) and the photocleavable fluorescent protein mEos2, that switches from its green-fluorescing to red fluorescent state only when it is simultaneously illuminated with violet light and exposed to spiking calcium levels.

An approach to gaining super-resolution imaging without super-resolution microscopy that recruits tissue-hydrogel formulations to expand tissue so that subcellular features are sized above the diffraction limit (ExM). 4--5-fold tissue swelling is granted through modifying a tissue-embedding hydrogel formulation to contain a superabsorber—sodium acrylate—in addition to the acrylamide monomers and bisacrylamide crosslinker used in CLARITY. Expansion significantly beyond CLARITY or PACT hydrogels is conferred by proteinase K digestion and water absorption. This version of ExM does not preserve endogenous fluorescence—however, updated protocols do (unpublished, personal communication with Prof. Ed Boyden).


A comprehensive review on the fundamentals of light interactions with biological matter and the tissue clearing field.


103. Ke MT, Imai T: Optical clearing of fixed brain samples using SeeDB. Curr Protoc Neurosci 2014, 66 Unit 2.22.


A detailed video on conducting 3DISCO-based tissue clearing, which provides an informative, user-friendly follow-up to the Nature Protocol on the 3DISCO clearing technique.


Describes Archer, an engineered genetically encoded voltage indicator (GEVI) based on Archaeorhodopsin-3 (Arch), a microbial rhodopsin proton pump, with improved baseline fluorescence and dynamic range. Together, these properties permitted the detection of neuronal activity in behaving C. elegans.


Expanding upon previous work (E. Lubeck and L. Cai.) using super-resolution microscopy to resolve a large number of mRNAs in single cells (emFISH), this correspondence highlights a sequential barcoding scheme to multiplex the detection and quantification of mRNA abundance for different mRNAs. Of note, these methods are compatible with mRNA labeling and detection in FACT-cleared thick tissues (Yang et al. and Shah et al., 2016, http://dx.doi.org/10.1242/dev.138560).


