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Extracting structural and functional features of widely distributed biological circuits with single cell resolution via tissue clearing and delivery vectors

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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.

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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

Table 1

Anticipated biomedical applications of modern clearing techniques

Application areas	Cleared tissue and complementary technologies
Assessing biodistribution of chemicals or biologicals; screening compound libraries [53••,91,145–151]	Whole-body clearing[a] of rodents (embryo through aged adult; see Figure 1) Excised whole-organ delipidation through major blood vessels in larger mammalian subjects (e.g., pigs, nonhuman primates) [102,152] CREATE platform for viral vector screening in cleared samples (Figure 1) [53••]
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[b] and excised organs Whole-body perfusion-clearing[a] in disease models
3D-tracing long-range fiber bundles (e.g., vagus nerve); lineage-mapping in neurodevelopment [168]	Viral or transgenic labeling technologies (e.g. Brainbow [7], TRIO [6], Confetti [169], MAGIC markers [170]; Box 1) followed by whole-body clearing
Monitoring the progression of cell death and tissue damage (e.g., stroke, infarcts), and the corresponding re-oxygenation [171,172]	Whole-organ or whole-body clearing Perfusion labeling to counterstain intact vasculature and surrounding tissues
Tracking nerve/axon regeneration and de/re-myelination; examining neuroplasticity at the synaptic level [28,173–180]	Whole-body perfusion-clearing[a] and perfusion-labeling, and bone-clearing for the vertebral column Co-registration of array tomography [181,182], light and electron microscopy datasets [8•,73,160,183–187]
Spectrally resolving subcellular labels (e.g., single molecule transcripts) within native tissue	Multiplexed labeling and/or sequential barcoding with FISH [119–121] and HCR [98••,125]; or neuronal positioning system (NPS) [8•] Hydrogel-embedding and expansion-clearing (ExM [72••], ePACT [41••])
Exploring topics in parasitology [188] and microbiology (e.g., biofilm formation, microbe distribution within a niche [189–192], host interaction with the microbiome [64•,193])	Hydrogel-embedding of fragile samples,[b] followed by gentle, passive whole-organ clearing to maintain bacterial colonization
Extending the imaging depth range and resolution for optical coherence tomography [194–197] and photoacoustic tomography [140–142,144,198–205]	Future prospects for optically clearing living tissue with optical and/or contrast clearing reagents (e.g., varying ratios of PEG-400, DMSO, and/or glycerol)

[a] For whole-body clearing and perfusion-labeling methods, see [31,32••,39•,41••], with detailed methods on PARS and perfusion-CUBIC in their respective *Nature Protocols*.
[b] For further advice, see troubleshooting instructions at http://www.nature.com/nprot/journal/v10/n11/fig_tab/nprot.2015.122_T5.html [41••].

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]).

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* *C*re-*RE*combination-based *A*AV *T*argeted *E*volution (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,[1] 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

[1] Novel AAV capsid: AAV-PHP.B was named in honor of Caltech Professor Paul H. Patterson (1943-2014).