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Light-microscopy mapping of connections
in the intact brain

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Mapping neural connectivity across the mammalian brain is a daunting and exciting prospect. Current approaches can be divided into three classes: macroscale, focusing on coarse inter-regional connectivity; mesoscale, involving finer focus on neurons and projections; and microscale, reconstructing full details of all synaptic contacts. It remains to be determined how to bridge the datasets or insights from the different levels of study. Here we review recent light microscopy-based approaches that may help to integrate across scales.

In neuroscience there has been recent debate regarding the potential value of, and methodologies for, full delineation of brainwide connectivity (the connectome). For this challenge, multiple approaches may be taken that operate at different spatial scales. The connectome at the macroscale involves visualizing distinct brain regions, correlated activity patterns, and putative connecting pathways [1]. While this approach holds the unique advantage of applicability to living human brains for capturing higher-order cognitive and affective processes, macroscale connectomics cannot directly resolve neural cell bodies and axonal fibers. At the other end of the spatial scale, electron microscopy (and specialized high-resolution light-microscopy methods) can visualize the connectome with synaptic resolution [2], but even with high-throughput microscopy these microscale approaches are still labor-intensive and costly, require morphological reconstruction methodologies, and are applicable to small tissue volumes.

Mesoscale connectomics bridges the gap between the two abovementioned approaches. Using neural tracers such as genetically encoded fluorescent proteins introduced by injected viruses, short- or long-range connections among distinct cell populations can be examined under a light microscope [3]. The most widespread form of this approach involves serial tissue sectioning followed by staining, imaging, and reconstruction of the 2-D image stacks into a 3-D volume. Generating thin brain sections has been necessary, due to limited penetration of photons into tissue (~150 μm below the brain surface for standard confocal microscopy and 500-800 μm for two-photon microscopy, chiefly constrained by scattering) and limited penetration of molecular probes into tissue (usually, less than 50 μm-thick thin tissues are used for this reason, but see [4] for developments in enhancing antibody penetration). Recent efforts to increase throughput have focused on automation of tissue sectioning and serial block-face microscopy (in some cases with molecular phenotyping) [5,6]. Ongoing challenges associated with many sectioning methods include obtaining precise alignment at the axonal level, achieving efficient and reconstruction of volumetric information, and avoiding damage due to mechanical disruption.

In a distinct approach, passive clearing techniques have emerged to render brain tissue transparent and thereby bypass the challenges associated with sectioning and reconstruction of the 3-D volume (Table 1) [5,6]. One approach is to reduce variations in refractive index (RI), and hence light scattering, by replacing water (RI=1.33) in the tissue with organic solvents that match the refractive index of membrane lipids (RI~1.5). Such experiments date back many decades [8]; a recent realization of this idea involved dehydrating the tissue with ethanol and subsequently incubation in high refractive-index organic solvents such as BABB (a mixture of benzyl-alcohol and benzyl-benzoate, also called Murray's clear) [9]. However, such organic solvents rapidly

quench most fluorescent protein signals [10]. This issue was recently partially overcome with a method called 3DISCO (3-D imaging of solvent-cleared organs, which extends GFP signal half-life to 1-2 days) [11]. For obtaining imaged volumes that are small or low resolution this is not a problem, but such organic solvent-based methods are not amenable to high-resolution mapping of large tissues (e.g. whole mouse brains, or portions of primate brains) that require prolonged imaging.

To address this issue, aqueous-based clearing methods have been developed. For example, the Sca*l*e solution (a mixture of urea and glycerol) renders tissue relatively transparent while preserving fluorescent protein signals [12]. However, myelin-rich brain regions remain opaque even after incubation on the timescale of weeks to months, and lasting tissue expansion is seen [10,13–15]. The subsequent ClearT method, consisting of formamide and polyethylene glycol, shows less tissue expansion and takes one day to clear an intact mouse embryo [14], though applicability to myelinated mature brain has not been established. Another aqueous solution, SeeDB (saturated fructose in water) [10] clears rapidly without tissue expansion, and the brain can be stored in SeeDB solution up to 1 week without fluorescent quenching, but clearing large pieces of tissue such as the whole brain of adult mice was reportedly difficult without incubating samples at higher temperatures (and some accompanying fluorescence loss [10]).

These optical tissue-clearing methods enable sectioning-free imaging of intact animal brain tissues, and together with genetic labeling of subpopulations of neurons, will undoubtedly facilitate mapping of neural connectivity. But unlike the serial sectioning methods, these chemical-based passive tissue clearing techniques are not readily compatible with immunophenotyping, which has restricted utility of these powerful methods to transgenic labels in animal models. Only photons can penetrate deep into the tissue, while molecular labels (such as antibody and RNA probes) crucial for characterizing neurons and connectivity patterns cannot reach deep inside the brain.

To help address these challenges, a fundamentally distinct approach that modifies the brain to be permeable to macromolecules as well as photons has been developed [13]. This technique (CLARITY) transforms intact brains into hydrogel-tissue hybrid constructs that are mechanically stable. Biomolecules (such as nucleic acids, proteins and small neurotransmitters) are secured at their physiological location by the hydrogel-crosslinked network, but lipids that cause light scattering and constitute antibody-impermeable barriers can be removed via solubilization with ionic detergents and subsequent active transport out of the tissue with electrophoresis. After two weeks of this CLARITY treatment (clarification) an intact adult mouse brain becomes transparent without losing fluorescence signals [13], which enables imaging of genetically labeled local and long-range circuits throughout the brain, and importantly allows for diffusion of molecular probes deep into the intact tissue. For both mouse and human brain tissue, a broad range of neuronal and axonal labels can be used to visualize cell bodies and their projections. Indeed, using postmortem brain tissue from an autistic patient, deep immunolabeling and visualization/tracing of individual axonal fibers across unsectioned tissue blocks was achieved, and within the three-dimensional arborizations of parvalbumin-positive interneurons in prefrontal cortex, topologically abnormal features of dendritic morphology could be readily observed by simple inspection [13]. This

property of CLARITY could be useful for integrating information about long-range connectivity, local wiring, morphological features, and molecular identity.

However, CLARITY (as with the other methods summarized here) is a newly introduced technique that requires much improvement. While CLARITY allows immunostaining of large-scale intact tissues, passive diffusion of antibodies into dense hydrogel-tissue hybrid is still slow and therefore requires high antibody concentrations and long incubation times for multiple antibodies/wash steps (in total, on the timescale of months in the case of whole mouse brain) [13]. Molecular phenotyping of human brain tissues is even more challenging as human samples from brain banks are typically already extensively crosslinked by fixation, and also have a great deal more myelin to be cleared than the mouse brain. Using CLARITY, immunostaining of only 500 μm-thick human samples has been demonstrated, and accelerated or active transport of antibodies remains a key goal. CLARITY also requires a custom-built apparatus (the electrophoretic tissue clearing chamber) and involves many steps even before antibody staining; therefore for researchers seeking to simply optically clear a transgenically-labeled mouse brain for volume imaging, other techniques might be preferred. In contrast, for molecular phenotyping of any intact tissue, and for any non-transduced animal preparation including human brains, clarification and labeling for detailed brainwide information may be a method of choice.

Clarified and immunophenotyped brain tissue also may be subsequently subjected to electron microscopy [13] (providing a link to microscale-level synaptic contacts), or previously subjected to MRI analyses (providing a link to macroscale-level structural and functional information), thereby vertically connecting data across different levels of connectome studies. And all of the techniques described here exhibit potential for accelerating the pace of brain mapping. However, a challenge common to all approaches remains efficient and fast analysis of massive imaging datasets. Moreover, understanding brain function and dysfunction at the circuit level will require integration of dynamic patterns of activity *in vivo* (observed in behavior, and causally tested in behavior) with brain-wide structural connectivity. Cross-registration of anatomical, functional, and causal information from these emerging techniques in the setting of *in vivo* experiments will be vital, posing many challenges and opportunities for the years to come.

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Table 1. Comparison of technologies for intact-brain analysis

Technique	Principle / composition	Optical clearing in intact brain	Molecular phenotyping	Processing time for whole adult mouse brain	Tissue size and morphology	Molecular integrity	Reversibility	Storage	Complexity
Chemical transformation of tissue									
CLARITY [13]	Formation of tissue-hydrogel hybrid followed by electrophoretic tissue clearing and optical clearing	Yes, shown in whole adult mouse brain, whole adult zebrafish brain, and postmortem human brain tissue.	Antibody staining and *in situ* hybridization demonstrated.	~2 weeks	Transient, reversible tissue expansion during the process.	No fluorophore quenching observed. Lipids are lost. Not compatible with lipophilic dyes.	Not reversible. The brain tissue is chemically transformed.	Many months	Involves custom setup assembly, and many steps of experiment
Optical clearing agent									
ClearT [14]	Formamide or formamide and polyethylene glycol.	Demonstrated in whole young mouse brain (<P11), and in sections from adult mouse brain.	-	No data available for adult mouse brain. 1 day for embryonic brains.	No [14] or mild sample expansion [10].	Compatible with lipophilic dye tracing (e.g. DiI), but not compatible with fluorescent proteins [10]. ClearT2 preserves GFP signal but renders the tissue less transparent and causes expansion [14].	Reversible with PBS	Unknown, but formamide is unsuitable for long-term tissue storage [14].	Incubation in solution
Scale [12]	Urea, glycerol and triton X-100	Demonstrated in whole young mouse brain [12], though myelin-rich white matter not fully clear.	-	3 weeks (ScaleA) to 6 months (ScaleU).	Large expansion in tissue volume. Tissue becomes fragile [10,12–15].	Partial denaturation and loss of proteins by urea. Not compatible with lipophilic dyes [10,14].	Not fully reversible due to protein denaturation and tissue deformation [10].	Unknown	Incubation in solution
SeeDB [10]	Saturated aqueous fructose solution with α-thioglycerol	Demonstrated in whole young mouse brain, but reported to be difficult in adult mice [10].	-	3 days for immature brains; clearing adult brain reportedly difficult [10].	No tissue expansion or fragility reported.	No fluorescent protein quenching observed. Lipophilic dyes well preserved.	Reversible with PBS multiple times.	Up to 1 week in SeeDB. Can be stored longer after reversing in PBS.	Incubation in solution
3DISCO [11,15]	Dibenzyl ether and tetrahydrofuran	Yes.	-	2-5 days [11].	No tissue expansion reported.	No fluorescent protein quenching observed within 1 day [11]. Not compatible with lipophilic dyes nor EM due to dehydration and loss of lipids [11,15].	Not reversible. The clearing agents dehydrate tissues and dissolve lipids.	1 day (half-life of GFP signal in cleared brains is 1-2 days).	Incubation in solution