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Light microscopy mapping of connections in the intact brain

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Mapping of 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 a 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 in integration 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 visualization of distinct brain regions, correlated activity patterns, and putative connecting pathways [1]. Although this approach holds the unique advantage of applicability to living human brains for capture of 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 labor-intensive and costly, require morphological reconstruction methodologies, and are only applicable to small tissue volumes.

Mesoscale connectomics bridges the gap between the two above-mentioned approaches. Using neural tracers such as genetically encoded fluorescent proteins introduced via 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 2D image stacks into a 3D volume. Generation of thin brain sections has been necessary owing 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) [7] and limited penetration of molecular probes into tissue (tissues of < 50 μm in thickness are normally used for this reason, but see [4] for developments in enhanced 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 precise alignment at the axonal level, efficient reconstruction of volumetric information, and avoiding damage due to mechanical disruption.

In a distinct approach, passive clearing techniques have emerged in which brain tissue is rendered transparent, thereby bypassing the challenges associated with sectioning and reconstruction of the 3D 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 RI of membrane lipids (RI≈1.5). Such experiments date back many decades [8]; a recent realization of this idea involved tissue dehydration with ethanol and subsequent incubation in high-RI 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 partly overcome with a method called 3DISCO (3D imaging of solvent-cleared organs) that extends the GFP signal half-life to 1–2 days [11]. To obtain imaged volumes that are small or low resolution this is not a problem, but such organic solvent-based methods are not suitable for 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

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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	Demonstrated 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; brain tissue is chemically transformed	Many months	Involves custom set-up assembly and many experimental steps
Optical clearing agent									
ClearT [14]	Formamide or formamide and poly(ethylene 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 brain	No [14] or mild sample expansion [10]	Compatible with lipophilic dye tracing (e.g. DiI)[10] but not with fluorescent proteins; 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], although 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 owing 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	Demonstrated in whole adult mouse brain and spinal cord segments	–	2–5 days [11]	No tissue expansion reported	No fluorescent protein quenching observed within 1 day [11]; not compatible with lipophilic dyes or EM owing to dehydration and loss of lipids [11,15]	Not reversible; clearing agents dehydrate tissues and dissolve lipids	1 day (half-life of GFP signal in cleared brains is 1–2 days)	Incubation in 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 a timescale of weeks to months, and lasting tissue expansion is seen [10,13–15]. The subsequent ClearT method, consisting of formamide and poly(ethylene glycol), shows less tissue expansion and takes 1 day to clear an intact mouse embryo [14], although 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 for up to 1 week without fluorescent quenching, but clearing of large pieces of tissue such as the whole brain of adult mice is reportedly difficult without sample incubation 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. However, unlike serial sectioning methods, these chemical-based passive tissue-clearing techniques are not readily compatible with molecular phenotyping, which has restricted the utility of these powerful methods to transgenic labels in animal models. Only photons can penetrate deep into tissue, and 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 has been developed in which the brain is modified so that it is permeable to macromolecules as well as photons [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 by electrophoresis. After 2 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 were achieved, and within the 3D 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 (similar to the other methods summarized here) is a newly introduced technique that requires much improvement. Although CLARITY allows immunostaining of large-scale intact tissues, passive diffusion of antibodies into dense hydrogel–tissue hybrids is still slow and therefore requires high antibody concentrations and long incubation times for multiple antibodies and washing 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 because human samples from brain banks are typically already extensively crosslinked by fixation, and also have a great deal more myelin to be cleared than 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 preferable. By contrast, for molecular phenotyping of any intact tissue and for any non-transduced animal preparations including human brains, clarification and labeling for detailed brainwide information may be the method of choice.

Clarified and immunophenotyped brain tissue can also 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. 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 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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