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Mesoscale connectomics

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Brain cells communicate with one another via local and long-range synaptic connections. Structural connectivity is the foundation for neural function. Brain-wide connectivity can be described at macroscopic, mesoscopic and microscopic levels. The mesoscale connectome represents connections between neuronal types across different brain regions. Building a mesoscale connectome requires a detailed understanding of the cell type composition of different brain regions and the patterns of inputs and outputs that each of these cell types receives and forms, respectively. In this review, I discuss historical and contemporary tracing techniques in both anterograde and retrograde directions to map the input and output connections at population and individual cell levels, as well as imaging and network analysis approaches to build mesoscale connectomes for mammalian brains.

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The mammalian brain — including the human brain as 'the most complex piece of organized matter in the known universe' (http://www.alleninstitute.org/what-we-do/brain-science/), has millions to billions of neurons with trillions of synapses connecting them. One way the nervous system manages complexity is by grouping neurons into canonical 'types'. Neurons can be grouped into an estimate of thousands of types, each located in specific brain regions. Brain regions and neuronal types are organized into four major functional systems: sensory, motor, cognitive and state [1]. Multiple subsystems exist within each major system, forming specific circuits and subserving specific functions. To understand how diverse forms of function emerge from the brain's structural architecture, it is essential to identify the cell type components of brain circuits, determine the structural connections between those components, measure the physiological signals that pass between synaptically-coupled neurons, and precisely manipulate specific components to investigate their functional roles [2,3].

Brain connectivity can be described at at least three levels [3–5]. It is generally considered that the macroscale connectome describes inter-areal connections that can be inferred from fiber tracts using techniques such as diffusion tensor imaging (DTI). DTI is especially useful for generating macroscale connectomes from living human brains, where more invasive approaches cannot be applied [6]. The mesoscale connectome describes connections at the cellular level, between neuronal types across different brain regions. The microscale and/or nanoscale connectomes describe connections between individual neurons at the synapse level.

Microscale connectomes often rely on electron microscopy (EM) to provide the clearest evidence about the presence and location of synapses. Although whole-brain EM connectomics has been done in *C. elegans* [7–9], and is ongoing in other species such as fruit flies [10–12] or zebrafish larvae [13], the extremely high resolution as well as other experimental and computational requirements make it prohibitive to obtain an EM-based microscale connectome for a mammalian brain in the foreseeable future, and currently it is confined to the investigation of local circuits [14–18].

Mesoscale connectomes can be built using a variety of anatomical tracing approaches reviewed below at the brain-wide level. The cell type-based connections can be amenable to functional probing using the same cell type-targeting genetic strategies driving functional monitoring or manipulating tools. Mesoscale connectomes also bridge information collected at macroscale or microscale connectomic levels. Thus, mesoscale connectomics allows multiscale and structural-functional integration, and has been widely employed in neural circuit studies. In the text that follows I discuss techniques that enable important new insights at this level of abstraction. Many of the powerful genetically-based techniques have been developed in and most applicable to the mouse brain. It is our hope that at least some of these methods can be extended in the near future to other, especially primate brains.

Traditional tracers

Historically, inter-areal connections are mapped using a variety of tract tracers. These tract tracers can be made of chemical compounds, glycoproteins, radioactively tagged amino acids or fluorescently conjugated beads that can be

transported along axon fibers in either directions (see [19,20] for a comprehensive overview). Commonly-used tracers that move principally in the anterograde direction, that is, from the soma to the tips of the axons, include biotinylated dextran amine (BDA), Fluoro-ruby (FR) and *Phaseolus vulgaris*-leucoagglutinin (PHA-L). Commonly used retrograde tracers, that is, tracers principally propagate from the axon terminals of a neuron back towards its soma and/or dendrites, include cholera toxin B fragment (CTB), Fluoro-gold (FG), and retrobeads. Wheat germ agglutinin (WGA) has been used as a trans-synaptic tracer that can be genetically targeted and can cross synapses in both retrograde (i.e., from postsynaptic to presynaptic side) and anterograde (i.e., from presynaptic to postsynaptic side) directions. Although not selective for neuronal types, these traditional tracers are still widely used in circuit studies to label axonal projections from specific regions (in the anterograde direction), or specific neuronal populations defined by projection targets (in the retrograde direction), often in conjunction with the use of newer generation of recombinant virus-based tracers that are more amenable to cell type specific targeting in genetic model organisms such as the mouse.

Virus-mediated anterograde and retrograde tracing

Harnessing the power of nature, a plethora of neurotropic viruses have been modified to support a wide range of neuroscience applications including neuroanatomical connectivity mapping. Readers should refer to an excellent recent review [19] that more systematically describes all the major types of recombinant viral vectors for neuroanatomical studies. Here I focus on the most recent technical advances in viral tools for mesoscale connectivity mapping.

In anterograde tracing, cells at the viral injection site are directly infected by the virus and express genes that code for fluorescent proteins, which travel through the entire extent of axon fibers revealing the target areas to which the infected neurons project (Figure 1a). In retrograde tracing, axon terminals at the viral injection site take-up the virus; the virus is transported back to the soma from the axon terminals and then leads to expression of fluorescent proteins in the infected cell (Figure 1b). The first virus widely used for anterograde tracing was adeno-associated virus (AAV). It can express fluorescent proteins in a Cre and/or other recombinase (e.g., Flp) dependent manner using double-inverted recombination site cassettes (called DIO or FLEx) [21–23]. In addition, a suite of Brainbow AAVs have been generated that could enable simultaneous multi-color labeling and tracing [24].

AAVs have different serotypes (capsid proteins) with different degrees of neurotropism that can be utilized for cell-specific and direction-specific targeting. For example, AAVs have low rates of retrograde transport; through directed evolution, a new capsid variant rAAV2-retro was developed that enables efficient retrograde labeling [25•]. Similarly, using a cell type-specific capsid selection method, AAV variants were developed that can be delivered systemically and infect majority of neuronal types throughout the brain (AAV-PHP.B and AAV-PHP.eB, with the latter having enhanced efficiency), or infect preferentially peripheral neurons (AAV-PHP.S) [26•,27•].

Other viruses that have been used in circuit tracing studies include sindbis virus, canine adenovirus-2 (CAV-2), herpes simplex virus-1 (HSV-1), and rabies virus. Sindbis virus allows robust labeling of individual neurons and their anterograde axonal projections [28,29]. CAV-2 displays potent retrograde transport but modest transgene expression level, and CAV-2 vectors carrying Cre or Flp have been used in retrograde tracing studies [30,31,32•]. Replication-incompetent HSV-1 and rabies virus have also been used as retrograde tracers and can deliver strong transgene expression to infected neurons [19].

While AAV and CAV-2 facilitate relatively safe and stable long-term expression of proteins in host cells, sindbis virus, HSV-1 and rabies virus display cytotoxicity within days or weeks after infection. Efforts have been made to engineer new viral vectors with reduced toxicity, such as the self-inactivating rabies virus [33•] and the double-deletion mutant rabies virus RVΔGL [34•]. Both versions lead to diminished transgene expression, and thus their best use is to express Cre (or other) recombinase which does not need to be abundant to work effectively in combination with Cre-dependent transgenic lines or viral vectors for retrograde tracing. It has also been observed that different retrograde viruses, for example, rAAV2-retro, CAV-2 and RVΔGL, have differential neurotropism and do not label all input cell types equally [34•]. Thus, it will be important to compare tracing results from different retrograde viruses in order to obtain a more comprehensive picture.

Trans-synaptic tracing

The anterograde and retrograde tracing techniques described thus far enable one to map connections between cell populations and regions. To establish connections between input and recipient cell populations requires trans-synaptic (or trans-neuronal) tracing, in which the viral tracer is transported across synapses in either the anterograde or retrograde direction and label both presynaptic and postsynaptic neurons simultaneously. Monosynaptic trans-synaptic tracing, that is, the viral tracer can only jump across one step of synaptic connections, is critical to reveal directly connected cell populations.

Both rabies and pseudorabies virus (PRV) have been used for polysynaptic retrograde trans-synaptic tracing.