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Mesoscale Connectomics

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Summary

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.

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

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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, *i.e.*, 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, *i.e.*, 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 (Fig. 1a). In retrograde tracing, axons 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 (Fig. 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 [30–32*]. 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 doesn’t need to be abundant to work effectively in combination with Cre-dependent transgenic lines or viral vectors in retrograde tracing. It has also been observed that different retrograde viruses, e.g. 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 pre- and post-synaptic neurons simultaneously. Monosynaptic trans-synaptic tracing, *i.e.*, 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. Monosynaptic, retrograde trans-synaptic tracing by EnvA-pseudotyped G-deleted rabies (RVdG) is a powerful tool that has been widely used to map interconnected neurons in a variety of circuits throughout the nervous system (see [35] for an overview) (Fig. 1c). For example, as a technical tour-de-force, rabies tracing from a single starter cell has revealed exquisite details of anatomical and functional correlations among interconnected neurons in microcircuits [36–38].

Recent efforts have focused on reducing cytotoxicity and improving trans-synaptic efficiency of the rabies virus. Replacing the glycoprotein of the original SAD-B19 strain with an optimized version oG [39*], or using the G-deleted CVS-N2c strain of rabies [40*], improves efficiency of trans-synaptic spread and, in the latter case, prolongs cell health. As mentioned above, self-inactivating [33*] or double-deletion mutant [34*] rabies virus can also reduce cytotoxicity; note, however, that these forms of rabies have yet to be modified to act in a trans-synaptic manner.

In the anterograde direction, the H129 strain of HSV-1 has been used to label neurons that are postsynaptic to the infected cells, *i.e.*, as a polysynaptic anterograde trans-synaptic tracer. A Cre-dependent, polysynaptic, anterograde trans-synaptic H129 variant was developed by replacing the endogenous viral TK gene with a floxed TK expression cassette [41]. A monosynaptic, anterograde trans-synaptic version of H129 was recently developed by also deleting the endogenous TK gene and supplementing it from a helper virus [42*]. The vesicular stomatitis virus (VSV) has been engineered to express either the rabies virus glycoprotein (RV-G) or its own glycoprotein (VSV-G), which can then trans-synaptically label neuronal circuits in either retrograde or anterograde direction, respectively [43]. A drawback of these approaches is that the rapid onset of toxicity in both H129 and VSV viruses has limited their widespread use. One potential way to avoid, or overcome, this drawback is to use versions of Cre-expressing AAV1 or AAV9 that exhibit anterograde trans-synaptic spread properties [44].

Viral expression of cross-synaptic molecular tools provides another means to visualize synaptic connections between defined cell populations. The pre- and postsynaptic components of the mammalian version of GRASP (GFP-reconstitution across synaptic

partners), mGRASP [45], can be expressed in two different neuronal populations and synapses between the two populations can be revealed through reconstituted GFP. Though currently only working in fruit flies, trans-Tango is a molecular anterograde trans-synaptic mapping system in which presynaptic ligand induced postsynaptic receptor activation is converted into reporter expression in postsynaptic cells through site-specific proteolysis [46*].

Combinatorial and functional circuit mapping

The array of anterograde, retrograde and trans-synaptic viral tools can be combined, and sometimes coupled with transgenic lines, to enable even more sophisticated circuit mapping approaches (for a comprehensive overview see [47]). For example, combining a retrograde tracer (e.g., CAV2-Cre) with an anterograde tracer (e.g., Cre-dependent AAV) can assess target-defined projection specificity [48] (Fig. 1d). The ‘tracing the relationship between input and output’ (TRIO) method combines CAV2-Cre with Cre-dependent rabies tracing, or CAV2-FLEX-Flp with Flp-dependent rabies (cTRIO), and enables trans-synaptic input tracing from specific subsets of neurons based on their projection and/or cell type [32*] (Fig. 1e). In a similar way, double retrograde and single-cell trans-synaptic tracing from visual cortex to dorsolateral geniculate nucleus to retina allowed examination of information encoding in directly connected circuit pathway across three distinct structures [49*].

Combining tracing with *in vivo* functional imaging (*e.g.*, wide-field, one- or two-photon calcium imaging, fiber photometry, or fMRI) allows mapping of circuit dynamics in behaving animals. Circuit components activated during behavior can also be mapped through immediate early gene (IEG) activation [50]. Optogenetic or chemogenetic perturbation can further probe behavioral or functional consequences of activation/inactivation of the specific circuit [47]. Optogenetic activation of the axon terminals of presynaptic neurons while recording from potential postsynaptic neurons in the target area, in methods like subcellular channelrhodopsin-assisted circuit mapping (sCRACM) [51], allows establishment of pre- and postsynaptic connections and further investigation of the strengths and other functional properties of such connections.

Imaging, image registration and data analysis

To facilitate standardized data generation and comparison, and to enable the creation of brain-wide connectomic maps, it is important to establish standardized imaging platforms. Given that mesoscale connectomes are largely fluorescence-based datasets, high-throughput fluorescence imaging platforms have been deployed, including section-based epifluorescence slide scanners and whole-brain serial two-photon (STP) tomography [52–55]. To enable more efficient whole-brain imaging, many tissue clearing methods have been developed (see [56] for an overview), including CLARITY [57], expansion microscopy (ExM) [58], iDISCO [59], etc. Related imaging techniques are also being developed, such as long working-distance objectives with high refractive index matching the clearing agent. Light-sheet microscopy is well suited for imaging large-volume cleared tissues at a much faster rate, albeit with lower spatial resolution, potentially enabling processing of much larger number of samples [60*,61]. Some clearing methods, *e.g.*, iDISCO [59], CLARITY

[62], ExM [63], and ExFISH [64], are compatible with antibody or mRNA labeling, further enriching data content and potentially allowing determination of cell type identity of mapped neurons.

The mammalian brain can be parcellated into hundreds of distinct anatomical regions, each expected to contain a unique set of cell types. To generate a meaningful connectivity map, the anatomical location of labeled cells and their inputs/outputs need to be accurately determined. Tissues with anatomical context, e.g. having counterstaining or intrinsic background fluorescence, are more readily amenable for anatomical annotation, whereas cleared tissues present a much greater challenge. To facilitate efficient annotation of the large numbers of datasets needed for comprehensive coverage, and especially to facilitate comparison across these datasets, co-registration of all the individual datasets into a common reference space is highly desirable. Commonly used mouse or rat reference atlases include the Paxinos' [65,66] and the Allen Reference Atlas [67]. By co-aligning 1,675 individual STP tomography whole-brain datasets from the Allen Mouse Brain Connectivity Atlas, a mesoscale connectome for the mouse brain [53], an average template brain was generated, from which a new 3-D reference atlas, a Common Coordinate Framework (CCF), was built (Fig. 1g). The current version, CCF v3, consists of 43 cortical areas with their associated layers, 330 subcortical gray matter areas, 82 fiber tracts, and 8 ventricle and associated structure volumes, all delineated natively in 3-D (http://atlas.brain-map.org/). Different labs have developed methods to register and map a variety of whole-brain multi-plane datasets into this CCF, including aMAP [68], ClearMap [60*], qBrain [69*], and WholeBrain [70*]. These approaches dramatically facilitate qualitative and quantitative comparison across circuit mapping datasets for the adult mouse.

Systematic analysis of the large-scale, mesoscale connectomics datasets requires signal segmentation in addition to registration with parcellated reference atlases described above. A raw connectivity matrix between source and target areas can then be generated from the quantitative signal strengths (Fig. 1h). Statistical or graph theory-based network analysis can be performed to identify network characteristics and organization, such as node degree, clustering, modularity and centrality [71]. Computational models can be built from structural connectivity to predict function and generate hypotheses [72,73].

Cell type characterization and development of cell type targeting tools

Mesoscale connectomics requires a solid foundation of cell types across all brain regions. However, our understanding of cell types in the brain remains far from complete. Large-scale efforts are underway to systematically characterize brain cell types in the mouse at transcriptomic, morphological and physiological levels [74]. Integrating these multi-dimensional properties of individual cells is essential for deriving a unified cell type taxonomy [75].

Single-cell transcriptomic profiling is powerful as it provides the genetic identity of cell types along with marker genes [76]. Targeting these marker genes to make driver lines provides genetic access to novel cell types, which can then be used for connectivity mapping and other functional studies. Single-cell epigenomic profiling [77,78] promises to identify

cell type specific enhancer elements, or more broadly cis-regulatory modules (CRMs), useful for targeting specific cell types. If these CRMs can be utilized in viral vectors, as shown in the case of GABAergic neuron specific mDlx enhancer [79], cell type specific targeting will become much more efficient and can also be applied to other genetically less accessible species.

Transcriptomic profiles can also be used for cell type identification in mesoscale connectomic studies through spatial transcriptomic approaches [80], such as multiplexed fluorescence in situ hybridization (FISH). This is particularly relevant for retrograde and trans-synaptic tracing studies where relevant input or output cells are only revealed after tracing; post hoc multiplexed FISH would then be useful to obtain their cell type identities.

Single cell projectome and barcode connectomics

Connectivity itself is also likely to be an important criterion for definition of a cell type. At the single cell level, the full extent of the dendritic and axonal morphology of a neuron can be considered a partial surrogate for its connectivity, as it contains substantial, though not all, input/output connectional information. In fact, it was the neuronal morphologies that Cajal used to define a ‘neuron’ and the polarity of its information flow, and to discover neuronal diversity [81]. However, our understanding of single neuron morphologies, especially for projection neurons with their axons extending much beyond the local area their somata reside in, is still extremely limited, hindering our progress in understanding cell types. Thus, it is critical to incorporate into mesoscale connectomes the single cell morphologies or single cell axonal projections throughout the entire brain, which can be properly named as a single cell projectome (Fig. 1f).

Recent development of high-throughput fluorescence microscopy such as the MouseLight [82*] and the fMOST [83] systems allows imaging of whole mouse brains at sub-micron resolutions. The imaging technologies combined with genetic sparse labeling methods can generate high-resolution whole brain datasets that enable the reconstruction of full morphologies of single neurons, already revealing rich diversity. A major challenge now is to develop automatic or semi-automatic reconstruction algorithms that will substantially reduce the time-consuming morphology reconstruction efforts. Obtaining a large-scale single cell projectome dataset that spans all regions of the brain will transform our understanding of neuronal diversity and connectional specificity.

An alternative approach is barcode connectomics [84], in which barcoded DNA sequences are incorporated into viral vectors for projection/connectivity mapping. In the MAPseq method [85*], using a barcoded sindbis virus library, each virally infected neuron expresses a unique barcode and each neuron’s projection pattern can be distinguished from that of other neurons through sequencing of the barcodes in micro-dissected brain tissues. Since thousands of neurons can be uniquely barcoded simultaneously within the same brain, this approach can potentially map the axonal projection patterns from a large number of neurons simultaneously in a highly efficient manner.

Outlook

Recent technological advances in virus engineering, imaging and genomics are rapidly transforming the landscape of connectomics and enabling us to conduct comprehensive and multi-faceted structure-function studies of brain-wide neuronal networks. I expect that mesoscale connectomics will be dramatically accelerated by our increased understanding of cell types in the brain, and through the mutually synergistic interaction between cell type characterization and connectomics, uncovering underlying principles for both is within sight.

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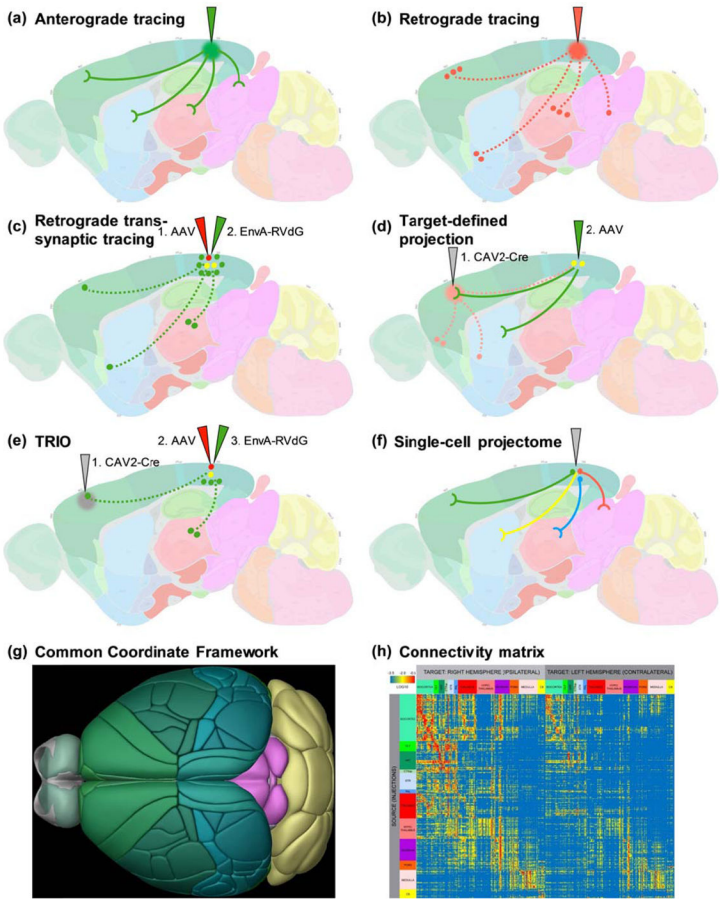

Figure 1.

Representative approaches for mesoscale connectomics. (**a**) In anterograde tracing, traditional or viral anterograde tracer is injected in the source region of interest and tracer-labeled long-range axonal projections and terminals in other parts of the brain are mapped. (**b**) In retrograde tracing, traditional or viral retrograde tracer is injected in the target region of interest and tracer-labeled somata or nuclei of input neurons in other parts of the brain are mapped. Dotted lines indicate that axon fibers of input neurons may or may not be labeled by the tracer. (**c**) In the most common scenario of monosynaptic, retrograde trans-synaptic tracing, AAV helper virus(es) (red) expressing TVA and RV-G proteins under Cre control is first injected into the target region of interest of a Cre-driver-containing animal, and then EnvA-pseudotyped, G-deleted rabies virus (RVdG) (green) is injected into the same region. Only neurons infected with both AAV and rabies virus (called starter cells, which are turned from red to yellow) will produce rabies viral particles that will be transported across synapses and label the somata of presynaptic input neurons (green cells) both locally and in the long distance. (**d**) In target-defined projection mapping, CAV2-Cre (or another retrograde virus, gray) is first injected into a target region of interest, and it will be taken up retrogradely by neurons projecting to the target region (pale red paths and cells); then a Cre-dependent AAV is injected into one of the source regions of interest, and it will label only the neurons (yellow) projecting to the initial target region as they contain CAV2-derived Cre. The axonal projections of these target-defined neurons will then be visualized (green fibers).

Pale red indicates that CAV2-Cre infected cells may or may not be visualized, depending on whether the animal contains a Cre-reporter or not. (**e**) In TRIO (tracing the relationship between input and output), two steps of retrograde tracing are involved. CAV2-Cre (or another retrograde virus, gray) is first injected into a target region of interest, and it will be taken up retrogradely by neurons projecting to the target region. Then a (set of) Cre-dependent AAV helper virus(es) is injected into one of the source regions of interest, to express TVA and RV-G proteins only in the neurons (red) projecting to the initial target region as they contain CAV2-derived Cre. Finally, EnvA-pseudotyped, G-deleted rabies virus (RVdG) (green) is injected into the same source region. Only neurons infected with CAV2-Cre, AAV and rabies virus (note these are target-defined starter cells, which are turned from red to yellow) will produce rabies viral particles that will be transported across synapses and label the somata of presynaptic input neurons (green cells) both locally and in the long distance. (**f**) In single-cell projectome, individual neurons (green, red and blue) in the source region of interest are sparsely and strongly labeled by transgenic or viral expression of a fluorescent protein, and their full extent of dendritic and axonal arbors are imaged and reconstructed to reveal each neuron's unique set of projection targets (green, red and blue fibers). Sometimes two neurons with otherwise different targets may also share one or more common target(s) (as indicated by the yellow fiber). Alternatively, individual neurons can also be densely labeled by barcoded anterograde viruses, and their individual projection targets resolved through the barcodes. (**g**) The mouse brain Common Coordinate Framework (CCF) is a digital 3-D reference atlas for image data registration and anatomical delineation. (**h**) Segmented and quantified mesoscale connectomic datasets can be used to generate connectivity matrix for neural network analysis (adapted from Ref [53]).