Cell, Volume *164*

Supplemental Information

Communication in Neural Circuits:

Tools, Opportunities, and Challenges

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Table S1. Tools for Neural Circuit Mapping

Selected techniques currently available for neural circuit mapping and covering a broad range of capabilities are summarized, with attention given both to major applications/advantages (particularly in terms of characterizing IODEs) and to major caveats. The terms IDE and ODE are defined only relative to the cell population of interest; hence transsynaptic markers are particularly useful in this regard for identifying the inputs to a genetically and anatomically specified starter cell population.

Method		Connectivity Information/ Tracing Directionality	Mechanism/ Marker	Properties Checklist						Species Compatibility	Major Applications/ Advantages	Major Caveats	References
				Long-range Connections	Cell-type specificity	Trans-synaptic	Mono-synaptic Restricted	Single Cell Resolution	Synapse Visualization				
Golgi staining		Detailed local cell morphology	Silver precipitate					✔		widely compatible	• Complete neuronal morphology and fine structure (e.g. spines) are visible • Sparse labeling allows single neurons to be distinguished	• Only sparse labeling is useful • Staining can only be applied to post-fixed samples • Difficult to establish connectivity patterns (esp. long-range)	Ranjan and Mallick, 2010 (modern updates)
Dyes	**DiX Lipophilic Tracers**	Non-specific membrane tracing	Fluorescent dye (variety of wavelengths available) incorporated into cell membranes								• Compatible with tracing in post-fixed brains as well as with live tissue imaging • Efficient transport via membrane diffusion	• No cell type specificity • No directional specificity for tracing	Honig and Hume, 1989
	Dextran Amines	Some anterograde vs retrograde specificity using different mW dextrans leading to preferential uptake by cell bodies vs axons	Fluorophore, biotin or other marker conjugated to the dextran amine.								• Wide variety of marker conjugates • Variety of MWs available to help achieve directional specificity • Can be used to identify ODEs	• No cell type specificity	Reiner et al., 2000
	FluoroGold	Retrograde (axonal uptake)	UV excitation gives gold or blue emission depending on pH	✔				✔ (sparse labeling can allow single cell tracing)		widely compatible	• Efficient uptake by axons • Can be used to identify ODEs • Visualization can be enhanced by immunostraining • Compatible with EM for ultrastructural studies	• No cell type specificity • One color option • High diffusibility can make local injections difficult	Naumann et al., 2000
	Retrobeads	Retrograde (axonal uptake)	Latex microbeads used to deliver red or green fluorescent dyes								• Limited local spread of beads allows local connectivity mapping or very precise ODE tracing • Beads are trafficked quickly, yet are non-toxic, allowing a very wide range of survival times post-injection	• No cell type specificity • Punctate appearance can make cell ID difficult • No labeling of cell morphology • Less efficient axonal uptake than other options (e.g. FluoroGold)	Katz et al., 1984 Katz and Iarovici, 1990

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	Method	Connectivity Information/ Tracing Directionality	Mechanism/ Marker	Properties Checklist						Species Compatibility	Major Applications/ Advantages	Major Caveats	References
				Long-range Connections	Cell-type specificity	Trans-synaptic	Mono-synaptic Restricted	Single Cell Resolution	Synapse Visualization				
Tracer Proteins	**HRP**	Retrograde (axonal uptake)	Diaminobenzidine (DAB) reaction								• One component system • Can be used to identify ODEs	• No cell type specificity • One staining option	LaVail and LaVail, 1972
	WGA	Retrograde (transsynaptic) and anterograde (transsynaptic): specific in some contexts	Fluorophore, biotin, HRP, cre or other marker conjugated to tracer protein	✔	✔ (optionally compatible with viral/genetic technqiues for cell specificity)	✔		✔ (sparse expression can allow single cell tracing)		widely compatible	• Transsynaptic labeling • Can be used to identify IDE or ODEs	• Transsynaptic labeling is not highly efficient • Direction of transsynaptic labeling can be mixed, variable, and circuit-dependent	Schwab et al., 1978
	PHA-L	Anterograde (transsynaptic)				✔					• Transsynaptic labeling • Can be used to identify IDEs	• Transsynaptic labeling is not highly efficient • Not strictly anterograde	Gerfen and Sawchenko, 1984
	CtB	Retrograde (axonal uptake)									• Retrograde labeling • Cell type specificity possible • Can be used to identify ODEs	• Not strictly retrograde	Conte et al., 2009
	TTC	Retrograde (transsynaptic)				✔					• Transsynaptic labeling • Can be used to identify IDE or ODEs	• Transsynaptic labeling is not highly efficient • Not strictly retrograde	Kissa et al., 2002
	AAV	Anterograde (axon tracing) Can also be used to express transsynaptic markers	XFP or cre expressed by virus	✔	✔	✔ (if encoded virus is engineered to express a transsynaptic tracer protein)		✔ (sparse expression can allow single cell tracing		mammals	• Versatile, relatively non-toxic package for delivery of numerous tracing components • Allows cell type specificity using specific promoters or when combined with recombinase expression strategies • Can be used to identify IDEs (e.g. when combined with transsynaptic tracer proteins) and/or ODEs (e.g. via axon tracing)	• Packaging size limited to ~5 kB • Inconsistent reports of retrograde transport, may require batch-by-batch characterization	Betley and Sternson, 2011 (review) Wang et al., 2014 (comparison with BDA) Oh et al., 2014 (Allen Mouse Connectivity Atlas)

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	Method	Connectivity Information/ Tracing Directionality	Mechanism/ Marker	Properties Checklist: Long-range Connections	Cell-type specificity	Trans-synaptic	Mono-synaptic Restricted	Single Cell Resolution	Synapse Visualization	Species Compatibility	Major Applications/ Advantages	Major Caveats	References
alpha-herpesviruses	**HSV-1**	Retrograde (transsynaptic)	XFP or cre expressed by virus	✔	✔	✔	✔ (Ba2000 variant; see References)			mammals, some evidence for fish (see References)	• Efficient retrograde viral tracer • Transsynaptic labeling • Can be used to identify IDEs or ODEs (as in Fenno et al., 2014)	• Toxicity • Careful characterization required to assure that spread is restricted to synaptically connected cells • Only particular strains are specifically retrograde	Ugolini et al., 1987 Zemanick et al., 1991 (strain specificity) LaVail et al., 1997 (strain specificity) Fenno et al., 2014 (cell-type specific approaches) Zou et al., 2014 (fish)
	PRV Bartha									non-primate mammals	• Efficient retrograde viral tracer • Transsynaptic labeling • Monosynaptic restriction may be possible • Less toxic than other HSV strains • Can be used to identify IDEs or ODEs	• Toxicity	Enquist, 2002 Ekstrand et al., 2008 De Falco et al., 2001 (Ba2001 cell-type specific strain) Callaway, 2008 (see comment on Ba2000 for monosynaptic restriction)
	H129 strain	Anterograde (transsynaptic)								mammals	• Anterograde viral tracer • Transsynaptic labeling • Can be used to identify IDEs	• Toxicity	Sun et al., 1996 Lo and Anderson, 2011 (cre-dependent cell-type specificity)
	VSV	Anterograde (transsynaptic) or retrograde (transsynaptic): Directionality is glycoprotein dependent	XFP label expressed by virus	✔	✔	✔	✔			widely compatible	• Anterograde viral tracer • Transsynaptic labeling • Can be used to identify IDEs • Can be retricted to monosynaptic labeling using G deletion • Cell type specificity using EnvA pseudotyping	• Toxicity • Poorly understood batch variability, requires careful batch-by-batch characterization (see Correction to Beier et al., 2011)	Beier et al., 2011 Mundell et al., 2015
	CAV	Retrograde (axon transducing)	cre or GFP expressed by virus	✔	✔					mammals	• Relatively non-toxic retrograde viral tracer. The lack of toxicity makes this virus particularly appealing for examining functional circuit elements in vivo. • Can be used to identify ODEs	• Not transsynaptic	Soudais et al., 2001 Junyent and Kremer, 2015 also see TRIO references

Method	Connectivity Information/ Tracing Directionality	Mechanism/ Marker	Properties Checklist						Species Compatibility	Major Applications/ Advantages	Major Caveats	References
			Long-range Connections	Cell-type specificity	Trans-synaptic	Mono-synaptic Restricted	Single Cell Resolution	Synapse Visualization				
Rabies	Retrograde (transsynaptic and axon tranducing)	XFP label expressed by virus	✔	✔ (EnvA variants; see References)	✔	✔ (G-deleted variants; see References)			mammals	• Specific, efficient retrograde viral tracer • Transsynaptic labeling • Can be used to identify IDEs or ODEs • Can be retricted to monosynaptic labeling using G deletion • Cell type specificity using EnvA pseudotyping	• Toxicity • Down-regulation of host gene expression	Wickersham et al., 2007 Callaway and Luo, 2015 (review)
TRIO/cTRIO	Retrograde (axon transducing; CAV) and Retrograde (transsynaptic; rabies): Allows three steps of a circuit to be examined	XFP label expressed by virus	✔	✔ (cTRIO variant; see References)	✔	✔			demonstrated in mice, likely compatible with other mammalian systems	• Same advantages of rabies (above) • Specification of inputs based on output target, allowing visualization of the relationship between IDEs and ODEs (IODEs)	• Toxicity • Down-regulation of host gene expression	Schwarz et al., 2015 Beier et al., 2015 Lerner et al., 2015
GRASP/mGRASP	Synaptic partners	Split GFP reconstituted at synapses	✔	✔				✔	currently optimized for worms (GRASP) and mammals (mGRASP)	• Synapse visualization from defined partners • Can be used to further characterize the fine structure of IDEs or ODEs	• Possible bias for false positives in synapse detection	Feinberg et al., 2008 Kim et al., 2011
SynView	Synaptic partners	Split GFP reconstituted at synapses	✔	✔				✔	currently optimized for mammals	• Synapse visualization from defined partners • Can be used to further characterize the fine structure of IDEs or ODEs	• Currently limited to examining synaptic contacts initiated by specific adhesion molecules	Tsetsenis et al., 2014
Brainbow	Anterograde (axon tracing)	Stochastic expression of 3 XFPs	✔	✔			✔		widely compatible - currently adapted for worms, flies, fish, mice	• Combination of single cell resolution and dense labeling is possible (up to 100s of colors) • Can be used to identify ODEs	• Imaging is a major challenge (chromatic aberrations, bleaching, etc can make analysis difficult)	Livet et al., 2007 Pan et al., 2011 (fish) Hampel et al., 2011 (flies) Hadjieconomou et al., 2011 (flies) Cai et al., 2013
Electron Microscopy	Ultrastructural cell morphology	HRP/Diaminobenzidine (DAB) reaction, electron-dense membrane contrast agents, and/or heavy metal-conjugated antibody labeling	✔ (can be combined with long-range techniques e.g. FluoroGold, GESEM)	✔ (limited multifeature immunostaining)			✔	✔ (synapses can be identified by morphology and/or limited immunostaining)	widely compatible	• The most complete picture of neuronal morphology and circuit structure is obtained • Can be used to identify or further characterize the fine structure of IDEs or ODEs	• Extensive time and cost, even for imaging very small tissue volumes	Jurrus et al., 2009 Kleinfeld et al., 2011 Ward et al., 1975 Bock et al., 2011 Briggman et al., 2011 Atasoy et al., 2014 (GESEM)

Method	Connectivity Information/ Tracing Directionality	Mechanism/ Marker	Properties Checklist						Species Compatibility	Major Applications/ Advantages	Major Caveats	References
			Long-range Connections	Cell-type specificity	Trans-synaptic	Mono-synaptic Restricted	Single Cell Resolution	Synapse Visualization				
Functional Magnetic Resonance Imaging (fMRI)	Functional connectivity	BOLD signal (correlation)	(inference by correlation, need not be direct)						Human, non-human primate, rodent	• Whole brain functional connectivity visible in a live subject • Non-invasive, compatible with human studies	• Indirect (non-anatomical) measure of connectivity precludes IODE identification • No cell type specificity	Friston, 2011 (functional and effective connectivity review) Poldrack and Farah, 2015 (recent review of human imaging methods, with a focus on fMRI)
Diffusion Weighted Imaging (DWI)	White matter tract structure	Visualization of water diffusion preferentially along white matter tracts	(inference by diffusion, need not be direct)						Human, non-human primate, rodent	• Whole brain structural pathways visible in a live subject • Non-invasive, compatible with human studies	• Resolution limited to large white matter tracts • No functional information • No cell type specificity	Le Bihan and Johansen-Berg, 2012

Table S2. Tissue Transparency Methods for Intact Analyses

Selected techniques currently available for achieving intact tissue transparency and covering a broad range of capabilities are summarized. In light of the focus of this primer, methods with demonstrated capacity to clear intact adult mouse brains are listed. We divided these published whole-brain transparency techniques into three main categories: hydrogel-based methods (e.g., CLARITY), organic methods (e.g., iDISCO/3DISCO), and aqueous non-gel methods (e.g., Scale, CUBIC). Under each general heading, we then list extensions, variations, and new directions, as well as published demonstrations of use and papers reporting biological discoveries made using these methods. N.D., not determined in the original literature as of this writing.

Tissue Transparency Method	Initial method references	Clearing mechanism	Optical quality (intact adult mouse brain)	Reversibility	Labeling				Extensions/ variations and new directions	Biological demonstrations and discoveries in the brain (beyond the initial papers)	Biological demonstrations and discoveries in non-brain tissues (beyond the initial papers)
					Protein (native fluorescence)	Protein (immunostaining)	Nucleic acid	Lipid dye			
CLARITY and hydrogel variations	Chung, 2013	Formation of a hydrophilic tissue-polymer composite, followed by aqueous solvent-based disruption and removal of unbound components such as lipids by diffusive, mechanical, thermal, electrical, or other means	Fully transparent	Irreversible gel transformation, reversible labeling and imaging	Yes	Yes	Yes	No	Passive CLARITY (Tomer 2014, Zheng 2015), PACT/PARS (Yang, 2014), COLM (Tomer, 2014), ExM (Chen, 2015a), Stochastic electrotransport (Kim, 2015), SWITCH (Murray, 2015), ACT-PRESTO (Lee, 2016), SPED (Tomer, 2015), EDC-CLARITY (Sylwestrak, 2016)	**Rodent brain:** Hsiang, 2014; Spence, 2014; Lerner, 2015; Menegas, 2015; Adhikari, 2015; Plummer, 2015; Zhang, 2014; Tomer, 2015; Unal, 2015; Sylwestrak, 2016 **Human brain:** Ando, 2014; Liu, 2015a	**Rodent:** Lung (Joshi, 2015; Saboor, 2015), Liver (Font-Burgada, 2015), Whole animals/embryo/multiple organs (Epp, 2015; Yang, 2014), Spinal cord (Zhang, 2014) **Plant:** Palmer 2015
3DISCO and hydrophobic (organic solvent) variations	Erturk, 2012	Organic solvent-based lipid removal by dehydration/rehydration and bleaching on native tissue	Fully transparent	Irreversible	Rapid quenching	Yes (especially with iDISCO)	N.D.	No	iDISCO (Reiner, 2014)	**Rodent brain:** Weber, 2014; Zapiec, 2015; Garofalo, 2015 **Human brain:** Theofilas, 2014	**Rodent:** Thymus (Ziętara et al, 2015), Skin (Maksimovic, 2014; Oshimori, 2015), Islets (Juang, 2015), Bone marrow (Acar, 2015), Lymph node (Liu, 2015c), Spinal cord (Papa, 2016; Soderblom 2015; Zhu, 2015) **Human:** Lung (Hoffmann, 2015)
Aqueous non-gel variations	Hama, 2011 (Scale) Susaki, 2014 (CUBIC)	Chemical cocktail-based lipid removal and decolorization on native tissue (also compatible with CLARITY/hydrogel variants)	Mostly transparent	Irreversible	Yes	Yes	N.D.	No	Whole body CUBIC (Tainaka, 2014); ScaleS (Hama, 2015)	**Rodent brain:** Singh 2015; Asai, 2015; Ozkan, 2015	**Rodent:** Lung (Noguchi, 2015; Peng, 2015; Jain, 2015), Heart (Machon, 2015; Chabab, 2016), Spinal cord (Hinckley, 2015), GI system (Higashiyama, 2016; Liu, 2015b), lymph node (Jafarnejad, 2015; Moalli, 2015), Whole animals/embryo (Huang, 2015; Roccaro, 2015; Hirashima, 2015; Dorr, 2015; Hartman, 2015) **Bird:** Botelho, 2015 **Xenopus:** Tsujioka, 2015 **Human:** Intestine (Clairembault, 2015)

Table S3. Activity Readouts for Functional Neural Circuit Analysis

Selected techniques currently available for achieving brain activity readouts and covering a broad range of capabilities are summarized. Three main categories are listed: electrophysiological, optical, and immediate early gene (IEG)-based. We also list fMRI as an important method for achieving whole-brain activity readouts, especially given compatibility with small mammals and optogenetics. For recent discussion of other activity readouts available for use in humans, beyond the scope of this review, see Poldrack and Farah (2015).

	Method	Species Compatibility	Compatibility with Awake Behavior	Major Applications/ Advantages	Major Caveats	References
Electrophysiological Readouts	Whole-cell in slice	primarily mice, rats	not compatible	• Experimenter control over ion concentrations • Easily controlled pharmacological manipulation • Intracellular access • Single cell resolution	• No behavioral context • Full circuits and circuit dynamics may not be preserved in slice	Walz et al., 2002 (Neuromethods textbook)
	Whole-cell in vivo	widely compatible	compatible	• Intracellular access in an intact circuit • Intracellular access during behavior • Single cell resolution	• Low throughput, technically demanding approach • Not currently compatible with behavior over days	Lee et al., 2006 Kitamura et al., 2008 ("shadow patching" of unlabeled cells) Kodandaramaiah et al., 2012 (automation) Munoz et al., 2014 (channelrhodopsin-assisted cell targeting)
	Extracellular in vivo	widely compatible	compatible	• Well-established method for monitoring neuronal activity during free behavior • Excellent temporal resolution • Multi- or single-unit recordings • Action potential collision tests can be used to establish projection targets	• Cell type identification (e.g. using juxtacellular labeling) is low throughput • Biased towards isolating active cells	Chorev et al., 2009 (review) Lipski et al., 1981 (collision testing)
	Extracellular in vivo with optotagging	mice	compatible	• Combines a well-established method for monitoring neuronal activity with a potentially higher throughput method of cell type identification	• Although cell type identification is higher throughput than juxtacellular labeling, it can be difficult to definitively ID cells. Arbitrary cutoffs are often employed.	Lima et al., 2009 Cardin et al., 2010
Optical Readouts	Voltage imaging	flies, mice	not yet tested	• An optical readout of neuronal activity that permits single cell resolution from many, even densely packed cells • Good temporal resolution • Access to subthreshold membrane voltage dynamics • Compatible with in vivo or slice preparations	• Sensors are still largely under development	Gong et al., 2015 (recent indicator improvement) St.-Pierre et al., 2014 (recent indicator improvement) Knopfel, 2012 (indiciator review) Hamel et al., 2015 (recent brain imaging review)
	Calcium imaging	widely compatible	compatible	• An optical readout of neuronal activity that permits single cell resolution from many, even densely packed cells • Compatible with in vivo or slice preparations • High signal-to-noise sensors available in green and red	• No access to subthreshold membrane voltage dynamics • Relatively slow kinetics compared to electrophysiology	Hamel et al., 2015 (recent brain imaging review)
	Fiber photometry	mice, rats	compatible	• An optical readout of neuronal activity from a genetically defined population of neurons • An easy-to-implement technique that is highly comptabile with freely moving behavior • Compatible with any optical indicator	• Lack of single cell resolution	Lutcke et al., 2010 Schulz et al., 2012 Cui et al., 2013 Gunaydin et al., 2014 (deep brain axonal signals relevant to ODEs) Lerner et al., 2015 (isosbestic control excitation wavelength) Guo et al., 2015 Kim et al., 2016 Zalocusky et al., 2016 (rat)
Immediate Early Gene (IEG) Readouts	IEG histology	widely compatible	compatible	• Allows a broad readout of recently activated neurons	• Poor temporal resolution (hours) • Post-mortem fixed-tissue readout	Guzowski et al., 2005 (review)
	IEG transgenic reporters (Fos-GFP, Arc-GFP)	mice	compatible	• Allows a broad readout of recently activated neurons • Compatible with whole brain measurement, in vivo imaging, slice electrophysiology	• Poor temporal resolution (hours)	Barth et al., 2007 (review)
	TRAP (FosTRAP, ArcTRAP)	mice	compatible	• Allows a broad readout of recently activated neurons • Compatible with whole brain measurement, in vivo imaging, slice electrophysiology • Readout occurs during a chemically-defined window	• Poor temporal resolution (hours)	Guenthner et al., 2013
	fMRI	Human, non-human primate, rodent	compatible with awake, but still, subjects	• Whole brain readout visible in a live subject • Non-invasive, compatible with human studies • in non-human studies, can be combined with optogenetic manipulation (ofMRI)	• Poor temporal resolution (seconds) • Lack of single cell resolution	Poldrack and Farah, 2015 (review) Lee et al., 2010 (ofMRI)

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