Studies of cortical connectivity using optical circuit mapping methods

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Abstract

An important consideration when probing the function of any neuron is to uncover the source of synaptic input onto the cell, its intrinsic physiology and efferent targets. Over the years, electrophysiological approaches have generated considerable insight into these properties in a variety of cortical neuronal subtypes and circuits. However, as researchers explore neuronal function in greater detail, they are increasingly turning to optical techniques to bridge the gap between local network interactions and behaviour. The application of optical methods has increased dramatically over the last decade, spurred on by the optogenetic

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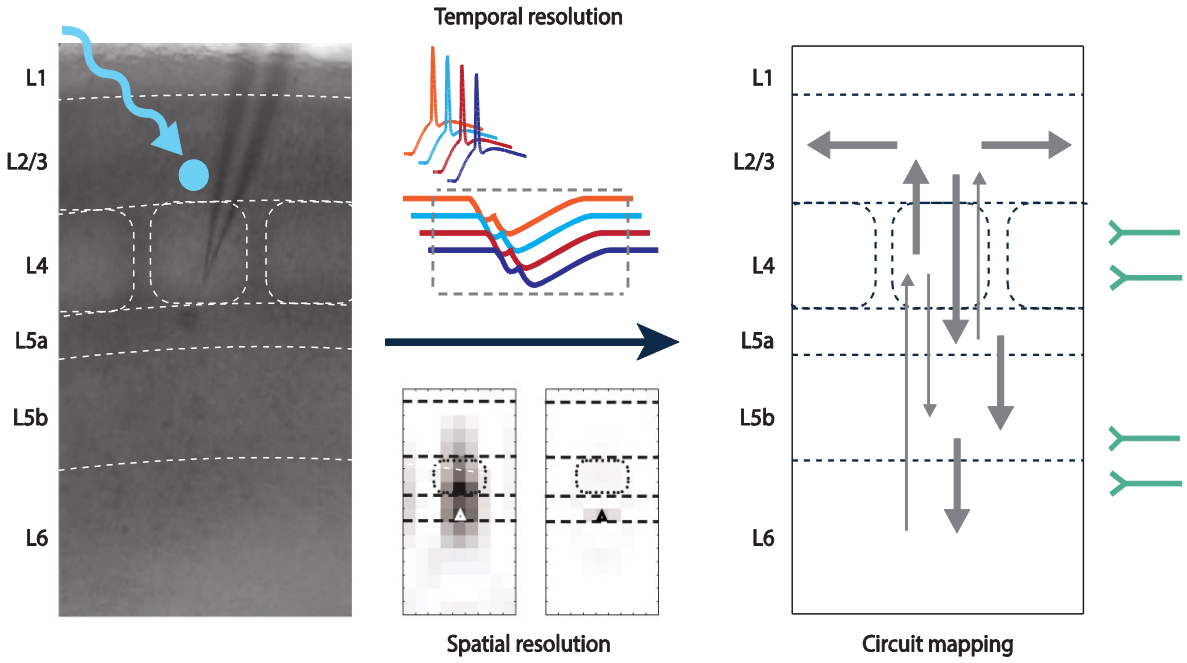
revolution. In this review, we provide an account of recent innovations, providing researchers with a primer detailing circuit mapping strategies in the cerebral cortex. We will focus on technical aspects of performing neurotransmitter uncaging and channelrhodopsin-assisted circuit mapping, with the aim of identifying common pitfalls that can negatively influence the collection of reliable data.



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Drs. Anastasiades, Marques-Smith and Butt have employed laser scanning photostimulation and optogenetic approaches to probe the earliest circuits in mammalian cerebral cortex. Dr. Anastasiades is a postdoctoral associate at the Center for Neural Science, New York University working in the laboratory of Professor Adam Carter. He studies the synaptic organisation and neuromodulation of the Prefrontal Cortex and retains an interest in the development of cortical circuits. Andre Marques-Smith received his PhD from the University of Oxford in 2014. During his PhD and first Post doctoral position, he investigated the development of cortical inhibitory circuits. He is now a researcher at the Sainsbury-Wellcome Centre (UCL), using high-density extracellular probes to investigate thalamocortical circuits in behaving rodents. Dr. Butt is an Associate Professor at the University of

Oxford with a keen interest in GABAergic interneurons and the emergence of early neocortical circuits involved in sensory perception.



Additional information section

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Introduction

Circuit neuroscientists are faced with a complex problem, namely resolving the dense interconnectivity of the mammalian neocortex. Not only must studies account for the diversity of the constituent cellular components (Molnar & Cheung, 2006; Hattox & Nelson, 2007; Petilla Interneuron Nomenclature *et al.*, 2008; Anastasiades & Butt, 2011; Harris & Shepherd, 2015), but also the vast number of possible synaptic connections. A single cortical column in the rodent contains somewhere in the region of 20,000 neurons, with each cell possessing thousands of synaptic inputs (Meyer *et al.*, 2010; Oberlaender *et al.*, 2012). This provides a significant challenge to our understanding, only slightly simplified by the presence of multiple synapses between connected partners and anatomical limitations to connectivity (Feldmeyer, 2012; Harris & Shepherd, 2015; Markram *et al.*, 2015). Historically both anatomical and physiological studies have contributed significantly to our understanding of cortical circuits (Mountcastle, 1957; Hubel & Wiesel, 1962; Woolsey & Van der Loos, 1970; Gilbert & Wiesel, 1989; Douglas & Martin, 2004). In particular, exploration of the laminar structure of the neocortex has proven particularly fruitful, as individual cell types, axons and dendrites often display layer-specific distributions (Thomson & Bannister, 2003; Binzegger *et al.*, 2004; Ma *et al.*, 2006; Gonchar *et al.*, 2007; Wimmer *et al.*, 2010; Oberlaender *et al.*, 2012; Hooks *et al.*, 2013; D'Souza *et al.*, 2016).

Recent advances in anatomical tracing techniques have greatly enhanced the specificity with which we can study the organization of cortical networks (Wickersham *et al.*, 2007; Kim *et al.*, 2011; Wickersham *et al.*, 2013; Xu & Sudhof, 2013; Wouterlood *et al.*, 2014; Beier *et al.*, 2015; DeNardo *et al.*, 2015). However, electrophysiology remains the gold standard for interrogating cortical circuits, enabling unequivocal determination of functional synaptic

connectivity and assessment of the dynamic properties of individual synapses with high temporal resolution. Electrophysiological methods have been applied successfully to resolve synaptic connectivity between small groups of neurons throughout the cortex (Reyes *et al.*, 1998; Thomson & Bannister, 2003; Song *et al.*, 2005; Feldmeyer *et al.*, 2006; Wang *et al.*, 2006; West *et al.*, 2006; Le Be *et al.*, 2007; Silberberg & Markram, 2007; Perin *et al.*, 2011; Jiang *et al.*, 2013; Jiang *et al.*, 2015). These studies highlight fundamental principles of synaptic organization, and identify certain connections that occur at frequencies unexplained by stochastic apposition principles alone (Song *et al.*, 2005; Morishima & Kawaguchi, 2006; Wang *et al.*, 2006; Brown & Hestrin, 2009; Morishima *et al.*, 2011). They have also shown that the probability of connectivity is low in many cases and decreases significantly as a function of distance (Thomson & Bannister, 1998; Song *et al.*, 2005; Morishima & Kawaguchi, 2006). This suggests a need for high-throughput approaches to map cortical connectivity with cell-type specificity. Such cell-type specific wiring diagrams will enhance our understanding of cortical circuits, while providing important reference points to compare inter-areal, or cross-species differences in circuit organization and ultimately function (Wang *et al.*, 2006; Hooks *et al.*, 2011; Katzel *et al.*, 2011; Harris & Shepherd, 2015).

Optical stimulation methods have emerged as an attractive approach to address this need for high-throughput assessment of cortical connectivity, enabling rapid and repeated mapping of synaptic inputs emanating from multiple locations across large regions of the cortical network (Dalva & Katz, 1994; Dantzker & Callaway, 2000; Shepherd *et al.*, 2005; Petreanu *et al.*, 2007; Petreanu *et al.*, 2009; Ashby & Isaac, 2011). Indeed, the array of currently available optical circuit mapping strategies allows researchers to greatly extend studies of cortical connectivity beyond assaying synaptic connections between individual neurons in the local circuit. Such studies include the investigation of long-range connections routinely severed in reduced acute *in vitro* preparations (Cruikshank *et al.*, 2007; Petreanu *et al.*, 2007; Petreanu *et al.*, 2009; Cruikshank *et al.*, 2010; Little & Carter, 2013), as well as the discovery of novel pathways whose scarcity had precluded detection using classical approaches (Pluta *et al.*, 2015), and transient connections restricted to certain periods of development (Anastasiades *et al.*, 2016; Marques-Smith *et al.*, 2016; Tuncdemir *et al.*, 2016). Optical approaches can also be combined with highly specific genetic tools to facilitate region, layer and cell-type specific targeting. Despite the considerable advantages

of these methods, rigorous circuit mapping depends upon many technical and practical considerations. The scope of this review is to outline the advances being made in the use of optical techniques to interrogate the structure of neocortical circuits, and highlight examples of best practice.

Optical approaches to study cortical circuits

Light is an excellent source of excitation as the strength, shape, wavelength and duration of the light beam can be tightly controlled by the experimenter (Callaway & Yuste, 2002; Jerome & Heck, 2011). Moreover, for circuit mapping applications a focused light beam can be rapidly shifted between different sites in the tissue. This laser-scanning photostimulation (LSPS) method allows relatively fast interrogation of connectivity across a large number of spatially distinct locations (Dantzker & Callaway, 2000; Shepherd *et al.*, 2003). Alternatively, wide-field illumination can be used to measure the net presynaptic input onto recorded postsynaptic neurons (Little & Carter, 2013; Suter & Shepherd, 2015; McGarry & Carter, 2016). However, because cortical neurons do not normally respond to direct illumination, a method to transduce light into a neural electrochemical signal is required. Photo-stimulation can be achieved by flash photolysis of caged compounds – where a neurotransmitter molecule (typically the excitatory amino acid glutamate) is bound to a caging moiety via a photo-scissile bond, producing an effect via endogenous receptors (Dalva & Katz, 1994; Ellis-Davies, 2007; Nikolenko *et al.*, 2007). Alternatively, expression of exogeneous light- or ligand-gated ion channels can be used to induce presynaptic excitation (Zemelman *et al.*, 2002; Nagel *et al.*, 2003; Boyden *et al.*, 2005; Szobota *et al.*, 2007; Miesenbock, 2011). These approaches, neurotransmitter uncaging and optogenetics, have yielded considerable information regarding the organization of cortical networks. However, although these approaches are highly complementary, there are subtle differences in their application which make them better suited to specific circuit mapping questions. Below we outline the different optical methods one can employ to map cortical connectivity, summarising the strengths and weaknesses of each approach.

Neurotransmitter uncaging

Single-photon uncaging

Early studies mapping cortical connectivity combined single-photon glutamate uncaging with LSPS to focally stimulate small clusters of neurons across the cortex (Dalva & Katz, 1994; Dantzker & Callaway, 2000; Shepherd *et al.*, 2003). Because ionotropic glutamate receptors are primarily restricted to the soma and dendrites of cortical neurons, glutamate uncaging does not activate *en passant* axons, making it well suited for mapping the dense, recurrent circuitry of the neocortex. The resolution of the approach is largely dependent upon the point-spread function of the light source, scattering of light by neuronal tissue, and appropriate calibration to restrict action potentials to the peri-somatic region (*see below*). If performed correctly, experimental resolutions in the order of ~50 μm are readily achievable (Shepherd *et al.*, 2003; Jerome & Heck, 2011; Anastasiades & Butt, 2012), activating somewhere in the region of 30-60 presynaptic neurons with each light-pulse (Shepherd *et al.*, 2005).

The sublaminar resolution of single-photon uncaging makes it well-suited to compare inter-laminar connectivity within a cortical column (Dantzker & Callaway, 2000; Schubert *et al.*, 2001; Bureau *et al.*, 2004; Shepherd & Svoboda, 2005; Xu & Callaway, 2009; Anderson *et al.*, 2010; Anastasiades & Butt, 2012; Yamawaki & Shepherd, 2015; Anastasiades *et al.*, 2016; Marques-Smith *et al.*, 2016). Mapping columnar connectivity allows the relative strength and laminar distribution of synaptic inputs to be determined onto postsynaptic neurons in all layers of cortex. Such connectivity matrices can be compared across distinct cytoarchitectonic subdivisions of neocortex to reveal conserved or unique connectivity motifs (Hooks *et al.*, 2011). LSPS can also be used to examine changes in the strength or distribution of synaptic inputs in the same cortical area but under variable experimental conditions. For example, alterations in network activity (Anastasiades & Butt, 2012; Kuhlman *et al.*, 2013; Meng *et al.*, 2015; Marques-Smith *et al.*, 2016; Meng *et al.*, 2017), changes in gene expression (Bureau *et al.*, 2008; Marques-Smith *et al.*, 2016; Sun *et al.*, 2016; Rajkovich *et al.*, 2017), or synaptic integration over the course of development (Bureau *et al.*, 2004; Anastasiades & Butt, 2012; Viswanathan *et al.*, 2012; Anastasiades *et al.*, 2016; Marques-Smith *et al.*, 2016).

One important consideration with glutamate uncaging is the ubiquitous expression of glutamate receptors throughout the mammalian CNS. This presents two immediate issues: first, local connections can be obscured by large direct glutamate responses at recorded neurons (*see section on assigning postsynaptic responses*). Second, there is no presynaptic cell-type specificity (**Figure 1A**). However, excitatory and inhibitory conductances can be isolated by adjusting the holding potential of voltage-clamped postsynaptic neurons to elucidate inhibitory or excitatory afferent input onto a postsynaptic neuron (Roerig & Chen, 2002; Shepherd *et al.*, 2003; Brill & Huguenard, 2009; Xu & Callaway, 2009). Furthermore, uncaging can be combined with numerous methods that allow classification of postsynaptic cell-types (**Table 1**) within both the interneuron (Xu & Callaway, 2009; Apicella *et al.*, 2012) and pyramidal neuron classes (Anderson *et al.*, 2010; Yamawaki & Shepherd, 2015). This approach allows researchers to compare the strength and distribution of synaptic inputs onto distinct populations within the local circuit.

Two-photon uncaging

Single-photon uncaging has been used with great success to compare intra- and inter-laminar connectivity across an entire cortical column. However, it lacks the necessary precision to stimulate a single cell per trial, which requires the greater spatial resolution attained from two-photon (2-P) uncaging (**Figure 1B**). This approach is similar to single-photon uncaging, but requires coincident arrival of two photons in order to release glutamate from the caging moiety (Furuta *et al.*, 1999). 2-P uncaging utilises a mode-locked laser light source which drastically increases the probability of coincident multi-photon excitation at the focal point of the objective (Denk *et al.*, 1990; Denk & Svoboda, 1997; Jerome & Heck, 2011). This provides much greater axial resolution compared to single-photon techniques, allowing stimulation of single neurons in a defined plane (Furuta *et al.*, 1999). The longer wavelengths used are also advantageous as they have much higher tissue penetrance and lower light scatter than UV lasers used for single-photon uncaging (Denk & Svoboda, 1997; Nikolenko *et al.*, 2011).

2-P glutamate uncaging can be used to assay hundreds of potential synaptic connections, providing a relatively high-throughput method to probe the underlying structure of local cortical networks. For example, 2-P uncaging has been used to determine connection probability between distinct cell-types, as well as the relative convergence or divergence of given inputs onto individual nodes in the network (Ashby & Isaac, 2011; Fino & Yuste, 2011; Packer & Yuste, 2011). As with single-photon methods, cell-type specificity is not explicitly achieved by 2-P stimulation itself (**Figure 1B**). However, it is possible to use fluorescent reporters to label and subsequently target specific presynaptic populations (Fino & Yuste, 2011; Packer & Yuste, 2011). Over the last few years researchers have applied single-cell 2-P uncaging to probe the emergence of recurrent connectivity between layer 4 stellate cells in somatosensory cortex (Ashby & Isaac, 2011) and the dense blanket of inhibition mediated by individual interneuron subtypes within superficial (Fino & Yuste, 2011; Packer & Yuste, 2011) and deep layers of neocortex (Packer *et al.*, 2013).

Despite advances in 2-P uncaging, it is not without limitations. While it can readily be applied to map connectivity within (Ashby & Isaac, 2011), or between layers (Viswanathan *et al.*, 2012), producing single-cell connectivity matrices across an entire column is challenging due to the vast number of potential connections. The low glutamate yield afforded by 2-P stimulation is a further issue, resulting in considerable variance in the suprathreshold activation of presynaptic neurons (Nikolenko *et al.*, 2007; Matsuzaki *et al.*, 2008). This is less critical in immature neurons, which possess higher input resistance (Ashby & Isaac, 2011). However, for adult cortical neurons beam-multiplexing is typically required to evoke action potentials in single neurons (Nikolenko *et al.*, 2007). This involves stimulating at multiple uncaging sites forming a concentric ring around the target neuron to evoke a localized increase in glutamate that is sufficient to drive spiking in the target neuron, but not adjacent cells (Nikolenko *et al.*, 2007). Because of difficulties evoking reliable responses, 2-P stimulation can be combined with calcium imaging to detect presynaptic firing (Nikolenko *et al.*, 2007). This helps reduce false negatives and ensures reliable assignment of connection probability. An additional consideration is that 2-P uncaging often requires concentrations of caged compound that either completely (in the case of MNI-glutamate), or partially (for Rubi variants) block GABAergic transmission (Fino *et al.*, 2009). MNI-glutamate should therefore be avoided for mapping GABAergic connections using 2-P uncaging. Rubi-glutamate has

been successfully used to map GABAergic connectivity from multiple interneuron subtypes onto pyramidal neurons (Fino & Yuste, 2011; Packer & Yuste, 2011). However, 300 μM Rubi-glutamate will produce a 50% reduction in IPSC amplitude (Fino *et al.*, 2009), which should be taken into account when mapping GABAergic inputs using this approach.

Optogenetics

Over the past decade the application of microbial opsins, most notably Channelrhodopsin-2 (ChR2) (Nagel *et al.*, 2003; Boyden *et al.*, 2005), to enable neuronal photostimulation has proven a powerful tool for studying cortical connectivity. In contrast to neurotransmitter uncaging, optogenetic approaches can be used to study both local and long-range inputs. The latter is possible because light sensitive opsins are expressed throughout axons and dendrites. Although many axons are severed during preparation of acute *in vitro* slices, synaptic terminals remain functional and presynaptic release can be evoked using brief pulses of light (**Figure 2A**) (Petreanu *et al.*, 2007; Petreanu *et al.*, 2009; Mao *et al.*, 2011). As with glutamate uncaging, optogenetics can be combined with methods to label and target specific postsynaptic neuronal populations. However, one advantage of optogenetics over classical glutamate uncaging is that it can also be combined with a wide range of methods to produce presynaptic input specificity (**Table 1**).

Long-range connectivity

To study long-range circuits, ChR2 is first expressed in a putative presynaptic brain region. After preparation of acute *in vitro* slices, photosensitive axons are then stimulated via illumination over the postsynaptic structure while recording from target neurons. Optogenetics can therefore confirm the presence, or absence of synaptic connectivity between presynaptic structures and postsynaptic neurons located throughout the nervous system (**Figure 2A**). One of the earliest studies to use this approach mapped long-range connectivity between neocortical pyramidal cells across the corpus callosum (Petreanu *et al.*, 2007). By restricting ChR2 expression to a subset of layer 2/3 neurons using *in utero* electroporation, it was possible to map the outputs of these cells both within and across hemispheres. This ChR2-assisted circuit mapping (CRACM) strategy allows inputs to be

compared onto target neurons in different layers. While the presence of photoexcitable ChR2 throughout the extent of neurons is an essential part of the utility of this approach, it can make it challenging to assign the origin of recorded synaptic inputs to a specific spatial location (Petreanu *et al.*, 2007; Lewis *et al.*, 2009). The methodology of the approach has been simplified over time, with wide-field LED illumination reducing the cost and technical requirements. This increased accessibility has yielded numerous studies examining the relative strength of synaptic inputs onto distinct components of the cortical network (Mao *et al.*, 2011; Cruikshank *et al.*, 2012; Hooks *et al.*, 2013; Little & Carter, 2013; Rock & Apicella, 2015; Suter & Shepherd, 2015; McGarry & Carter, 2016).

When performing optogenetic experiments, it is important to account for the considerable variation in ChR2 expression that can occur when using viral vectors, even in slices obtained from the same brain (Mao *et al.*, 2011). This is achieved by normalizing input amplitude to a consistent component of the cortical circuit in each slice experiment, for example a pyramidal neuron of a given cell-type, or located in a specific layer (Mao *et al.*, 2011; Lee *et al.*, 2013; McGarry & Carter, 2016). This normalization process allows inputs to be compared onto cells of different subtypes (Lee *et al.*, 2013; Little & Carter, 2013; Yang *et al.*, 2013; Lee *et al.*, 2014; Rock & Apicella, 2015; Yamawaki & Shepherd, 2015; McGarry & Carter, 2016), located in different layers (Mao *et al.*, 2011; Hooks *et al.*, 2013; Suter & Shepherd, 2015; Yamawaki & Shepherd, 2015), across experimental conditions (MacAskill *et al.*, 2014; Xue *et al.*, 2014; Rajkovich *et al.*, 2017), or during development (Tuncdemir *et al.*, 2016)**.** To compare inputs between individual cells in a slice ideally one should use wide-field illumination, consistent stimulus intensity, and focus the light-beam on the same location across trials. This ensures that equivalent synapses are stimulated to a similar degree across the entire postsynaptic dendrite, allowing direct comparison of input strength for neurons contained within the spatial extent of the light beam (**Figure 2 A-C**).

To enhance mechanistic understanding of observed differences in connectivity, optogenetics can be used in a similar manner to classical studies using electrical stimulation. If ChR2 positive axons are optically stimulated in the presence of extracellular strontium, quantal analysis of evoked, ChR2-driven mini-postsynaptic current (PSC) amplitude and frequency

can be performed (Silver, 2003; Little & Carter, 2013; MacAskill *et al.*, 2014). This is possible because strontium interferes with presynaptic release (Goda & Stevens, 1994; Xu-Friedman & Regehr, 1999), such that asynchronous PSCs recorded within a defined window after a given synaptic stimulus can largely be attributed to the stimulated axons (Hull *et al.*, 2009). An alternative strategy employs a focused blue laser to limit stimulation to a single axon (Ye *et al.*, 2015; Morgenstern *et al.*, 2016; Del Pino *et al.*, 2017); an approach that is analogous to minimal electrical stimulation (Finnerty *et al.*, 1999; Hull *et al.*, 2009). Additional properties such as AMPA/NMDA ratios (Little & Carter, 2013; McGarry & Carter, 2016), E/I balance (Lee *et al.*, 2014; Xue *et al.*, 2014; Rock & Apicella, 2015; Yamawaki & Shepherd, 2015; McGarry & Carter, 2016) and presynaptic release probability (Little & Carter, 2013; Lee *et al.*, 2014; Crandall *et al.*, 2015; McGarry & Carter, 2016) can also be examined. However, it should be noted that in some cases synaptic currents evoked by optical stimulation have been reported to depress more than those evoked electrically (Zhang & Oertner, 2007; Cruikshank *et al.*, 2010; Schoenenberger *et al.*, 2011; Olsen *et al.*, 2012; Jackman *et al.*, 2014). This disparity could be explained by the slow kinetics of some ChR2 variants broadening the action potential waveform or by the permeability of ChR2 to Ca^{2+} enhancing its influx directly into synaptic boutons, both of which are situations predicted to artificially inflate release probability (Zhang & Oertner, 2007; Olsen *et al.*, 2012). A detailed study of this issue revealed that the method of ChR2 expression seems to be mainly responsible for the disparities observed, with AAV serotypes influencing synaptic depression in a synapse-dependent manner (Jackman *et al.*, 2014). Expression of ChR2 by usage of transgenic animals was the method that least altered release probability, when compared to electrical stimulation, followed by AAV9 expression vectors (Jackman *et al.*, 2014).

Subcellular connectivity

Dendritic location has a significant influence over the functional impact of individual synapses (Yuste *et al.*, 1994; Williams & Stuart, 2002). To determine the subcellular location of ChR2 positive axon terminals, Svoboda and colleagues took advantage of the ability of ChR2 to evoke release of neurotransmitter from presynaptic terminals to determine the subcellular location of afferent input on the postsynaptic dendrite of cortical pyramidal cells. This refinement of their CRACM method was made possible by adding tetrodotoxin (TTX) to

block the fast transient sodium channel and the potassium channel blocker 4-AP to enhance photostimulation-induced neurotransmitter release. This pharmacological manipulation blocks action potential conductance along the axon, while restoring presynaptic release, effectively restricting photoexcitability to presynaptic terminals (Petreanu *et al.*, 2009). TTX and 4-AP can therefore be used to ensure postsynaptic responses are monosynaptic (Cruikshank *et al.*, 2010; Little & Carter, 2013). However, when combined with LSPS it is possible for light evoked responses to be assigned to specific stimulation sites across the postsynaptic dendrite. Svoboda and colleagues termed this approach *subcellular* ChR2 assisted circuit mapping (sCRACM) and applied it to study the subcellular distribution of inputs from individual layers, thalamus and motor cortex (Petreanu *et al.*, 2009). sCRACM has subsequently been utilised to study subcellular targeting across numerous inputs and neuronal subtypes (Mao *et al.*, 2011; Hooks *et al.*, 2013; Marlin & Carter, 2014; Suter & Shepherd, 2015). However, a caveat to the sCRACM approach is that electrotonic filtering yields an underestimation of distal input when recorded at the soma (Williams & Stuart, 2002; Williams & Mitchell, 2008; Dembrow *et al.*, 2015). This issue is particularly relevant in large dendritic arbor layer 5 pyramidal neurons, where the approach has been applied most frequently. This is not unique to sCRACM, occurring for synaptic responses evoked using both electrical or optogenetic stimulation (Dembrow *et al.*, 2015). Regardless, it remains important to consider dendritic cable properties when interpreting sCRACM data. The attenuation of distal inputs can be reduced slightly through pharmacological blockade of resting conductances (Williams & Mitchell, 2008). Alternatively, input maps can be adjusted to account for dendritic location (Petreanu *et al.*, 2009).

Finally, the resolution of sCRACM (~60 μm)(Petreanu *et al.*, 2009) is insufficient to examine inputs at single spines, where typical inter-spine distances are ~1 μm (Konur *et al.*, 2003). To achieve this, optogenetics can be combined with 2-P calcium imaging to map the dendritic distribution of synaptic inputs at the level of the single spine (Little & Carter, 2012; MacAskill *et al.*, 2012). This approach is particularly useful when examining small neurons with compact dendrites where the lower resolution of sCRACM may limit the ability to discriminate differences in synapse distribution. It also allows researchers to pose more complex questions, such as examining input specific synaptic clustering on individual dendritic branches (Gokce *et al.*, 2016).

Local connectivity

Optogenetics can also be used to map local connectivity. This is advantageous as it allows selective stimulation of specific presynaptic cell-types within the local network (**Figure 1C**)(Katzel *et al.*, 2011; Lee *et al.*, 2013; Pfeffer *et al.*, 2013; Pluta *et al.*, 2015). In addition to microbial opsins, local connectivity can be assessed through selective expression of ligand-gated ion channels (Anastasiades *et al.*, 2016; Marques-Smith *et al.*, 2016). In this approach, light evoked uncaging, or photo-isomerism, causes selective depolarization of neurons induced to express an optogenetic actuator (Szobota *et al.*, 2007; Miesenbock, 2011). Regardless of the method used, optogenetics can be combined with conditional genetic approaches (Katzel *et al.*, 2011; Olsen *et al.*, 2012; Bortone *et al.*, 2014; Crandall *et al.*, 2015; Pluta *et al.*, 2015), or *in utero* electroporation (Petreanu *et al.*, 2007; Petreanu *et al.*, 2009) to study layer, or cell-type, specific inputs. These inputs can be compared within and across layers, in a similar manner to glutamate uncaging and long-range connectivity. Although axonal expression is advantageous for studying long-range inputs, it is detrimental to the study of local networks, as it hinders the reliable assignment of inputs to specific sites (Petreanu *et al.*, 2007). To circumvent this problem optogenetic actuators can be restricted to specific subcellular compartments (Lewis *et al.*, 2009; Grubb & Burrone, 2010; Baker *et al.*, 2016), effectively producing responses at the soma but not stimulating distal dendrites or local axons. This approach allows cortical circuits to be mapped in a similar manner to glutamate uncaging, with the spatial location of presynaptic neurons resolved to sublaminar resolutions (Baker *et al.*, 2016).

In theory 2-P optogenetics can be used to study local connectivity with single-cell resolution (**Figure 1D**). In practice, as with uncaging, this approach suffers difficulties in generating suprathreshold photocurrents in postsynaptic neurons to drive reliable, time-locked action potentials necessary to reliably assay connectivity (Packer *et al.*, 2012). Novel stimulation paradigms such as temporal focusing, or spiral scanning may help overcome this limitation (Rickgauer & Tank, 2009; Papagiakoumou *et al.*, 2010), while red-shifted opsin variants with larger single channel conductance at wavelengths typically used for 2-P stimulation may also enhance the applicability of this approach (Packer *et al.*, 2012; Chaigneau *et al.*, 2016). However, to date the number of studies utilizing 2–P optogenetics are few, and largely

limited to technical accounts (Rickgauer & Tank, 2009; Andrasfalvy *et al.*, 2010; Packer *et al.*, 2012; Prakash *et al.*, 2012). We remain hopeful that these recent advances will prove decisive in the success of this potentially powerful technique.

Spatial and temporal calibration

For each of the photostimulation approaches described above an important initial requirement is the calibration of the light to selectively stimulate the presynaptic input. For interrogation of dense local circuits, such as those found in neocortex, this focuses on controlling the number and spatial extent of light-evoked action potentials in presynaptic neurons. This applies equally to circuit mapping using optogenetics or glutamate uncaging. For long-range optogenetic studies, it is essential that the vector used to drive opsin expression is constrained to the presynaptic region of interest. In both cases calibration ensures that the recorded postsynaptic input can be attributed to the spatial location, brain region or cell type interrogated.

Controlling presynaptic firing

For local circuit mapping the photoresponse of the presynaptic population is crucial to correctly interpret experimental data. When using glutamate uncaging, the presynaptic response will vary significantly based on the concentration of caged-glutamate, while for optogenetic experiments opsin expression will strongly influence firing. Both factors should be tightly controlled to ensure reliable calibration. The presynaptic population is recorded from first to calibrate a photostimulus that produces reliable, spatially restricted excitation. Recordings should ideally be made in cell-attached mode, to avoid perturbing the intracellular state of the neuron (**Figure 3A**) (Shepherd *et al.*, 2003). Adjustments to the duration, intensity and shape of the light-pulse are made until optical stimulation evokes a series of ≤ 3 time-locked action potentials, spatially restricted to sites adjacent to the soma (Ashby & Isaac, 2011; Anastasiades & Butt, 2012). Evoking multiple (2 - 3) presynaptic action potentials can compensate for potential failures in synaptic transmission (Dantzker & Callaway, 2000). For 2-P experiments calibration involves testing for the absence of evoked firing in response to optical stimulation at proximal or distal spines and dendrites (Ashby &

Isaac, 2011). For single-photon experiments the calibration process is repeated across all layers of the cortex creating a response map to the photostimulus (**Figure 3B**). Either the light-pulse should be adjusted until firing properties are similar across layers (Anastasiades & Butt, 2012), or differences in presynaptic excitability can be corrected for *post hoc* (Bureau et al., 2004).

This calibration process ensures the somatic source of synaptic inputs can be confidently assigned to a given stimulation site. When performing calibration experiments, care should be taken to avoid evoking suprathreshold conductances in distal dendrites, particularly the dendritic tufts of pyramidal neurons (Dantzker & Callaway, 2000). We have found that mapping using carefully titrated laser power and long duration pulses can effectively limit photostimulation to the immediate soma, even in immature pyramidal cells with relatively simple dendritic arbors (**Figure 3C**) (Anastasiades & Butt, 2012). Minimizing axo-dendritic firing is more challenging for optogenetic stimulation, owing to the expression of ChR2 throughout the axon and dendrite (Petreanu *et al.*, 2007). To circumvent this, ChR2 can be restricted to perisomatic, regions, using temporally restricted expression (Katzel *et al.*, 2011) or chimeric proteins that contain targeting sequences for Kv2.1 or other subcellularly restricted proteins (Wu *et al.*, 2013; Baker *et al.*, 2016). Alternatively, “sculpting” the photostimulus to create ramped rather than square pulses can help restrict firing to perisomatic stimulation sites (Adesnik & Scanziani, 2010; Pluta *et al.*, 2015) (Marques-Smith, *unpublished observations*).

Intrinsic physiology, neuronal morphology and glutamate receptor expression can change significantly during development (McCormick & Prince, 1987; Monyer *et al.*, 1994; Picken Bahrey & Moody, 2003; Maravall *et al.*, 2004)**,** or after experimental manipulations (Greenhill *et al.*, 2015; Mowery *et al.*, 2015; Santello & Nevian, 2015), such as sensory deprivation. When comparing input maps across periods of development it is important to use age-appropriate photostimuli that endeavor to keep presynaptic stimulation levels constant (Anastasiades & Butt, 2012). Similarly, experimental manipulations that might affect the way neurons respond to photostimulation must be controlled for if putative alterations in synaptic input are to be interpreted with any certainty (Shepherd *et al.*, 2003; Del Pino *et al.*, 2017). In

such studies, one key consideration is if the sensitivity of neurons to light varies between control and test samples. To account for differences in presynaptic excitability, it is possible to adjust light intensity to give uniform firing, or alternatively scale the afferent input maps *post hoc* based upon recorded differences in presynaptic spike number (Bureau *et al.*, 2004). Furthermore, for optogenetic experiments it is important to control for variation in expression of optogenetic actuators, within and across conditions (Mao *et al.*, 2011; Arruda-Carvalho *et al.*, 2017)**.** In essence, the extraneous variable to be controlled is biological, namely presynaptic output, rather than illumination intensity *per se*.

Assigning postsynaptic responses

A fundamental requirement of circuit mapping is to correctly identify light-evoked postsynaptic responses. A number of confounds exists that must be controlled for in order for accurate response profiles to be mapped. These responses typically fall into three categories. First, light evoked firing may yield polysynaptic activity via distal sites that synapse onto the target cell. Second, across the network there will be a degree of baseline spontaneous activity that must be subtracted from evoked response. Third, "direct" responses occur due to uncaging, or opsin stimulation, at the soma or dendrite of recorded neurons, which can obscure local inputs (**Figure 3C**). Polysynaptic activity can be attenuated by standard physiological adjustments, for example, recording at room temperature in artificial cerebrospinal fluid (ACSF) containing high divalent cation concentrations (Shepherd *et al.*, 2003; Jin *et al.*, 2006). Separating direct responses from light evoked synaptic input is relatively trivial, as direct responses can be readily distinguished from synaptic responses based on two criteria: (1) the amplitude of the direct response is remarkably constant; (2) direct response onset is coincident with the start of the light pulse. The impact of direct responses can be further reduced by using long, weak light pulses, which are lower in amplitude and decay more slowly, enhancing the ability to distinguish synaptic responses from large direct responses (**Figure 3C**). In contrast, synaptic inputs always occur at a delay from light onset, representing the time taken to fire the presynaptic neuron plus the lag required for synaptic transmission to occur (**Figure 3C**) (Shepherd *et al.*, 2003; Nikolenko *et al.*, 2011). Using the data for spike firing from calibration experiments, the monosynaptic detection window is calculated from the onset of the first

light-evoked action potential until a set time after the last, or n^{th} spike (typically around 100 ms - **Figure 3D**). The breadth of this detection window accounts for the cell-to-cell variability in light-evoked action potential generation and allows the experimenter to extract and measure the totality (or a very high proportion) of putative light-evoked postsynaptic currents, while enhancing the ability to distinguish these synaptic events from direct responses (**Figure 3E**) (Dantzker & Callaway, 2000; Shepherd *et al.*, 2003; Anastasiades & Butt, 2012). The impact of spontaneous events is reduced by averaging across multiple trials and/or accepting only responses that occur in a set proportion of trials (Ashby & Isaac, 2011; Anastasiades & Butt, 2012; Anastasiades *et al.*, 2016). When recording from cells with high rates of spontaneous synaptic activity, narrowing the postsynaptic detection window (Bendels *et al.*, 2010), or subtracting a value for baseline spontaneous events recorded from trials lacking photostimulation (Dantzker & Callaway, 2000; Xu & Callaway, 2009) can minimise the impact of false positives. For cells with a number of large direct responses, for example neurons with dense dendritic arbors, one can bath apply TTX to the slice perfusate and re-run the uncaging procedure to produce a map of direct responses (Dantzker & Callaway, 2000; Roerig & Chen, 2002). This approach is also helpful in 2-P uncaging, and helps rule out false positives where postsynaptic dendrites may run in close proximity to the uncaging site (Nikolenko *et al.*, 2011). These can be subtracted from the synaptic input map to help provide a better estimation of local connectivity.

Ensuring restricted and reliable opsin expression for Optogenetics

Expression of optogenetic actuators, such as ChR2, can be achieved in numerous ways. The most common approach – which allows good spatial restriction to ChR2 expression – is to inject a viral vector (typically AAV or lentivirus) encoding the relevant opsin into the presynaptic region of interest. An important step for the investigator is to calibrate the injection volume prior to performing circuit mapping experiments (Arruda-Carvalho *et al.*, 2017). The expression efficiency of the virus can vary based on serotype (Aschauer *et al.*, 2013), and will also depend upon the duration of expression, so either injection volume, viral serotype, or survival time post injection should be adjusted to ensure opsin expression is restricted to the presynaptic structure of interest. Researchers should also check for retrograde transfection, which can occur with AAVs taken up at axon terminals (Aschauer *et*

al., 2013; Rothermel *et al.*, 2013). This is particularly important in the neocortex where many connections between regions are often reciprocal. Retrograde infection may be dependent upon serotype, viral payload (for example Cre dependent, or independent expression), duration of expression and occur in a region, or projection specific manner (Aschauer *et al.*, 2013; Rothermel *et al.*, 2013). It is important that researchers rule-out retrograde infection wherever possible and adjust their viral strategy accordingly.

Transgenic reporters (Madisen *et al.*, 2012) and *in utero* electroporation (Petreanu *et al.*, 2007; Petreanu *et al.*, 2009; Adesnik & Scanziani, 2010) offer alternatives to viral injections and confer certain advantages and disadvantages. *In utero* electroporation at defined embryonic time points restricts opsin expression to excitatory projections of individual cortical layers (Petreanu *et al.*, 2007; Petreanu *et al.*, 2009). However, long-term expression after *in utero* electroporation (> 40 days), or similar approaches that promote particularly strong opsin expression in neurons, may cause deficits in axonal morphology and synaptic connectivity (Miyashita *et al.*, 2013). Layer, or cell-type specific expression can also be achieved using Cre driver lines crossed with optogenetic reporters (Madisen *et al.*, 2012). Reporter lines are advantageous as they help limit variability between experiments by providing consistent expression levels (Madisen *et al.*, 2012; Hooks *et al.*, 2015). However, a potential disadvantage of this approach is that off-target recombination has been described for some driver lines when crossed with reporters (Hu *et al.*, 2013). For subcortical reporter lines, it should be noted that brain regions typically comprise neurons belonging to multiple subtypes. Some neurons can co-release multiple neurotransmitters (Tritsch *et al.*, 2012; Saunders *et al.*, 2015), while Cre driver lines may label multiple presynaptic populations (Lammel *et al.*, 2015). Consequently, receptor specific pharmacology can be applied to isolate inputs from neurons that utilise a particular neurotransmitter.

Regardless of the approach used, restricted expression methods facilitate mapping intra-cortical and long-range connections emanating from distinct cell-types, or cortical layers (Petreanu *et al.*, 2007; Petreanu *et al.*, 2009; Olsen *et al.*, 2012; Bortone *et al.*, 2014; Pluta *et al.*, 2015). Cre driver lines combined with optogenetic reporters are particularly useful for studying the connectivity of local interneurons, where it can be assumed that the majority of

GABAergic inhibition is from the local circuit (Katzel *et al.*, 2011; Lee *et al.*, 2013; Pfeffer *et al.*, 2013); however see (Basu *et al.*, 2016; He *et al.*, 2016; Rock *et al.*, 2017). This is more problematic for Cre driver lines expressed by broad swaths of neurons – for example the *Emx1*-Cre line that captures all projection neurons – as many intra-cortical projections are recurrent (Mao *et al.*, 2011; Suter & Shepherd, 2015), obscuring regional specificity. However, projection specific driver lines can be combined with viral expression of Cre-dependent opsins to overcome this problem (Olsen *et al.*, 2012; Crandall *et al.*, 2015). Alternatively, more complex viral strategies exist to restrict opsin expression based on specific projection, or connection target (**Table 1**). The tools available to study circuits with optogenetics are considerably more complex than for uncaging and require care to implement them correctly. However, with this complexity comes greatly enhanced specificity, which in turn has yielded new and exciting insight into cortical structure and function.

Discussion- future developments

This review focuses on approaches to map the structural organisation of the neocortex in a cell-type, input and layer specific fashion. Although the field has progressed rapidly in recent years, technological advances will undoubtedly yield additional possibilities to map cortical connectivity in greater detail. In addition to mapping the structural organisation of cortical networks, circuit mapping has proven adept at uncovering plasticity related changes in network architecture (Shepherd *et al.*, 2003; Bureau *et al.*, 2008; Qiu *et al.*, 2011; Marques-Smith *et al.*, 2016; Rajkovich *et al.*, 2017). It has also been possible to use circuit mapping combined with paired recordings to probe shared inputs amongst reciprocally connected neurons, giving greater insight into the organisation of cortical subnetworks (Yoshimura & Callaway, 2005; Yoshimura *et al.*, 2005; Morgenstern *et al.*, 2016). Recent advances have begun to extract the synaptic connectivity rules that give rise to sensory experience from data recorded *in vivo* (Ko *et al.*, 2011; Ko *et al.*, 2013). Combining optical circuit mapping of cortical subnetworks with recording of neural activity *in vivo* will greatly enhance our ability to determine how given neurons form active ensembles and ultimately our functional understanding of neural circuits. Recent advances have begun to bridge the study of circuits *in vitro* and *in vivo* by using 2-P microscopy to combine excitation of putative pre-synaptic neurons with calcium imaging of postsynaptic responses (Packer *et al.*, 2015). Though this approach is limited to supra-threshold responses, simultaneous whole-cell patch-clamp

recordings or advances in genetically-encoded voltage-sensitive indicators could open a window into mapping subthreshold connectivity *in vivo* (Marshall *et al.*, 2016). All-optical mapping strategies may be especially advantageous *in vivo*, inasmuch as they circumvent space-clamp and electrotonic attenuation of distal synapses, issues compounded by the low input resistance of neurons and the challenges involved in obtaining sufficiently low-access resistance recordings *in vivo (*Margrie *et al*., 2002; Williams & Mitchell, 2008*)*.

Since early applications of ChR2, the toolkit available to modulate neuronal activity has been growing steadily (Zhang *et al.*, 2011). Tools now exist that provide faster (Gunaydin *et al.*, 2010), or slower (Berndt *et al.*, 2009; Yizhar *et al.*, 2011) on-off kinetics, optical inhibition (Gradinaru *et al.*, 2008) and red shifted activation (Yizhar *et al.*, 2011; Lin *et al.*, 2013; Klapoetke *et al.*, 2014). Many of these have useful application for studying behaviour (Witten *et al.*, 2010; Yizhar *et al.*, 2011; Nieh *et al.*, 2013), but are less practical for studying connectivity. Optical inhibition has been useful for removing certain inputs evoked by a given stimulus, for example feed-forward inhibition mediated via certain inhibitory neurons (Delevich *et al.*, 2015; Rock & Apicella, 2015). In theory, red shifted opsins should allow for the interrogation of multiple inputs onto a given postsynaptic neuron. In practice, however the opsin variants available are not sufficiently spectrally distinct to allow isolated multi-channel stimulation- see however (Klapoetke *et al.*, 2014; Hooks *et al.*, 2015). In the future, novel optogenetic tools and expression systems will surely provide greater specificity with which to probe cortical networks.

Finally, as we increase our understanding of how individual cell types connect with other neurons, layers or regions of the brain, it will be of interest to uncover the molecular determinants of specific connectivity patterns. Recent advances in single cell genomic analysis (Fuccillo *et al.*, 2015; Usoskin *et al.*, 2015; Zeisel *et al.*, 2015; Foldy *et al.*, 2016; Poulin *et al.*, 2016; Romanov *et al.*, 2017) combined with local circuit mapping methods outlined herein, may offer a reliable, relatively high-throughput approach to achieve such a goal (Pfeffer *et al.*, 2013). Combining these approaches will provide significant insight into the molecular mechanisms that produce the complex wiring patterns observed within neocortical circuits. It will also provide novel markers, allowing researchers to continue probing the function of cortical microcircuits with ever greater specificity.

Figure legends

Figure 1 Overview of methods to optically stimulate neuronal populations

A) Single-photon uncaging indiscriminately activates multiple populations of neurons within the local network. Firing (orange trace) is induced in all neurons within the spatial extent of the light beam.

B) Two-photon uncaging selectively activates individual neurons with single cell resolution. Firing (orange trace) can be induced in target cells, but not adjacent neurons.

C) Single-photon optogenetics selectively activates multiple neurons within the local network. Firing (orange trace) is induced in all neurons within the spatial extent of the light beam so long as they express the construct encoding the optogenetic actuator.

D) Two-photon optogenetics selectively activates individual neurons with single cell resolution. Firing (orange trace) can be induced in the target cell, but not adjacent neurons, so long as target neurons express the construct encoding the optogenetic actuator.

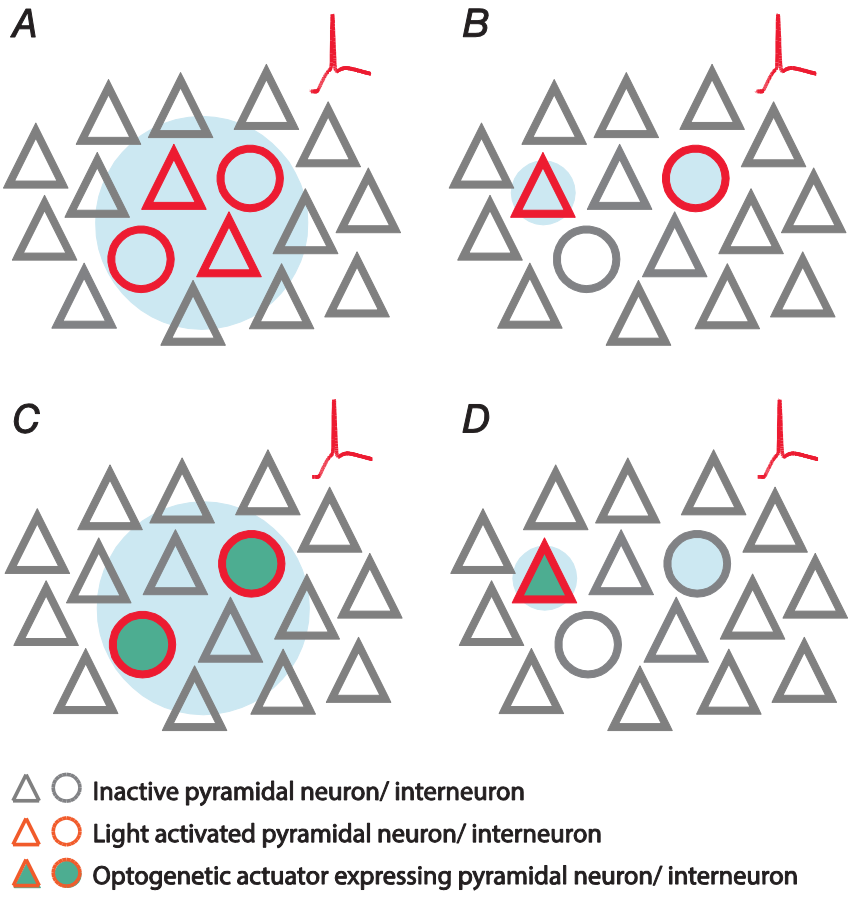

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Figure 2 Strategy to examine long-range inputs with wide-field optogenetics

A) Presynaptic axons (green) expressing the optogenetic construct are present within cortical network and can be selectively activated by light. Wide-field illumination must be correctly aligned so the light beam covers the entire extent of the dendritic arbor of both recorded neurons. Input from the presynaptic region expressing the optogenetic actuator can be reliably compared at the two postsynaptic neurons.

B) Wide-field illumination is too focused so the light beam only covers a portion of the dendritic arbor of the recorded neurons. Inputs that synapse onto dendrites outside the extent of the light beam are not activated, so total input from the presynaptic region expressing the optogenetic actuator cannot be reliably compared at the two postsynaptic neurons.

C) Wide-field illumination is incorrectly aligned as the light beam only covers the dendritic arbor of one of the recorded neurons. Inputs that synapse onto dendrites outside the extent of the light beam are not activated, so inputs onto the leftmost cell cannot be reliably measured.

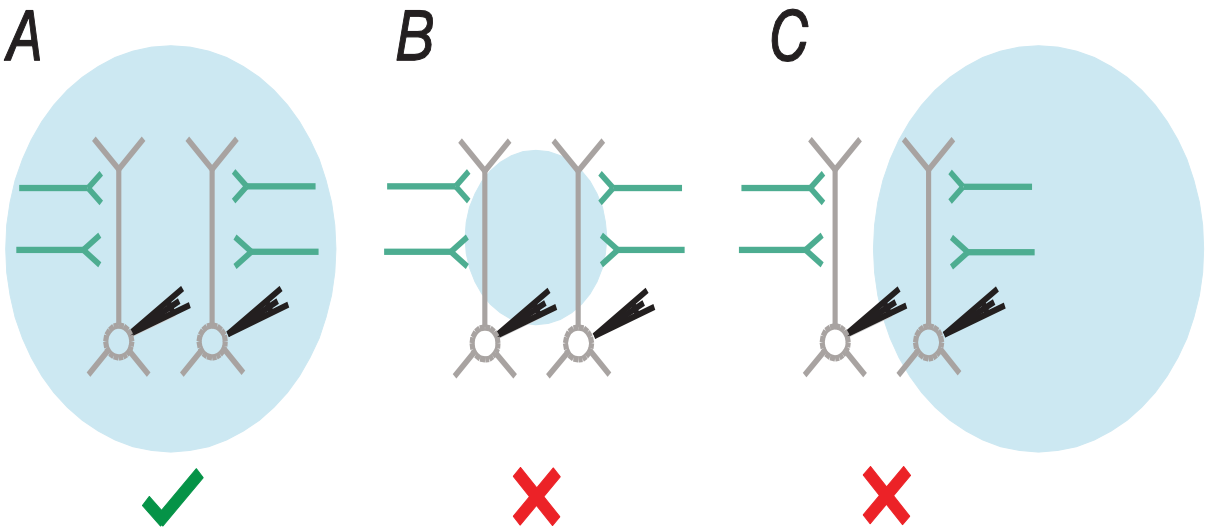

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Figure 3 Optimising LSPS laser calibration and event detection

A) Loose cell-attached recordings of action potentials (APs) evoked by glutamate uncaging in a layer 5a pyramidal cell in primary somatosensory cortex at postnatal day 8 (P8). The laser was fired (1 Hz) across a 50 μm resolution grid that covered the depth of a cortical column at this age. Decreasing laser intensity reduced the number of sites at which APs were elicited.

B) Average maps showing the distribution of points at which APs can be evoked. At high laser power (left) APs can be evoked across layers 4 to 5b as well as at a single point in layer 2/3. Using a low laser power (right) confined action potential generation to sites in the immediate layer 5a, providing good spatial resolution.

C) Identifying light-evoked synaptic responses is straight forward for distal presynaptic neurons (*left*). However targeting presynaptic neurons close to the recorded cell (*middle*) invariably results in a direct glutamate response (grey line, bottom traces) with an onset locked to the start of the laser pulse. Large direct responses can obscure evoked synaptic responses. Using a long duration, low intensity laser pulse (*right*) leads to a slower direct glutamate response from which synaptic responses are more readily extracted (see panel E).

D) Action potentials evoked from the same neuron upon laser stimulation across the entire depth of the cortex at the two laser intensities shown in panel B. The monosynaptic event windows (black dashed line box) begins at the earliest spike and ends 100 ms after the last spike is detected. Top trace recorded at 8.2 mW/cm^2, bottom trace, 1.1 mW/cm^2.

E) Example traces recorded from a layer 5a fast spiking (FS) interneuron: top trace, direct glutamate response evoked when the laser was fired at the cell body of the FS interneuron. Despite the careful calibration and slow laser pulse no EPSC could be extracted from this single spot. Second trace, EPSCs are evoked as a delay from the laser onset and can be extracted from the low amplitude direct response. Third trace, an EPSC evoked from a pyramidal cell distal from the dendritic arbor of the FS cell. Bottom trace, a spontaneous or polysynaptic EPSC (asterisk) that falls outside of the monosynaptic event detection window.

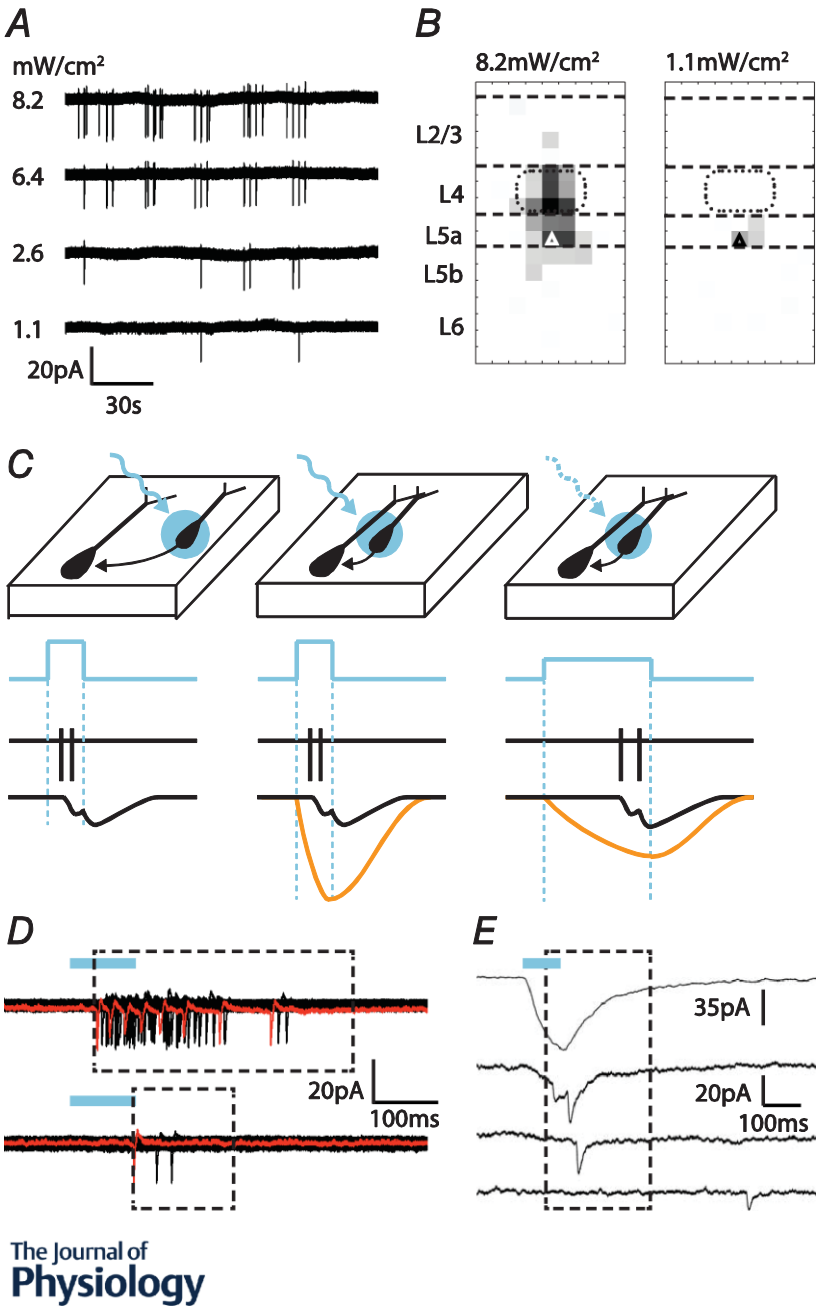

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Table 1. Methods to selectively label presynaptic or postsynaptic cells in neural circuits

Different methods that allow for the labelling and optogenetic manipulation of individual neuronal populations are indicated along with the types of neurons that can be labeled. The

suitability of each approach to study presynaptic inputs of postsynaptic responses along with the ability of each method to provide optogenetic access to specific or broad populations of presynaptic neurons is shown.

Abbreviations: PYR – Pyramidal neuron, IN- Interneuron, PRE- suitable for studying presynaptic cells, POST – suitable for studying postsynaptic cells. **References: 1** (Tang *et al.*, 2015); **2 (**Gong *et al.*, 2003) (see also www.gensat.org); **3** (Apicella *et al.*, 2012); **4** (Xu & Callaway, 2009)**; 5** (Taniguchi *et al.*, 2011); **6** (He *et al.*, 2016); **7** (Crandall *et al.*, 2015); **8** (Olsen *et al.*, 2012); **9** (Madisen *et al.*, 2012); **10** (Anastasiades *et al.*, 2016); **11** (Katzel *et al.*, 2011); **12** (Madisen *et al.*, 2010); **13** (Petreanu *et al.*, 2007); **14** (Petreanu *et al.*, 2009); **15** (Cruikshank *et al.*, 2010); **16** (Dimidschstein *et al.*, 2016); **17** (Little & Carter, 2013); **18** (Saunders *et al.*, 2012); **19** (Saunders & Sabatini, 2015); **20** (Wickersham *et al.*, 2007); **21** (Osakada *et al.*, 2011); **22** (Zingg *et al.*, 2017);

23 (Xu *et al.*, 2016); **24** (McGarry & Carter, 2016)**; 25** (Anderson *et al.*, 2010); **26** (Guenthner *et al.*, 2013).

Method	Cell types	Presynaptic/ postsynaptic	Optogenetic access (Y/N)	Specificity	References
BAC Lines	PYR/IN	POST (Mostly)	N (Y with virus)	Various interneuron and projection neuron classes	**1**, **2**, **3**, **4**
Cre/Flp Drivers	PYR/IN	PRE/POST	Y	Various interneuron and projection neuron classes	**5**, **6**, **7**, **8**
Optogenetic Reporters	PYR/IN	PRE	Y	Dependent on driver line	**9**, **10, 11**
Fluorophore Reporters	PYR/IN	POST	N	Dependent on driver line	**10, 12**
***In utero* electroporation**	PYR/IN	PRE/POST	Y	Dependent on stage of embryogenesis and target location	**13**, **14**

Viral Vector	PYR/IN	PRE/POST	Y	Non-specific, promoter specific, or Cre-dependent	**14, 15, 16**, **17**
Cre-Off Virus	PYR/IN	PRE/POST	Y	Non-Cre expressing neurons	**18**, **19**
Rabies Virus	PYR/IN	PRE/POST	Y	Mono-synaptically connected neurons	**20**, **21**
Anterograde-Cre Virus	PYR/IN	PRE	Y	Cells receiving input from presynaptic region	**22**
Retrograde-Cre Virus	PYR (Mostly)	PRE/POST	Y	Projection class	**23**
Retrograde Tracer	PYR (Mostly)	POST	N	Projection class	**3**, **17**, **24**, **25**
TRAP	PYR/IN	PRE/POST	Y	Active neurons	**2**

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