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An optogenetics- and imaging-assisted simultaneous multiple patch-clamp recording system for decoding complex neural circuits

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Abstract

Deciphering neuronal circuitry is central to understanding brain function and dysfunction, yet it remains a daunting task. To facilitate the dissection of neuronal circuits, a process requiring functional analysis of synaptic connections and morphological identification of interconnected neurons, we present here a method for stable simultaneous octuple patch-clamp recordings. This method allows physiological analysis of synaptic interconnections among 4–8 simultaneously recorded neurons and/or 10–30 sequentially recorded neurons, and it allows anatomical identification of >85% of recorded interneurons and >99% of recorded principal neurons. We describe how to apply the method to rodent tissue slices; however, it can be used on other model organisms. We also describe the latest refinements and optimizations of mechanics, electronics, optics and software programs that are central to the realization of a combined single- and two-photon microscopy-based, optogenetics- and imaging-assisted, stable, simultaneous quadruple–viguple patch-clamp recording system. Setting up the system, from the beginning of instrument assembly and software installation to full operation, can be completed in 3–4 d.

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AUTHOR CONTRIBUTIONS G.W. and J.J.Z. developed the optogenetics- and imaging-assisted simultaneous multiple patch-clamp recording system with inputs from D.R.W., T.L., X.J. and Y.S. Q.-Q.S. developed the laserspritzer technique. G.W., D.R.W. and W.Y. performed the experiments with technical assistance from Y.W., L.C.M., Q.-Q.S. and J.J.Z. G.W., D.R.W. and J.J.Z. wrote the manuscript with contributions from all other authors.

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INTRODUCTION

Modern electrophysiological tools have been used in all aspects of neuroscience research owing to their unparalleled high sensitivity and temporal resolution¹. The initial development of the simultaneous multiple (i.e., dual, triple and quadruple) patch-clamp recording method has greatly facilitated the investigation of communication between neurons and neuronal subcellular compartments *in vitro* and *in vivo*²⁻¹⁰. However, deciphering complex interconnected neuronal circuits, a process requiring functional analysis of multisynaptic and trans-synaptic connections and morphological identification of the cell types of many different interconnected neurons¹¹⁻¹³, remains a difficult endeavor. Because the testable connectivity pattern, C , increases exponentially as the number of simultaneously recorded neurons increases, n , or $C = 4^{n(n-1)/2}$, simultaneous patch-clamp recordings from four or more neurons exponentially increases the chance to decode and interrogate complex neuronal circuits. This principle was first verified by the Markram and Petersen groups¹⁴⁻¹⁶, who investigated the synaptic interconnections of excitatory neurons in the cortex with simultaneous hextuple–duodecuple patch-clamp recordings. By working with engineers and technicians in commercial companies and local university workshops, we have recently overcome various mechanical, electronic and software barriers to develop a stable simultaneous octuple patch-clamp recording technique that allows the recovery of the detailed morphology of >99% of recorded excitatory neurons and >85% of recorded interneurons^{17,18}. The technique thus makes it easy to study and identify inhibitory neurons, which frequently possess a dense intricate axonal arborization, in complex neuronal circuits. With this technique, we have recently deciphered two trans-synaptic disinhibitory and inhibitory neuronal circuits that span multiple layers and columns in the rat cortex^{17,18}, further validating the advantage of making simultaneous patch-clamp recordings from four or more neurons. In this protocol, we describe the octuple patch-clamp recording technique, as well as the latest refinements and optimizations of mechanics, electronics, optics and software programs that may allow the realization of an optogenetics- and imaging-assisted stable simultaneous multiple (from quadruple up to viguple) patch-clamp recording system for functional interrogation of more complex neuronal circuits.

Mechanics

To accommodate eight or more motorized manipulators in a simultaneous multiple patch-clamp recording setup, compact manipulators are essential. In addition, these manipulators need to be stable enough for long-lasting recordings (1–2 h) to be achieved. We found that it typically took ~1–2 h to thoroughly investigate all synaptic connections formed among eight recorded neurons and to load a sufficient amount of cell marker (e.g., biocytin) to recover the complete cell morphology for the identification of recorded neurons, particularly interneurons. In addition, stable manipulators allow easy replacement of new patch pipettes, and thus they facilitate the sequential multiple patch-clamp recordings⁹. A large variety of motorized manipulators are currently available on the market. We chose L&N motorized manipulators (Luigs-Neumann), because over the years the company has been continuously improving the manipulators to keep up with our increasing demands with respect to stability and compactness. The MINI series of L&N manipulators were initially invented in 1992. These manipulators had their motor resolution refined to 9.8 nm per step and movement

reproducibility improved to $<1\ \mu\text{m}$ when we started to build our simultaneous octuple patch-clamp recordings setup in 2008 (**Fig. 1a**). We found that the MINI manipulators produced very smooth and highly reproducing movements, ideal for multiple stable recordings, and they were small enough to fit eight of them around a standard microscope. However, we soon realized that the mechanical disruption associated with electrode replacement became a more significant issue with the increased number of neurons recorded. In response to our technical request and prototype suggestion for improvement, Luigs-Neumann developed multiple generations of adapters for the electrode headstage in the following 2 years. Our tests showed that the third generation of adapters, the guide rails, performed far better than the two older versions: the first generation of turning adapters and second generation of backfolding adapters. The guide rail exchange system allows the electrode holders to slide backward and forward along the manipulators to replace electrodes, thus largely eliminating the mechanical disruption associated with the turning or backfolding adapters. Therefore, the system allows replacement of multiple patch pipettes multiple times to record many additional neurons without jeopardizing the existing recordings^{17,18}.

To minimize the size of the manipulators, Luigs-Neumann developed the smaller JUNIOR manipulator in 2002 (**Fig. 1b**). However, the movement and stability of the first version of JUNIOR manipulators were not ideal. In 2010, the company completely redesigned the JUNIOR manipulator, and the new JUNIOR manipulator has the motor resolution of 7.8 nm and reproducibility of $<1\ \mu\text{m}$. We used the new JUNIOR manipulators in simultaneous multiple patch-clamp recordings. We found that the JUNIOR manipulators had movement resolution and stability comparable to the MINI manipulators. Therefore, both the MINI and JUNIOR L&N manipulators are excellent choices for simultaneous multiple patch-clamp recordings.

In 2013, Luigs-Neumann released a modified version of its JUNIOR manipulator, the JUNIOR COMPACT manipulator. The y axis width of the manipulator was further minimized to $\sim 50\ \text{mm}$, which is much smaller than the $\sim 100\text{-mm}$ -wide JUNIOR manipulators or $\sim 150\text{-mm}$ -wide MINI manipulators (**Fig. 1b**). Our recent tests showed that the JUNIOR COMPACT manipulator had the same movement resolution and stability as the MINI and JUNIOR manipulators. The exceptionally small size of JUNIOR COMPACT manipulators makes it possible to accommodate 8–14 manipulators (octuple–quattuordecuple patch-clamp recordings) at a standard microscope or to realize a 20-patch (viguple patch-clamp recordings) system on a 360° ring structure with a modified microscope. Moreover, the JUNIOR COMPACT manipulators alleviate the space competition among the instruments for electrophysiology, two-photon laser-scanning imaging and optogenetics. The reduction in space competition should be particularly substantial in terms of improving the simultaneous multiple *in vivo* patch-clamp recording technology because two-photon imaging can improve the targeting of *in vivo* patch-clamp recordings^{19,20}, and optogenetics may help in searching and investigating synaptic connections in brain tissue slices and intact brains of anesthetized and behaving animals.

Electronics

Stable patch-clamp recordings have paved the way to effectively manipulate and detect neuronal activities, yet the manipulation and detection of neuronal activity are best achieved with high-quality and low-noise amplifiers. Typically, one amplifier is needed for each recorded neuron. We recommend the Axoclamp 2A/B and Axopatch 200B amplifiers (Molecular Devices) for voltage (current-clamp) and current (voltage-clamp) recordings, respectively, owing to their unsurpassed low-noise performance and high compatibility with customizable operation software programs. Alternatively, one may choose the newer versions of Axon amplifiers, MultiClamp 700A/B. The MultiClamp 700A/B amplifiers are versatile amplifiers with two primary head-stages, which are intended to support many electrophysiology applications, including voltage or current recordings from two neurons. These amplifiers are computer-controlled and they may only run under its designed program, the Axon MultiClamp Commander.

To control the operation of an amplifier and to receive the experimental data collected by the amplifier, a computer is needed. To realize the communication between the multiple amplifiers and the computer, data acquisition (DAQ) interface boards with multiple digital-analog output (D/A) and analog-digital input (A/D) channels are preferred. In general, at least three channels are needed for operation control and data collection of an amplifier, with one D/A channel dedicated to its external command port and two A/D channels dedicated to its current and voltage output ports, respectively. Thus, for a simultaneous octuple patch-clamp recording setup, a DAQ board with eight D/A channels and 16 A/D channels would be ideal. However, to the best of our knowledge, no such single interface board is commercially available. Therefore, in general, combining multiple interface boards is necessary to achieve eight or more simultaneous patch-clamp recordings (**Fig. 2**). An InstruTECH ITC-18 DAQ board (HEKA Instruments) has four D/A channels and eight A/D channels, which can thus support simultaneous recordings from four neurons. The electronics of the ITC-18 board are optically isolated from the amplifiers and computer, which is ideal for low-noise performance. We found it possible to use one computer to control multiple ITC-18 boards to realize operation and data collection of eight or more amplifiers. In principle, we operated one ITC-18 board as the ‘master’ board. When this ‘master’ ITC-18 board started to send operation commands and acquire data, it also sent out a digital trigger signal to one or multiple other ITC-18 board(s) set to the external trigger mode. In this way, the computer could simultaneously control operation and data collection (both voltage and current data) of eight or more amplifiers.

Alternatively, one may use DAQ boards manufactured by National Instruments. The National Instruments Corporation offers a large variety of DAQ boards (NI boards) with various numbers of A/D and D/A channels. Given the fast sample rate (up to 10 MHz), NI boards are perfect for imaging studies, yet they are also commonly used for electrophysiology recordings. Again, a combination of two or more NI boards is required to run eight or more simultaneous patch-clamp recordings. As with ITC-18 boards, one may use the computer to activate one NI board and then use this NI board to trigger itself, as well as other NI boards, to control operation and data collection of eight or more amplifiers. Combining ITC-18 and NI DAQ boards is also possible. We have verified that one computer

can control multiple ITC-18 and NI boards to operate simultaneous electrophysiology recordings, two-photon laser-scanning imaging and/or optogenetics.

The most recent versions of DAQ boards from Molecular Devices are the Axon Digidata 1440 board, which has four D/A channels and 16 A/D channels, and the Axon Digidata 1550 board, which has eight D/A channels and eight A/D channels. As with single ITC-18 and NI boards, one Axon Digidata 1440 board may run simultaneous dual–quadruple patch-clamp recordings because it can control the operation of up to four primary headstages of two MultiClamp amplifiers via four D/A channels. The Axon Digidata 1550 board can simultaneously support the operation and data collection of either voltage or current with up to four Axon MultiClamp amplifiers under Axon MultiClamp Commander. Therefore, the Axon Digidata 1550 board can run four Axon MultiClamp amplifiers to perform simultaneous octuple patch-clamp recordings collecting either voltage or current data. However, according to Molecular Devices, the Axon Digidata boards are not designed to operate in combination with other boards from the same or different companies.

Optics

To perform optogenetics experiments, stimulation lasers can be delivered to the tissues via optical fibers and/or objective lenses. A modified optical fiber approach is the laserspritzer, which can improve the spatial resolution of light illumination spots. An alternative way to improve the spatial resolution is to combine single- and two-photon stimulation. We found that it was possible to steer the light beam of diode-pumped solid-state (DPSS) lasers into the light path of a two-photon laser-scanning microscope. This enabled the optogenetic activation of neurons with single-photon laser pulse and/or two-photon laser-scanning stimulations in the same experiments. The single- and two-photon laser-focusing spots could be aligned in the same focal plane and controlled by a single pair of scanning mirrors. To obtain the smallest laser spots, laser beams should be expanded by a telescope consisting of scanning and tube lenses to fill the entire rear aperture of the objective.

Software

IGOR Pro (WaveMetrics) is an interactive software environment that was chosen to carry out experiment and data analysis when the simultaneous dual, triple and quadruple patch-clamp recording techniques were initially developed. Over the years, many IGOR-based programs have been custom-written to handle the operation, online and off-line data analysis of the experiments involved in multiple patch-clamp recordings and imaging applications. As IGOR Pro can support multiple DAQ boards by the XOP file released by HEKA Instruments, we upgraded an IGOR-based program used for quadruple patch-clamp recordings to support multiple ITC-18 boards and eight or more amplifiers (**Fig. 3a**). However, for ITC-18 boards running in the external trigger mode, acquisition would not stop automatically even when the designated amount of data had been acquired. Thus, a stop command must be sent in time to prevent the FIFO memory of ITC-18 boards from overflowing. Moreover, the available sample interval setting in ITC-18 boards is related to the number of channels in operation. Therefore, to match the sample interval of multiple ITC-18 boards, one should ensure that the same number of channels in ITC-18 boards is operating the entire time. Finally, the sample rate of ITC-18 boards is too slow to support

fast DAQ—e.g., two-photon laser-scanning imaging. To accommodate two-photon imaging, one may use the NIDAQ Tools MX package, which adds support for DAQ directly into IGOR Pro. With the NIDAQ Tools MX package, we were able to use a single IGOR-based program to simultaneously run multiple NI boards to control two-photon laser-scanning imaging (and optogenetics) in addition to ITC-18 boards that operate multiple patch-clamp recordings (**Fig. 3b,c**).

Besides IGOR, MATLAB (MathWorks) is another widely used interactive software environment. MATLAB-based Ephus has been developed for cellular electrophysiology applications²¹. We found that Ephus could run simultaneous dual recordings with its nonstandard customized routines. The program is structured in a way that it can, in principle, support multiple recordings, including octuple patch-clamp recordings, but to the best of our knowledge this particular application has not yet been officially verified. Currently, the programmers of Ephus are working on a successor version of Ephus to include routines that will make it easy for the application of Ephus in controlling eight or more simultaneous patch-clamp recordings. It is worth noting that Ephus can be easily linked with ScanImage, a coevolved, powerful dedicated software package for two-photon laser-scanning microscopy²². Adapting Ephus to run eight or more simultaneous patch-clamp recordings should be an excellent alternative to satisfy the desire of combining electrophysiological recordings, two-photon laser-scanning imaging and photostimulation.

It seems to be possible to use IGOR- and MATLAB-based software programs to communicate with Axon MultiClamp amplifiers using boards other than the Axon Digidata boards (e.g., ITC-18 and NI boards)²³⁻²⁷. The approaches provide alternative solutions to run multiple Axon MultiClamp amplifiers to achieve simultaneous multiple patch-clamp recordings with two-photon laser-scanning imaging and optogenetics.

Experimental design

Although simultaneous multiple patch-clamp recordings are powerful in decoding complex neuronal circuits, the technique only works when all the components of the circuits (i.e., presynaptic neurons, postsynaptic neurons and their synaptic connections) are intact in the tissue preparations. Therefore, it is essential to have optimized brain slice preparations and high-quality patch-clamp recordings, which permit the relatively unbiased interrogation of local neuronal circuits with their components located as far as ~500–1,000 μm apart^{4,8,9,14-18}. To optimize brain slice preparations, we recommend the use of a high-quality microslicer that can generate large-amplitude and high-frequency movements of the cutting blade in a horizontal axis with minimal vibrations in the vertical axis, which may confine tissue damage to within ~10 μm below the cutting surface and thus produce brain slices with the best quality²⁸. We also recommend cutting tissues at an angle closely parallel to the projecting dendrites and/or axons, such as the apical dendrites of cortical layer 5 (L5) pyramidal neurons²⁹⁻³¹, which is crucial for avoiding the truncation of selective populations of dendrites and axons^{17,18}. To achieve high-quality recordings, we recommend using the low-noise amplifiers, proper arrangement of connecting electric circuits and extensive practice of patch-clamp recording skill before the actual experiments. The averaged somatic whole-cell recording traces obtained from the high-quality recordings will have a solution to

detect ~ 10 μV (in the current-clamp mode) or ~ 0.1 pA (in the voltage-clamp mode) events^{14,15,17,18}. Considering the conductance of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and γ -aminobutyric acid (GABA) receptor channels, as well as dendritic filtering effects²⁹, the smallest excitatory and inhibitory synaptic inputs from the distal dendrite would be ~ 50 μV or ~ 0.5 pA when they arrive at the soma. Thus, high-quality patch-clamp recordings can detect these smallest synaptic events, as has been experimentally verified^{14,15,17,18}. We do not recommend the use of a low- or no-sodium slicing solution as a substitute for a high-quality microslicer and/or a proper slicing procedure. This is because many superficial neurons, although they may survive the slicing process in the low- or no-sodium solution, have their dendrites and axons severely amputated. Recording from such neurons will interfere with achieving a more accurate measurement of synaptic connectivity and *post hoc* cell identification. Although programming the motorized manipulators to move patch pipettes to positions just above the targeting tissue areas helps speed up experiments, we also do not recommend the use of automated patch-clamp systems, as there is currently no patch-clamp algorithm designed to match the flexibility of a skilled patch-clamp experimenter. We noted that on the basis of the slightly different conditions of individual neurons, skilled electro-physiologists are able to make subtle modifications of parameters of all patch-clamp steps (e.g., size of pipette, position to target pipette, amount of positive and suction pressure applied, speed of suction and time to wait on sealing before break-in) to achieve high-quality recording from every single neuron.

Of course, preparing healthy brain slices containing intact presynaptic neurons, postsynaptic neurons and their connections, particularly when long-distance circuits are examined, is not always possible. In these cases, or to further investigate the incoming axonal fibers originated from other brain areas or subareas, adding optogenetics to the procedure would be a solution^{32,33}. Furthermore, the multiple *in vivo* patch-clamp recordings technique, which can be combined with extracellular recordings³⁴, may be used to reveal and verify the key features of the organization of neuronal circuits¹⁷. As with the *in vitro* preparations, high-quality animal preparation is essential, as injury, for example, at the cortical surface, could preclude high-quality recordings (and imaging) from cortical neurons and apical dendrites in the superficial layers in these *in vivo* experiments^{17,35–37}. At the moment, the yield for detecting neuronal circuits with multiple *in vivo* patch-clamp recordings is fairly low^{17,38}. However, we expect this to be improved when two-photon imaging and/or optogenetics are combined to help search for the connections.

Combination with other techniques

The power of simultaneous multiple patch-clamp recordings in decoding complex neuronal circuits can be boosted when combined with electrophysiological, genetic, optogenetic and/or imaging monosynaptic connection ‘search’ techniques in actual *in vitro* and *in vivo* experiments. The electrophysiological search technique was initially developed by Feldmeyer and colleagues⁶. This approach uses a relatively higher impedance (6–10 M Ω) patch pipette as a search electrode to form a loose seal (~ 30 –300 M Ω) on potential presynaptic neurons and elicit action potentials in the neurons with high-intensity (~ 0.2 –2 nA), short (~ 5 –10 ms) current pulses. When the current pulse stimulation induces coincident

unitary excitatory or inhibitory postsynaptic potentials in recorded postsynaptic neurons, an indicator of putative synaptic connection, the search electrode will be repositioned to test other potential presynaptic neurons or it will be removed. The normal patch pipettes will then move in to patch the putative presynaptic neurons and to fully characterize the synaptic connections between these putative presynaptic and postsynaptic neurons. Given the improved stability associated with the updated electrode exchange/motorized manipulator system, electrophysiological search techniques can be applied repetitively and intermittently with simultaneous multiple patch-clamp recordings during individual experiments to reveal more complex (e.g., trans-synaptic) neuronal circuits.

The first genetic approach, invented by the Callaway laboratory, uses the rabies virus–based monotrans-synaptic tracing technique^{39,40}. The technique uses a modified rabies virus that can only retrogradely cross single synapses to label a small population of presynaptic neurons. This technique anatomically identifies a few monosynaptically connected neurons that can be used as the starting point for simultaneous multiple patch-clamp recordings to reveal more complex neuronal circuits involved in these and other neurons. The other genetic approach, developed by Kim and colleagues^{41,42}, is the GFP reconstitution across synaptic partners technique (GRASP)^{41,42}. GRASP is based on the functional complementation between two nonfluorescent GFP fragments expressed at the presynaptic and postsynaptic neurons, respectively. If the nonfluorescent GFP fragments can be highly expressed only in single presynaptic or postsynaptic neurons within designated areas, GRASP may be potentially used as a way to identify the putative monosynaptically connected neurons for physiological analysis, although this idea has not yet been validated.

A recently invented optogenetic search technique uses a fine optic fiber–based laserspritzer to locally deliver laser light to activate presynaptic neurons⁴³. The search laserspritzer is manufactured by pulling the core optic fiber of a multimode fiber optic patch cable under a fire to generate an ~5–10- μm -diameter tip (**Fig. 4a**). The optic fiber tip is then coated with the glass thawed from a patch pipette with an ~30–50- μm -diameter tip (**Fig. 4b**). With 0.1–0.8 mW/mm² laser power, the optic fiber tip produces an ~10–30- μm -diameter light spot that can effectively activate single neurons expressing channelrhodopsin 2 (ChR2) and induce unitary excitatory/inhibitory postsynaptic potentials in postsynaptic neurons (**Fig. 4cd**). With a laser-spritzer, a large number of potential presynaptic neurons may be quickly scanned. Simultaneous multiple patch-clamp recordings can then be applied to make a full investigation of the putative and other potential synaptic connections.

The newest channelrhodopsin actuator, CheRiff, produces approximately twofold-larger maximal photocurrent (~2 nA), and it has an approximately ninefold increased photon sensitivity compared with ChR2 (ref. 44). This makes it possible to use high-magnification objective lenses (i.e., 40 $\times$ or 60 $\times$) to focus the laser on the soma of CheRiff-expressing neurons to elicit action potentials without applying the high laser illumination intensity that can injure neurons. Specifically, we found that to elicit action potentials in all L5 pyramidal neurons the illumination intensity for ChR2-expressing neurons was larger than 2 mW, whereas that for CheRiff-expressing neurons was less than 200 μW (**Fig. 5a**). Two-photon laser-scanning stimulations provide a better spatial resolution of laser illumination spots than single-photon laser pulse stimulations. However, we found that even by applying the various

scanning techniques (e.g., spiral scanning, temporal focusing, lower numerical aperture objective and their combinations) to increase the scanning area, two-photon laser-scanning stimulations could only produce a maximal photocurrent of ~200–400 pA in CheRiff-expressing L5 pyramidal neurons, which was insufficient to elicit action potentials in the majority of L5 neurons. This is consistent with the notion that two-photon laser-scanning stimulations only stimulate a small fraction of the somatic membrane at the z -axis compared with single-photon stimulations. Nevertheless, the increased CheRiff photocurrent makes it possible to combine single- and two-photon laser stimulations to increase the spatial resolution of laser activation areas. Our experiments showed that the combined single- and two-photon laser stimulation had increased half-height spatial resolution (~30 μm) compared with the single-photon stimulation (~60 μm ; **Fig. 5b,c**). By using the combined single- and two-photon laser stimulation, we could search the putative presynaptic neurons and then confirm the synaptic connections with subsequent multiple patch-clamp recordings (**Fig. 5d–f**). It should be pointed out that the search method was only effective in areas with sparse neurons expressing CheRiff, because we frequently had false-positive connections owing to the activation of bypassing axons of other expressing neurons. Further improving the actuator construct to express CheRiff only in the soma and/or increase its photocurrent (permitting the use of more two-photon-dependent better spatial resolution photonstimulations) should make this an efficient search approach for identifying putative monosynaptic connections.

Finally, imaging techniques have also been used to identify monosynaptic connections. One early technique used current injections to evoke a train of action potentials in ‘trigger’ neurons while optically monitoring a large number of neurons loaded with calcium indicators to identify ‘follower’ neurons⁴⁵. This method allowed the detection of the putative postsynaptic neurons receiving strong facilitating excitatory synaptic connections large enough to produce action potentials in the follower neurons. To detect weaker excitatory synaptic connections or inhibitory synaptic connections typically observed in central neuronal circuits, scientists have since developed a large number of GFP-based and rhodopsin-based genetically encoded voltage indicators. The most recently developed voltage indicators, QuasArs, Archers and ASAP1, have a voltage sensitivity F/F of ~30–90%/100 mV, and they can thus detect postsynaptic voltage responses of ~1–5 mV, thereby enabling the optical recording of single action potentials^{44,46,47}. However, QuasArs show a rapid reduction in voltage sensitivity after a few repetitive-light illustrations⁴⁴, whereas Archers and ASAP1 have a slow response time of multiple milliseconds^{46,47}. These imperfections hamper the use of the averaging strategy that permits the detection of smaller postsynaptic responses that are commonly found at neuronal connections.

In summary, these searching techniques can identify anatomical or putative functional monosynaptic neuronal connections. However, the currently available search methods usually do not recover the cell morphology to unambiguously define the neuron identity. Moreover, both the spatial (e.g., selectively activating presynaptic neurons) and signal (e.g., detecting common small postsynaptic responses) resolution of the optogenetics and imaging search methods remain to be improved (by ~10–100-fold) to be comparable to simultaneous multiple whole-cell recordings. Therefore, although the imaging and optogenetics methods

can sometimes help quickly premap the putative neuronal connections, at the moment, they serve more as an aid than a replacement of simultaneous multiple patch-clamp recording technique in deciphering complex neuronal circuits. In the other situations, for example, when the connectivity of neuronal circuits is investigated, directly applying the multiple patch-clamp recording technique to randomly target all neurons in the entire area without referring any connectivity clues (e.g., those from the search techniques) would be a less biased and more productive approach^{17,18}.

MATERIALS

REAGENTS

- Experimental animals: rodents (neonatal, developing or mature male and female Sprague-Dawley rats and C57BL/6J mice)
- **! CAUTION** Animal experiments must conform to all relevant governmental and institutional regulations.
- Carbogen (95% oxygen, 5% carbon dioxide)
- Sodium pentobarbital
- Artificial cerebrospinal fluid (ACSF; see Reagent Setup)
- Biocytin (Sigma-Aldrich, cat. no. B4261-5MG)
- CaCl₂ (Sigma-Aldrich, cat. no. 223506-2.5KG)
- Cesium methanesulfonate (CH₃O₃SCs; Sigma-Aldrich, cat. no. C1426-5G)
- D-(+)-Glucose (dextrose, Sigma-Aldrich, cat. no. G8270-1KG)
- EGTA (Sigma-Aldrich, cat. no. E3889)
- HEPES (Fisher, cat. no. BP310-1)
- KCl (Fisher, cat. no. BP366-1)
- Potassium gluconate (Sigma-Aldrich, cat. no. P1847)
- KOH (Sigma-Aldrich, cat. no. 319376)
- MgATP (Sigma-Aldrich, cat. no. A9187-100MG)
- MgCl₂ (Sigma-Aldrich, cat. no. 208337-1KG)
- Na₃GTP (Sigma-Aldrich, cat. no. G8877-10MG)
- NaH₂PO₄ (Sigma-Aldrich, cat. no. S8282)
- NaHCO₃ (JT Baker, cat. no. JT3506-5)
- Phosphocreatine disodium salt (C₄H₈N₃O₅PNa₂, Sigma-Aldrich, cat. no. P7936-5G)
- Double-distilled water
- Acrolein (Sigma-Aldrich, cat. no. 110221-25ML)

- Paraformaldehyde (Sigma-Aldrich, cat. no. P6148-500G)
- OsO₄ (Electron Microscopy Sciences, cat. no. 19105)
- Uranyl acetate (Electron Microscopy Sciences, cat. no. 22400-1)

EQUIPMENT

- Antivibration air table (e.g., Newport)
- Dissection tools (e.g., Fine Science Tools; scissors, forceps, scalpel and spatula)
- High-quality vibrating tissue slicer (e.g., Ted Pella, Microslicer DTK-1000)
- Razor blades for slicing (e.g., Gillette)
- Cyanoacrylate glue (e.g., Krazy glue)
- Filter paper (e.g., Fisher Scientific)
- Incubation chamber: ideally this should be a submerged chamber, often in a glass beaker, which allows for sufficient oxygenation of the slices during the recovery period along with dividers to keep the slices separate
- Water bath
- Microscope
- Water-immersion objective (40×)
- Intermediate magnification to achieve sufficient magnification
- Platinum ring covered with a grid of nylon strings
- Heater
- Osmometer (e.g., Wescor, Vapro 5600)
- Horizontal electrode puller (e.g., Sutter Instruments, Brown-Flaming P-87)
- Stable motorized micromanipulators (see INTRODUCTION)
- Patch-clamp amplifiers (e.g., Axoclamp 2A/B and Axopatch 200B amplifiers, see INTRODUCTION)
- Interface adapters (e.g., USB-18 and/or PCI-18, see INTRODUCTION)
- Computer interface (e.g., ITC-18 and/or NI BNC, see INTRODUCTION)
- Video monitor
- Software (e.g., IGOR Pro, see INTRODUCTION)
- Thick-walled borosilicate glass (e.g., Sutter Instrument, cat. no. B200-116-10)
- 473-nm DPSS laser (Coherent, OBIS 473 LX)
- Femtosecond Ti:sapphire laser (Coherent, Chameleon Ultra)
- Fiber optic patch cable (Thorlabs, cat. no. M38L02, diameter 200 μm)

- Scanning mirrors (Cambridge Technology, cat. no. 6210H)
- Shutters (Uniblitz, cat. nos. UHS1 and CS45)
- Pockels cell (Conoptics, cat. no. M350-80)
- Photodiode detector (Thorlabs, cat. no. PDA100A)
- Polarizing beamsplitter (e.g., Newport, cat. no. 05FC16PB.5)
- Mirrors (e.g., Newport, cat. no. 10Z20ER.2)
- Transmission electron microscope (e.g., EOL, JEOL-1230 transmission electron microscope)

REAGENT SETUP

ACSF—ACSF is 125 mM NaCl, 2.5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 1.25 mM NaH₂PO₄, 25 mM NaHCO₃ and 25 mM dextrose (pH 7.4). Use double-distilled water. Measure the osmolarity using a vapor pressure osmometer. ACSF should be ~300 mmol/kg. Throughout the procedure, ACSF must be saturated with carbogen. ACSF should be stored at 4 °C and used within 2–3 weeks.

Intracellular solution—Intracellular solution is 135 mM CH₃O₃SCs, 10 mM HEPES, 2.5 mM MgCl₂, 4 mM Na₂ATP, 0.4 mM Na₃GTP, 10 mM disodium phosphocreatine, 0.6 mM EGTA, 0.1 mM spermine and 0.5% (wt/vol) biocytin, for current recordings, and 120 mM potassium gluconate, 10 mM HEPES, 4 mM KCl, 4 mM MgATP, 0.3 mM Na₃GTP, 10 mM disodium phosphocreatine and 0.5% biocytin, for voltage recordings (pH 7.25, osmolarity ~310 mmol/kg for both solutions). Intracellular solutions are made in 1-ml aliquots and stored at –20 °C for up to 6–12 months.

Patch-pipette solution for searching in cell-attached mode—This solution should be based on the intracellular solution (in case of accidental break-through) but devoid of biocytin and very high potassium⁶. This solution can also be made in 1-ml aliquots and stored at –20 °C for up to 6–12 months.

EQUIPMENT SETUP

Patch-clamp setup—A description of the equipment needed for patch-clamp recording experiments can be found in the Axon Guide from Molecular Devices and a previous protocol³⁰ (see also the INTRODUCTION).

Platinum ring—This is a flattened platinum ring covered with a grid of nylon strings used for holding down the slice during recording⁴⁸.

Laserspritzer—A laserspritzer can be fabricated from a multimode fiber optic patch cable⁴³. The core optic fiber is exposed by stripping off the optic patch cable cladding; the fiber is then heated by a homemade syringe gas burner and gently pulled to make a tip size of ~5 μm (**Fig. 4a**). The pulled end of the optic fiber is inserted through a glass pipette with an opening of ~20–30 μm in diameter, pulled through the opening by ~30–50 μm under a

microscope, and the tip of the glass pipette is then melted by a heat gun to seal the optic fiber (**Fig. 4b**).

Optical setup—To enable single- and/or two-photon stimulation, we combined the beams of a 473-nm DPSS laser and a femtosecond Ti:sapphire laser using a polarizing beamsplitter. The laser beams then were coupled into the light path of a custom-made two-photon laser-scanning microscope. The power of the femtosecond laser was controlled by a Pockels cell. The dichroic mirrors in the microscope were used to aid the fine adjustment of DPSS laser intensity in the low-power range. The Pockels cell and/or Uniblitz shutters were used to control the laser pulse durations, and a pair of scanning mirrors were used to control the position of laser spots.

PROCEDURE

Hardware wiring ● TIMING ~4–12 h

1| Mount DAQ boards into the computer.

For Instrutech ITC-18 DAQ boards, mount USB-18 or PCI-18 host interface adapters into a computer and connect them to ITC-18 interfaces. For NI DAQ boards, mount boards into the computer by proper data bus and connect them to compatible BNC adapters.

2| According to your trigger strategy, wire all DAQ boards together.

3| Connect all patch-clamp amplifiers to DAQ interfaces, with BNC cables for current and voltage output ports connected to A/D ports, and external command ports connected to D/A ports of DAQ interfaces (**Fig. 2**).

4| Connect all headstages to the patch-clamp amplifiers.

5| Mount micromanipulators around the recording chamber and adjust their positions.

6| Mount all headstages onto the micromanipulators.

7| Connect the micromanipulators to their controllers and control pads/wheels/joysticks. For L&N manipulators, connect controllers together and set them as master or slave properly so that all manipulators can be controlled by a single SM7/8 pad. All the connections can be double-checked during the break period.

▲ **CRITICAL STEP** Proper arrangement and wiring of instruments reduces the noise level, and thus it decreases the number of recording traces needed to detect synaptic connections.

Software installation ● TIMING ~4–12 h

8| Install drivers for DAQ boards.

9| Install recording software, including IGOR Pro 6.0, XOP files for DAQ boards and custom-written DAQ and analysis programs. The operation of the system can be tested with model cells during the break period.

? TROUBLESHOOTING

Brain dissection and preparation of acute slices ● TIMING ~75–120 min

10| Fill a 100-ml beaker with ACSF and place it on ice with salt. Pour ice water around the stage of the microslicer.

In a 250-ml beaker, insert a submerged slice chamber with dividers. Fill the beaker with ACSF to just below the top of the chamber and place it in a 37 °C water bath. Bubble the ACSF in both beakers to saturation with carbogen.

▲ CRITICAL STEP Allow sufficient time for ACSF to cool (until the ice in ACSF is visible) and saturate with carbogen.

▲ CRITICAL STEP While waiting for the ACSF to cool, you can proceed to the next steps to prepare the animal and the microslicer.

11| Anesthetize the animal (e.g., with an intraperitoneal (IP) injection of sodium pentobarbital at 90 mg/kg of body weight).

! CAUTION Follow appropriate guidelines and regulations for animal experiments.

12| Once deep anesthesia has been established, decapitate the animal with large scissors or a guillotine. Cut the skin along the midline to reveal the skull. By using small scissors, cut the skull with slight upward pressure. Peel the skull back with tweezers. Immediately pour icy ACSF over the brain. By using a scalpel, cut along the midline of the brain and extract the brain into the 100-ml beaker filled with icy ACSF. Depending on the size of the tissue block extracted, one should allow 10–30 s for the brain to cool down at this point.

13| Apply a thin layer of glue onto the platform in the chamber of the microslicer. Use a spatula to transfer the brain onto a piece of filter paper to remove excess ACSF. Make sure that the midline of the brain is sufficiently dry.

14| Gently place the brain on the platform in the cutting chamber. After the brain is sufficiently glued in place, submerge the brain in the microslicer chamber with icy ACSF.

▲ CRITICAL STEP The time from decapitation to submersion of the brain in icy ACSF, along with the time the brain is out of solution, must be kept to a minimum.

15| Begin slicing the brain. After removing a small initial section to examine the region of interest, cut slices at ~300–400 µm. Ensure that the speed of the slicer, along with the vibration amplitude and frequency, are optimal for slicing without compressing the brain. The optimal settings will depend on the type of slicer used.

▲ CRITICAL STEP Optimizing the slicing settings will facilitate the production of the highest-quality tissue slices. The slicing speed should be lower for both the softer neonatal and harder adult and aged brain tissues²⁹.

16| Transfer slices to the incubated chamber after each slice is obtained. Keep track of the order of each slice. The use of a partition helps keep the slices separated and ordered. Incubate slices in oxygenated ACSF for 30–60 min at 37 ± 0.5 °C.

Visualization and determination of the optimal slice ● TIMING ~15–30 min

17| Transfer a cortical brain slice to the recording chamber on the stage of an upright microscope. The recording bath should be continuously perfused with oxygenated ACSF and held at 34.0 ± 0.5 °C. Orient the brain to help identify neurons from distinct layers. Hold the slice in place on the chamber by placing a platinum ring (see Equipment Setup) covered with a grid of nylon strings on top of the slice.

? TROUBLESHOOTING

18| By using a 40× water-immersion lens with a 1.5× magnifier, assess the overall health of the tissue and cells. Identify a L5 pyramidal neuron in the cortex and visually track its apical dendrite toward L1.

▲ **CRITICAL STEP** In an ideal slice, the entire apical dendrites of many L5 pyramidal neurons can be visually tracked with minimal focusing through the slice. Typically, only one or two slices per animal meet this requirement.

? TROUBLESHOOTING**Octuple patch clamping ● TIMING ~30–45 min (~4–5 min per cell)**

19| Once the ideal slice has been chosen, examine the area of interest for cells. Identifying healthy cells and then determining which electrode would best patch which cell will help expedite the patching process. When search techniques are used, we recommend capturing transmitted light images of the cells in the area with the landmarks to help find the putatively connected cells (compare with Le Be and Markram¹⁴).

20| By using a needle and filter, fill a glass electrode with enough intracellular solution to cover the electrode wire. Insert the electrode into the pipette holder and firmly seal the holder.

21| Apply positive pressure (~20–60 mbar) to the pipette holder chamber via the connected tubing attached to the side of the pipette holder. Maintain the pressure by closing a valve. Repeat this step for all electrodes.

22| Move a pipette into the bath and underneath the objective. Make sure that the tip of the pipette is not clogged, and that there is a steady stream of ACSF from the tip.

23| Decrease the positive pressure until there is a very small stream of ACSF out of the tip. This will reduce the chances of clogging the pipette and decrease the amount of high-potassium solution flowing onto the slice.

24| With the electrode in voltage-clamp, apply a voltage step (e.g., 1 mV, 10 ms square pulse) to determine the current deflection. The resistance of the pipette tip can be calculated by using Ohm's law ($R = V/I$). This resistance gives a good indication of the tip size and can verify that the pipette is not clogged. The resistance of the pipette should be between 3 and 7 MΩ.

25| Lower the pipette toward the slice.

26| Repeat Steps 20–23 for the remaining electrodes. The use of a preinstalled or custom-written program to control the motorized manipulators in the steps can be time-saving. Note that using automation increases the risk of the electrodes colliding with each other or with the slice or objective.

27| Zero any voltage offsets.

28| Move the first pipette into position and increase the positive pressure. Lower the pipette into the slice. Ensure that there is adequate positive pressure to blow debris away from the tip.

▲ CRITICAL STEP Cleaning off the debris surrounding the targeting cell is a prerequisite for a high-quality patch recording⁴⁸. The exact amount of positive pressure needed, ranging from ~20 to 60 mbar, will depend on many factors, including the electrode tip size, depth of the targeting cells and tissue health. Application of proper pressure can be crucial for cleaning off the debris and achieving a >1-GΩ seal (~10 GΩ is ideal) patch-clamp without interfering with other recordings. We recommend sufficient patch-clamp recording practice before the actual experiments.

29| Slowly advance the electrode toward the cell. An increase in resistance (seen in the test pulse), along with the appearance of a dimple on the cell, should occur as the tip approaches the neuron. At this point, release the positive pressure and apply negative pressure to the electrode. Clamp to a negative potential (approximately –70 mV) to facilitate the formation of the high-resistance seal. Release the negative pressure and withdraw the electrode slightly if the pipette has advanced too far into the cell. If a tight GΩ seal has formed, apply pulses of negative pressure to break into the cell, bringing the electrode to a whole-cell patch configuration with access to the intracellular milieu.

30| Repeat Steps 27–29 with the remaining electrodes. At this point different strategies can be used, such as searching for connected cells with the techniques described in the INTRODUCTION. A searching pipette can typically be used to test several (5–10) presynaptic neurons. However, it should be exchanged once a loose seal cannot be established. As with the patching electrodes, the amount of positive pressure (~20–60 mbar) needed for searching electrodes should be just enough to clear away debris in front of the electrode but not disturb the other electrodes.

▲ CRITICAL STEP High-quality patch recordings with minimal damage of neurites in the recording area help achieve a more accurate neuronal connectivity measurement and cell identification. Multiple attempts and side movements of electrodes should be avoided when approaching the targeted neurons. Proper training and sufficient practice of animal/tissue preparation and patch recording skills before the actual experiments can be extremely beneficial^{9,35,49}.

? TROUBLESHOOTING

Identifying and examining synaptic connections ● TIMING ~1–4 h (~15–60 min per two cells)

31| Place the cells into current-clamp mode. Inject a depolarizing current step (~10 ms) into one of the cells to reliably induce an action potential in the presynaptic neuron. To

speed up the investigation, an alternative approach is to inject a depolarizing current step into one of the cells, and another current step into another cell ~1 s later in the single tests. Monitor the current or voltage of the other cells for a postsynaptic response.

? TROUBLESHOOTING

32| Record 50–250 sweeps. The number of sweeps necessary to establish a postsynaptic response will depend on the strength of the response and the noise level⁵⁰.

33| After characterizing the physiology and allowing sufficient time for filling the neurons with biocytin, slowly withdraw a pipette (ideally you should be able to see the membrane stretch away from the cell). Once the pipette is ~5–10 μm away, retract the pipette rapidly out of the recording chamber.

▲ CRITICAL STEP Depending on the extent of the dendritic tree and axon, recordings must last at least 15–30 min to allow for the sufficient diffusion of biocytin into the neuron. We suspect the failure to reseal the membrane after the electrode removal to be the primary contributor to loss or incomplete recovery of the morphology of a small percentage of cells.

34| Fix the slice (lying flat) in 3% (vol/vol) acrolein/4% (wt/vol) paraformaldehyde in 0.1 M PBS at 4 °C for 24–48 h.

! CAUTION Acrolein and paraformaldehyde are toxic and/or carcinogenic. They should be handled according to approved institutional biosafety protocols.

▲ CRITICAL STEP Including acrolein in the fixation solution helps preserve the ultrastructure of the brain slice tissues.

■ PAUSE POINT Fixed tissues can be kept at 4 °C for up to ~2–3 weeks.

Morphological reconstruction ● TIMING ~2–7 d

35| After the 24–48-h incubation period, process the slice using the avidin-biotin-peroxidase method to elucidate the morphologies of the recorded cells. An example protocol can be found in a previous publication⁵¹.

▲ CRITICAL STEP If the ultrastructure examination is planned in order to validate synaptic connections, overreaction of avidin-biotin-peroxidase should be avoided, as it prevents visualization of presynaptic and postsynaptic ultrastructure. The fixation and immunostaining processing may need to be modified if the investigator is interested in further examining the neuronal connections of recorded and other neurons with the CLARITY procedure⁵².

? TROUBLESHOOTING

36| Use a computerized reconstruction system, such as NeuroLucida (MBF Bioscience), to reconstruct the recorded neurons.

Electron microscopic examination ● TIMING ~4–8 weeks

37| Resect the slice into 60- μm sections and postfix it in 1% (wt/vol) OsO_4 .

38| Counterstain the sections with 1% (wt/vol) uranyl acetate.

39| Choose areas of interest ($\sim 50 \times \sim 50 \mu\text{m}$) that contain putative synaptic boutons from single synaptic neurons. Flat-embed the sections in resin.

40| Carefully excise and then resect the slice into 80-nm ultrathin sections with an ultramicrotome. Do not excise and resect if the synaptic boutons originating from different presynaptic neurons are too close to separate.

41| Examine the ultrathin sections in sequence by following the labeled dendrites using a transmission electron microscope. These will typically lead to all microscopic synapses except those very few synapses that were either destroyed during electron microscopic processing or hidden behind grids. The order in which each synapse is identified should be predicted by the Neurolucida reconstruction.

▲ CRITICAL STEP Because it is not possible to unambiguously identify the origins of the presynaptic terminals under an electron microscope, it is important not to include the synaptic boutons originating from different presynaptic neurons in the same ultrathin sections. In addition, reconstructing the presynaptic and postsynaptic neurons and marking their putative contact sites with Neurolucida, and examining the ultrathin sections in sequence, will facilitate the recovery of synapses under the electron microscope.

? TROUBLESHOOTING

Troubleshooting advice can be found in **table 1**.

● TIMING

Steps 1–7, hardware wiring: $\sim 4\text{--}12 \text{ h}$

Steps 8 and 9, software installation: $\sim 4\text{--}12 \text{ h}$

Steps 10–16, brain dissection and preparation of acute slices: $\sim 75\text{--}120 \text{ min}$

Steps 17 and 18, visualization and determination of the optimal slice: $\sim 15\text{--}30 \text{ min}$

Steps 19–30, octuple patch clamping: $\sim 30\text{--}45 \text{ min}$ ($\sim 4\text{--}5 \text{ min per cell}$)

Steps 31–34, identifying and examining synaptic connections: $\sim 1\text{--}4 \text{ h}$ ($\sim 15\text{--}60 \text{ min per two cells}$)

Steps 35 and 36, morphological reconstruction: $\sim 2\text{--}7 \text{ d}$

Steps 37–41, electron microscopic examination: $\sim 4\text{--}8 \text{ weeks}$

ANTICIPATED RESULTS

With a stable octuplet-recording system that is properly set up as described above, simultaneous recording from eight neurons is fundamentally similar to recordings from one or two neurons. A skilled electrophysiologist can expect to master the technique and make it as a routine within $\sim 1 \text{ month}$. Afterward, the investigator can typically achieve 6–8

successfully patched neurons per slice on the first attempt. Next, the investigator may decide whether it is necessary to replace the failed pipette(s) to get the missed or new neurons. The success rate of patching additional neurons by replacing pipettes without losing those already recorded is ~90%. The recordings of 6–8 neurons usually last for 2–4 h, permitting the full investigation of all synaptic connections and cell identification. Compared with previous experiments^{9,29,31}, both the success rate of re-patching neurons and the recording time of high-quality *in vitro* and *in vivo* recordings are substantially improved^{17,18}. We attribute these achievements primarily to the recently improved mechanical stability of the L&N manipulators and associated guide rail electrode exchange systems. The expected yield for the number of synaptically connected neurons will depend on the actual connectivity of recorded neurons, as well as other factors, such as the quality of the preparations and whether the searching techniques outlined above are performed.

Simultaneous patch-clamp recording from four or more neurons allows the examination of complex neuronal circuit patterns (e.g., reciprocal, transitive, circular, parallel, converging and/or diverging circuits and their combinations) that are involved in multiple excitatory and inhibitory neurons, which seems to be common in the brain^{14–18}. Although we focus on the setup of simultaneous octuple patch-clamp recordings and exemplify applications in rat cortical brain slices in this protocol (**Fig. 6**), we anticipate that the approach can be adapted to set up other simultaneous multiple (quadruple–viguple) patch-clamp recording systems as well. Moreover, the technology can be used to investigate the functional organization of neuronal circuits (and the other aspects of neuroscience research, e.g., synaptic transmission and plasticity^{31,53}) in many different brain regions across a broad range of species (from mice to monkeys) at all developmental ages *in vitro* and *in vivo*. For the wide range of neonatal, developing and adult animal *in vitro* and *in vivo* preparations, we refer to other previous publications and protocols^{17,19,20,29,30,38,49,54–56}. As discussed above, multiple patch-clamp recording with single- and two-photon laser-scanning microscopy and optogenetics is instrumentally possible; together these techniques would work complementarily or synergistically in the investigation of complex local and long-range neuronal circuits.

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Figure 1. Manipulators for simultaneous multiple patch-clamp recordings. **(a)** Photograph of an array of eight MINI L&N motorized manipulators at a standard microscope. **(b)** Photographs of the side (top) and top (bottom) views of a 151-mm W $\times$ 151-mm D $\times$ 197-mm H MINI L&N motorized manipulator first invented in 1992 (left), a 104-mm W $\times$ 114-mm D $\times$ 130-mm H JUNIOR L&N motorized manipulator redesigned in 2010 (middle) and a 49-mm W $\times$ 114-mm D $\times$ 157-mm H JUNIOR Compact L&N motorized manipulator developed in 2013 (right) on a 25-mm-grid breadboard. Note ~50 mm width of the JUNIOR Compact manipulator in the y axis. Note that the red adapters can be custom-removed to further reduce the width of the manipulators.

Figure 2.

Hardware wiring for simultaneous multiple patch-clamp recordings. Schematic sketch shows the hardware wiring of the computer, interfaces and manipulators for the simultaneous multiple (octuple) patch-clamp recordings setup. Inset image shows the arrangement of the eight patch pipettes and recording chamber. ADC, analog-to-digital converter; DAC, digital-to-analog converter; Ext. Comm., external command; I , current; PCI, peripheral component interconnect; TRIG, trigger; TTL, transistor-transistor logic pulse; V , voltage.

Figure 3.

IGOR-based program for simultaneous multiple patch-clamp recordings. (a) Screenshot of IGOR-based program testing synaptic connections formed among eight recorded neurons. (b) Screenshot of the IGOR-based program measuring calcium transients at synapses of two neurons. Note that IGOR-based program displayed on multiple monitors can simultaneously run electrophysiology, two-photon laser-scanning imaging and/or optogenetics routines. (c) Flowchart showing generic sequence of operations during experiments. The laser light for optogenetic stimulation is delivered through the objective of the microscope (when no image data acquisition is required) and/or an optic fiber.

Figure 4.

Laserspritzer-based synaptic connection ‘search’ technique. **(a)** Schematic graph shows the fabrication of laserspritzer fiber probe. **(b)** The tip of a laserspritzer (top) and light spot produced by the laserspritzer with laser illumination (bottom) under a microscope. **(c)** 3D transparent rendering of a sensorimotor cortical brain slice prepared from a 2-month-old VGAT-YFP-ChR2 (green)-positive mouse. Bottom left, L2 neuron (red) filled with Alexa Fluor 594 (0.2 mg/ml; Invitrogen, cat. no. A10438). ChR2-expressing cells (bottom middle) and positions of the soma of ChR2-expressing cell 3 in the red circle and stimulating laserspritzer (bottom right) under differential interference contrast and/or fluorescence microscopy. Arrowheads indicate the somata of interneurons expressing YFP-ChR2, and numbers 1–8 indicate cells stimulated by laserspritzer. Scale bar, 20 μm (top, bottom left, bottom middle) and 10 μm (bottom right). **(d)** Laserspritzer stimulation (8 ms; 0.1 mW/mm²) at two of eight ChR2-expressing cells elicited inhibitory postsynaptic potentials (IPSCs) with short (<6 ms) and fixed latencies in postsynaptic L2 neuron. Note ten consecutive recording traces shown in black, average subthreshold and suprathreshold responses shown in red and laser stimulation indicated by blue bars. These animal experiments were approved by the University of Wyoming Institutional Animal Care and Use Committee.

Figure 5.

Single- and two-photon laser-based synaptic connection search technique. **(a)** Action potential (AP) thresholds for cortical L5 pyramidal neurons expressing either ChR2 ($20\ \mu\text{W}$, $n = 1$; $20\text{--}200\ \mu\text{W}$, $n = 7$; $200\text{--}2,000\ \mu\text{W}$, $n = 11$; $>2\ \text{mW}$, $n = 4$ out of 23 neurons tested) or CheRiff ($<20\ \mu\text{W}$, $n = 33$; $20\text{--}200\ \mu\text{W}$, $n = 4$ out of 37 neurons tested) in response to a 5-ms 473-nm single-photon laser pulse. **(b)** Top, schematic graph shows the photostimulation started with a 20-ms 920-nm two-photon spiral laser scanning followed by a 5-ms 473-nm single-photon laser pulse. Bottom, responses in a CheRiff-GFP-expressing cortical L5 pyramidal neuron to a 5-ms 473-nm suprathreshold single-photon laser pulse stimulation (stim) alone (cyan trace), a 20-ms 920-nm subthreshold two-photon laser spiral scanning stimulation alone (red trace), a 5-ms 473-nm subthreshold single-photon laser pulse stimulation alone (blue trace) and the combination of the subthreshold two-photon laser spiral scanning and single-photon laser pulse stimulation (pink trace) under a $40\times/0.8$ numerical aperture (NA) Olympus objective lens. **(c)** Top, responses of a CheRiff-GFP-expressing cortical L5 pyramidal neuron to the suprathreshold single-photon laser pulse stimulation (cyan traces), and the combination of the subthreshold two-photon laser spiral scanning and single-photon laser pulse stimulation (pink traces) with the objective lens focusing point moving away from the soma. Bottom, plot of the average suprathreshold action potential responses of CheRiff-GFP-expressing L5 pyramidal neurons against the distances between the laser-focusing spot and soma of L5 pyramidal neurons. Note the error bars showing the standard errors of action potential incidences in L5 pyramidal neurons ($n = 21$), and note that the half-height spatial resolution of the combined single- and two-photon stimulation ($\sim 30\ \mu\text{m}$) is smaller than that of the single-photon stimulation alone ($\sim 60\ \mu\text{m}$). **(d)** Reconstruction of four L5 pyramidal neurons recorded simultaneously from an acute cortical slice superimposed on the transmitted light image captured during the recordings. The double-colored dots indicate the putative synaptic contacts identified by light microscopy. **(e)** The schematic drawing shows symbolically the synaptic connections. **(f)** Top, the combined single- and two-photon optogenetic stimulation of CheRiff-GFP-expressing cortical L5 pyramidal neurons (green) evoked unitary excitatory postsynaptic currents (uEPSCs) in one of the L5 pyramidal neurons (orange), but not in two others (blue and red). The monosynaptic connection was confirmed after the CheRiff-GFP-expressing

neuron was patched and electrically stimulated in the whole-cell configuration. Note the slightly smaller amplitude and longer kinetics of the average uEPSC evoked by the optogenetic stimulation compared with that evoked by the current injection in postsynaptic neuron owing to the slight jittering of optogenetically evoked action potentials in the presynaptic neuron. The majority of the unconnected axonal branches of the pyramidal neurons are not reconstructed for simplicity, and these animal experiments were approved by the University of Virginia Institutional Animal Care and Use Committee.

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Figure 6.

Example of simultaneous multiple patch-clamp recordings. **(a)** An experiment of the rat sensorimotor cortical brain slice performed by a trainee with 3 weeks of experience on the simultaneous octuple patch-clamp recordings setup shown in **Figure 1a**. Single action potentials elicited in the presynaptic pyramidal neuron (red) evoked unitary excitatory postsynaptic potentials (uEPSPs) in the postsynaptic pyramidal neuron (orange), and single action potentials elicited in presynaptic interneuron (cyan) evoked unitary inhibitory postsynaptic potentials (uIPSPs) in postsynaptic pyramidal neurons (blue and pink) and interneuron (green). Scale bars apply to all recording traces with 80 and 2 mV bars applied to traces with and without action potentials, respectively. **(b)** Reconstruction of the eight recorded neurons reveals six pyramidal neurons and two interneurons, including a basket cell and an aspiny neuron that could not be morphologically identified owing to a large truncation of its axonal arborization. The double-colored dots indicate the putative synaptic contacts based on anatomical reconstruction. **(c)** The schematic drawing shows symbolically the synaptic connections. The majority of the unconnected axonal branches of the pyramidal neurons are not reconstructed for simplicity, and these animal experiments were approved by the University of Virginia Institutional Animal Care and Use Committee.

TABLE 1

Troubleshooting table.

step	problem	possible reason	solution
9	Lack of signal/command operation	Cables are not connected correctly	Connect the cables correctly (Fig. 2)
	Software will not load/acquire data/execute commands	Program is not installed correctly	Check that the program is installed correctly
17,18	Cannot identify the slice containing dendrites parallel to the plane of the slice; cannot trace L5 apical dendrite to L1	Did not cut the slice at the correct angle	Make sure that the brain tissue is flat on the ramp
		Slice is unhealthy	Follow the correct slicing procedure
30	Losing one cell after patching another	Optics are not optimal	Optimize the optics ³⁰
		Moving the pipette within the slice disrupts the slice	Only move forward/backward diagonally through the slice
		Pipette tip has drifted from the cell	Move previously patched electrodes back to their original locations on the cell they have moved
31	Lack of postsynaptic response	Too much positive pressure	Adjust the pressure to the pipette
		Slice is not healthy	Correct slicing procedures must be followed
		Slice does not contain parallel dendrites	Make sure that the pyramidal apical dendrites run parallel to the surface of the slice
		Cells are too superficial and connections are severed	Patch cells deeper in the slice
		Cells patched do not connect <i>in situ</i>	Try searching for connections
		The holding potential of the postsynaptic neuron is too near the reversal potential	Modify the internal and holding potential
35	Morphology is not recovered	Release probability is too low; axon failure	Stimulate three or more action potentials
		Noise level is too high	Reduce the noise level
		Cell dies while recording	The cell must be healthy when retracting the electrode
		Cell membrane breaks down when the electrode is removed from the cell	Remove the electrode more slowly
		Problem with the biocytin filling and/or labeling procedure	Follow the correct biocytin labeling and diaminobenzidine-based procedures ⁵¹