Quantum dot-based multiphoton fluorescent pipettes for targeted neuronal electrophysiology

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Abstract

Targeting visually-identified neurons for electrophysiological recording is a fundamental neuroscience technique; however, its potential is hampered by poor visualization of pipette tips in deep brain tissue. We describe a technique whereby quantum dots coat glass pipettes providing strong two-photon contrast at deeper penetration depths than current methods. We demonstrate utility in targeted patch-clamp recording experiments and single cell electroporation from identified rat and mouse neurons *in vitro* and *in vivo*.

Electrical recording from individual neurons in brain tissue using patch-clamp techniques provides the most direct information on neuronal activity[1,2], and will be critical to success of the brain mapping initiatives[3,4]. Advances in genetic labeling of specific cell types open the possibility of targeted patch-clamp recordings from individually-identified fluorescent neurons in living brain tissue[5,6]. However, direct access to neurons, both labeled and unlabeled, is hampered by a lack of methods for visualizing thin pipettes tips as they are advanced through the brain to contact the targeted neuron. Visualization, especially deeper within the brain, is currently accomplished using two-photon (2P) imaging of fluorophores (*e.g.* Alexa Fluor dyes)[5-7] that are continuously expelled from the pipette during the approach, thereby creating a "shadow" around a labeled or unlabeled neuron. Though such dyes have been successfully used for many years, their applicability is still limited by low 2P excitation action cross-sections (absorption of two photons of identical frequency) requiring potentially damaging higher laser powers, susceptibility to photobleaching, and dye accumulation which causes increased background fluorescence or light absorption, especially after multiple descents. As an alternative method for targeted single-cell recordings, we developed a technique for robust fluorescent labeling of standard borosilicate glass pipettes allowing their 2P visualization far deeper within brain tissue than current methods.

From a photophysical perspective, their unique properties make semiconductor quantum dots (QDs) ideal for this imaging challenge. These nanocrystals, whose photoluminescence (PL) can be tuned *via* core size and composition, display desirable optical properties including high quantum yields (ϕ), resistance to photo and chemical degradation, narrow and symmetrical PL

emission (full-width-at-half-maximum ~25-35 nm), broad absorption spectra coupled to large one-photon (ε = 10^4-10^7 $M^{-1}cm^{-1}$) and some of the highest two-photon absorption cross-sections (σ_2 = 10^3-10^4 Goeppert-Mayer or GM units) available[8,9]. QD utility for 2P imaging in tissue has been repeatedly confirmed[8-10]. Here, we show QD-labeled glass pipettes provide outstanding contrast of the pipette tip even in deep brain for targeted electrophysiological recordings without compromising electrical properties of the pipette or neuronal activity.

For optically targeting labeled neurons (typically expressing a red or green fluorescent protein), we coated pipettes with green (ϕ 19%, 530 nm), yellow (ϕ 33%, 550 nm) or red (ϕ 45%, 625 nm) emitting CdSe-ZnS core-shell QDs (**Fig. 1a**). These QDs were cap-exchanged with polyethylene glycol modified- or zwitterionic-terminated dihydrolipoic acid ligands for optical characterization (**Supplementary Fig. 1**)[11] or diluted in hexane with native phosphine-hexadecylamine ligands still present on their surface for pipette coating. We determined QD 2P action cross-section ($\phi\sigma_2$) spectra using a two-photon spectrometer[12]. QD $\phi\sigma_2$ were measured in comparison to Alexa Fluor 488 (ϕ 92%), Alexa 546 (ϕ 79%), and Alexa 594 (ϕ 66%) dyes (**Fig. 1b-d**). Comparative $\phi\sigma_2$ at 880 nm were ~400 GM units for 530 QDs *versus* 8 GM for Alexa 488, 752 GM for 550 QDs *versus* 6 GM for Alexa 546, and 16470 GM for 625 QDs *versus* 12 GM for Alexa 594. Assuming a pipette could be uniformly coated with equal amounts of 625 QD or Alexa Fluor 594 dye, and using a simplistic extrapolation of $(\phi\sigma_2)_{QD}/(\phi\sigma_2)_{dye}$ at equal 880 nm 2P excitation, the 625 QD probe should be >900X brighter.

To coat pipette tips with QDs, native QDs were first washed in organic solvent several times to remove the excess synthetic ligands then dried down and re-solubilized in hexane. The tip of the borosilicate pipette was then repeatedly dipped into the QD-hexane solution until a desirable PL was reached (visualized under UV light). To prevent QDs from clogging the pipette tip, we applied positive air pressure during the coating. Since native-capped QDs are completely insoluble in aqueous solutions, they remain attached to the glass pipette, providing 2P contrast in the presence of any physiological buffer, internal pipette solution or dyes. Comparing the standard approach for pipette visualization using a soluble fluorescent dye against the QD-coated pipette shows substantial intensity differences in the area of the pipette tip (**Fig. 1e,f**). When Alexa Fluor 488 is ejected from the pipette, measured fluorescence intensity is lowest at the tip, whereas QD-coated pipettes show the brightest fluorescence at the tip. This is ideal for

accurately determining pipette tip location in brain tissue, especially since this very structure will first contact neuronal membranes. To determine the detection limits of coated pipettes in deep brain tissue, we compared both methods in anaesthetized mice using 2P imaging and measured the intensity of fluorescence signals down to 500 µm depth at various laser powers (**Fig.1 g-l**)[5,13]. While Alexa Fluor 594 fluorescence ejected from the pipette deteriorated rapidly below 300 µm, QD-coated pipettes were still clearly visible at penetration depths of 500 µm, while using 77% less laser power (**Fig. 1i-l**). Even at the maximum excitation wavelength (800 nm), the Alexa Fluor 594 signal was still lower compared to the QD coated pipettes (**Supplementary Fig. 2**). Such extended imaging depths at lower laser power can expand experimental access *in vivo*.

To evaluate electrochemical and optical properties of QD-coated patch pipettes *in situ*, we performed patch-clamp recordings in brain slices. The pipette resistance of QD-coated pipettes did not differ from uncoated control pipettes, whereas the capacitance was slightly decreased (**Fig. 2a-c**). QD-coated pipettes formed gigaseal contacts similarly to uncoated patch pipettes[5-7,13], when using the standard "blow-and-seal" technique. We patched different fluorescently-labeled cell types in brain slices, including hippocampal Ds-Red-labeled cholecystokinin positive interneurons and GFP labeled parvalbumin-positive interneurons (**Fig. 2d**, **Supplementary Fig. 3a**). The fluorescence intensity of QD-coated pipettes was consistently higher than the endogenously-expressed fluorescent markers. Indeed, sensitivity of the photomultiplier detecting the QD-coated pipette signal needed to be scaled down to avoid saturation at the laser power required for visualizing the fluorescent proteins. Basic electrophysiological properties of neuron types patched with QD-coated pipettes were similar to those recorded using uncoated pipettes (somatic firing, voltage responses to a series of positive-negative current injections), confirming the QD coating did not interfere with neuronal electrophysiological properties nor affected viability. Furthermore, 2P Ca^{2+} imaging from CA1 pyramidal neurons loaded with the Ca^{2+}-sensitive dye Oregon Green BAPTA-1 (OGB-1) through the QD-coated pipette revealed normal dendritic and spine Ca^{2+} and voltage signals in response to backpropagating action potentials (APs, **Fig. 2e**) as well as to direct synaptic stimulation by 2P glutamate uncaging (**Fig. 2f** and **Supplementary Fig. 3b**)[13].

Under *in vivo* conditions, we recorded with QD-coated patch pipettes from cortical L2/3 pyramidal neurons of anaesthetized mice expressing the genetically encoded Ca^{2+} indicator

GCaMP6 (**Fig. 3a** and **Supplementary Videos 2** and **3**). QD-coated pipettes could be clearly visualized within the intact brain even after penetrating the dura. Spontaneous electrical activity and corresponding somatic GCaMP6 Ca^{2+} signals were measured in the patched cells and appeared normal. Recordings from channelrhodopsin-(ChR2) expressing interneurons using QD-coated pipettes verified that activation of ChR2 with 470 nm light produced robust and precisely driven firing as expected (**Fig. 3b**)[6,14]. QD-coated pipettes also successfully electroporated[7] with Alexa Fluor 594 dye and a Ds-Red encoding plasmid into individually identified L2/3 pyramidal neurons at ~300 μm depth (**Supplementary Fig. 4** and **Supplementary Video 1**). The challenge of sequentially electroporating multiple cells *in vivo* did not alter the QD coating nor produced QD adsorption to the brain parenchyma demonstrating the reliability of this method. Importantly, GFP-expressing neurons were successfully electroporated *in vivo* at 760 μm depth using 625 QD coated pipettes (**Fig. 3c-e** and **Supplementary Video 4**). Superb visibility of the QD coated pipette tips also improved access to small cellular structures, such as local dendritic regions *in vitro* (**Fig. 3f**).

In summary, we introduce a simple technique to fabricate permanently-labeled fluorescent glass pipettes which facilitate visually targeted recordings from individual (labeled or unlabeled) neurons at great depth and with high precision both *in vitro* and *in vivo*. Pipettes have been labeled previously with fluorophores; however the dyes utilized did not provide the required 2P properties for deep tissue imaging[15,16]. Our approach is an alternative or complement to the current "gold standard" method[5-7] while removing the need to perfuse dye into the extracellular space continuously which reduces visibility and contrast. High quality imaging with QD-coated pipettes is possible even at depths of ~500-800 μm within *in vivo* brain tissue (**Fig. 1** and **Fig. 3**). We note that the low intrinsic 2P properties of the currently used fluorescent proteins expressed in labeled neurons may still require higher laser power for their visualization. QD coating does not preclude use of fluorescent dyes, in fact, it allows the advantage of combining both visualization modalities simultaneously for specific applications (*e.g.* for monitoring pipette clogging or cell loading). Furthermore, narrow, size-tunable QD PL allows access to coatings of various colors across the spectrum as experimentally required[9]. Critically, QD-coated pipettes did not interfere with physiological functions monitored throughout our experiments for ≤ 3 hours, suggesting they can be used for a wide array of biological experiments. While we tested

QD coatings for electrophysiological recording pipettes in neuroscience, we expect that they can be applied to coat any probe type wherever improved visualization in tissue is needed.

METHODS. Methods and any associated references are available in the online version of the paper. *Note: Supplementary information is available in the online version of the paper.*

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AUTHOR CONTRIBUTIONS. B.K.A., M.B., J.J.M. and I.L.M. conceived the idea of using QDs for coating patch pipettes. B.K.A. and J.K.M. performed and analyzed *in vitro* experiments. G.L.G. and D.H. performed and analyzed *in vivo* experiments. J.J.M., K.S., J.B.D., A.L.H. and I.L.M. produced the QDs or characterized them. I.L.M., B.K.A., G.L.G., D.H. and J.K.M. wrote the paper with comments from all authors.

COMPETING FINANCIAL INTERESTS. B.K.A., M.B., J.J.M., K.S., J.B.D., A.H. and I.L.M. have filed a patent application for production of QD-coated probes based on the results reported in this paper. The rest of the authors declare no competing financial interest.

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Figure Legends.

Figure 1. QD photophysical properties and *in vivo* imaging. **(a)** Normalized absorption and PL of QDs. Molecular extinction coefficients ε at wavelengths corresponding to the first excitation peak are: 530 QD 159,092 M^{-1} cm^{-1} at 501 nm; 550 QD 120,000 M^{-1} cm^{-1} at 533 nm; 625 QD 500,000 M^{-1} cm^{-1} at 610 nm. (**b-d**) 2P action cross-section spectra ($\phi\sigma_2$) in GM units for **(b)** 530-, **(c)** 550- and **(d)** 625 QDs in phosphate buffered saline, superimposed over spectra of Alexa Fluor 488, Alexa Fluor 546 and Alexa Fluor 594 in water, respectively. Inset in **d** shows the enlarged spectrum of Alexa Fluor 594. (**e**) Image of a 625 QD-coated pipette (upper) and an uncoated pipette ejecting Alexa Fluor 488 (lower). Intensity measurements were performed in the white rectangles within the pipette tips. (**f**) Fluorescence intensity as measured in (e) for 625 QD, 550 QD and Alexa Fluor 488 dye. **(g, h)** Schematics of the **(g)** classic approach for pipette visualization during shadow patching and the new **(h)** approach using QD-coated pipettes (red). Alexa Fluor 594 filled pipettes **(i)** or 625 QD-coated pipettes **(j)** were imaged at different depths (D) in the mouse brain at the indicated laser power (LP). Images are the average of 10 frames, except for Alexa Fluor at 500 µm (100 frames average). Average gray value (10 frames) in arbitrary units of either the Alexa Fluor 594 **(k)** or the 625 QD **(l)** pipette's fluorescence as a function of laser power at 940 nm and depth.

Figure 2. Electrical properties of QD-coated patch pipettes. **(a-c)** Comparison of uncoated and QD-coated patch pipettes, **(a)** resistance (unpaired t-test, n = 7/6, P = 0.979), **(b)** capacitance (unpaired t-test, n = 7/8, P = 0.020), and **(c)** access resistance (one-way ANOVA test, n = 4/7/6 cells in 6 animals, P = 0.454). Black: mean ± S.D. (**d**) 2P monitoring of QD-coated pipettes (green, 530 nm QD) during patching of hippocampal neurons (red) in acute brain slices from a BAC-CCK-Ds-Red mouse. Representative for 13 cells in 5 animals. Panels from left: 2P images (1-3); voltage responses to positive and negative current injections (200 pA) in the same cell (4). **(e)** Rat hippocampal CA1 pyramidal neuron loaded with Ca^{2+} sensor OGB-1 (green) through 625 QD-coated patch pipette (red) in acute brain slice. Circles: dendritic regions used for recording backpropagation AP evoked Ca^{2+} signals induced by +50-150 pA current injections. Ca^{2+} signals for each location are plotted on the right (n = 1). **(f)** Rat hippocampal CA1 pyramidal neuron loaded with Alexa Fluor 594 (red) through a 550 QD-coated patch pipette in acute slice. Box inset: dendritic region and 12 spines selected for 2P glutamate uncaging. Right, top: uncaging-evoked excitatory postsynaptic potentials (gluEPSPs) at indicated spines with inter-spine stimulation interval (IsSI) of 200 ms. Right, bottom: simultaneous glutamate uncaging at all 12 spines (IsSI = 0.3 ms) evokes dendritic spike (arrow, n = 8/9 dendrites in 4 neurons from 2 animals, patched with various QD-coated pipettes). Black: voltage trace, red: dV/dt trace.

Figure 3. Neuronal manipulations with QD-coated pipettes. (**a**) Left: GCamp6f expressing cortical L2/3 pyramidal neuron (green) patched with 625 QD-coated pipette (red) *in vivo* at 207 µm depth. Right, GCamp6f Ca^{2+} signals (top) during spontaneous spiking activity (bottom). Representative of n = 5 cells. (**b**) Left: mouse cortical interneuron (green) expressing CHR2-YFP under the control of the vesicular gamma-aminobutyric acid (GABA) transporter (VGAT) promoter, patched with 530 QD-coated pipette (green) *in vivo*. After recording spiking activity in cell-attached mode, the cell was loaded with Alexa Fluor 594 (red). Right: 40 Hz sine wave-modulated 470 nm LED light stimulation (top blue; delivered through 2P microscope optical

path) and electrical activity of the same patched neuron (middle: single trial trace; bottom: raster plot of light evoked action potentials, 10 trials). (**c-e**) Deep layer targeting in Thy1-EGFP mouse. (**c**) Top: Z-projection (80 μm) of targeted neuron soma (arrowhead) at 760 μm depth pre-electroporation. Bottom: 3D reconstructed orthogonal view, corresponding to ~800 μm. Green: GFP fluorescence, arrowhead: site of pipette contact to the neuron. (**d**) Targeted neuron during electroporation. Red: 625 QD. 40 mW laser power at 940 nm. Frames are averaged 10x. (**e**) Targeted neuron expressing DsRed (red) and GFP (green) 2 days post-electroporation. **(f)** Fluorescence directed dendritic patching, representative of n = 3 dendrites in 2 animals. The apical trunk of an *in vitro* CA1 pyramidal cell preloaded with Alexa Fluor 488 patched with 625 QD-coated pipette using fluorescent visualization. Dendritic patch formation (top). Synaptic gluEPSPs (bottom) after uncaging at nearby spines.

Supplementary Materials

Online Methods

Materials and Methods

Preparation of hydrophobic QDs. Native organic QDs[11] were washed twice to remove the excess ligands present from synthesis. QD samples in toluene or decane were precipitated by the addition of several milliliters of an acetone:methanol 50:50 mixture in a 15 or 50 mL Falcon tube. The QDs were then centrifuged to a pellet and the supernatant decanted and discarded. The pellet was dried under nitrogen and the QDs were again resuspended in hexane or toluene. This was followed by another round of washing and precipitation with drying under nitrogen for storage. The QDs were resuspended in hexane for probe coating.

Two-photon action cross-sections of QDs and Alexa Fluor dyes. Action cross sections were measured with an inverted microscope using a Ti:sapphire laser as an excitation source, as described earlier[12]. Briefly, QD or dye solutions at micromolar concentration (or 0.1 μM for 625 QD) were contained in coverslip-bottomed dishes (MatTek) and 2P excitation spectra from 710 nm to 1080 nm were obtained at a constant laser power at the sample of 0.5 mW. 530 QD, 550 QD, and 625 QD were measured in phosphate-buffered saline, Alexa Fluor 488, 546, and 594 were measured in water (for comparison to published values), and the 2P reference dye fluorescein was measured at pH 11. Spectra obtained from the buffers alone were used as background correction for the fluorophore spectra. No emission filters were used other than two short-pass filters (720/SP, Semrock). The absolute two-photon action cross section of the reference dye fluorescein was taken from Xu and Webb (1996)[17], with corrections made for small differences in the quantum efficiency of the detector (avalanche photodiode detector model PDF; Micro Photon Devices) for the different emission wavelengths of the fluorophores.

***Pipette coating with QDs*.** Borosilicate pipettes were pulled with a standard puller. Positive air pressure was applied through the back of the pipette with a 10 ml syringe and submerged into methanol to determine the bubble number[18]. After methanol evaporation, the tip of the pipette was dipped into the QDs solution keeping the positive pressure to prevent clogging. After 0.5 to 2 seconds, the pipette was allowed to dry in the air to form a layer of QDs on the glass surface. The coating procedure was repeated several times until reaching a desirable photoluminescence determined under UV light. See **Supporting Protocol** for a stepwise QD coating procedure with more details and some notes.

In vitro and *in vivo* experiments

Note: all animal usage and all experiments were performed in strict accordance with institutional IRB approval and met all applicable regulations.

In vitro experiments

Slice preparation. Acute transverse hippocampal slices were prepared from either 8-9 week-old male Sprague-Dawley rats (400 μm thick slices) as described previously[19], or from P18-24 BAC-CCK-Ds-Red[20] or PV/GFP BAC[21] mice of both sexes (300 μm thick slices), according to methods approved by the Institute of Experimental Medicine, Hungarian Academy of Sciences, in accordance with DIRECTIVE 2010/63/EU Directives of the European Community and Hungarian regulations (1998. XXVIII. section 243/1998, renewed in 40/2013, II.14.). Briefly, rats were deeply anaesthetized with isoflurane and transcardially perfused with ice-cold cutting solution containing (in mM): sucrose 220, $NaHCO_3$ 28, KCl 2.5, NaH_2PO_4 1.25, $CaCl_2$ 0.5, $MgCl_2$ 7, glucose 7, Na-pyruvate 3, and ascorbic acid 1, saturated with 95% O_2 and 5% CO_2. Mice were deeply anaesthetized with isoflurane and decapitated without transcardial perfusion. The brain was quickly removed and sectioned with a vibratome (VT1000A, VT1000S or VT1200S, Leica). Slices were incubated in a submerged holding chamber (rat slices) or in an interface chamber (mice slices) in artificial cerebrospinal fluid (aCSF) at 37°C for 30 min and then stored in the same chamber at room temperature. For recording, slices were transferred to

the submerged recording chamber of the microscope where experiments were performed at 33-35 °C in aCSF containing (in mM): NaCl 125, KCl 3, $NaHCO_3$ 25, NaH_2PO_4 1.25, $CaCl_2$ 1.3, $MgCl_2$ 1, glucose 25, Na-pyruvate 3, and ascorbic acid 1, saturated with 95% O_2 and 5% CO_2.

Pipette property measurements. Pairs of pipettes were pulled from the same borosilicate glass. QD-coated and un-coated pipettes were filled with internal solution leaving a blocking bubble at the tip of the pipette, then submerged into the aCSF-containing recording chamber. Pipette capacitance was measured in voltage-clamp mode using 10 mV step command with a HEKA Amplifier at 100 kHz filtering. After the removal of the blocking bubble from the pipette, the pipette resistance was measured using the same protocol.

Electrophysiology. Cells were visualized using a Zeiss AxioExaminer epifluorescent microscope equipped with infrared Dodt optics and a water immersion lens (63X, 0.9 NA, Zeiss). Current-clamp whole-cell patch-clamp recordings were performed with a Dagan BVC-700 amplifier (Dagan) in the active 'bridge' mode, filtered at 3 kHz and digitized at 50 kHz. Patch pipettes were filled with a solution containing (in mM): K-gluconate 134, KCl 6, HEPES 10, NaCl 4, Mg_2ATP 4, $Tris_2GTP$ 0.3, Na-phosphocreatine 14, pH 7.25. In some experiments (as indicated in the text) the pipette solution was complemented with either 100 μM Alexa Fluor 488, 50 μM Alexa Fluor 594, or 100 μM Oregon Green 488 BAPTA-1 (OGB-1, for Ca^{2+} measurements; all dyes were from Invitrogen). Series resistance was <30 MΩ.

Two-photon imaging and uncaging. Two ultrafast pulsed laser beams (Chameleon Ultra II; Coherent) and a dual galvanometer-based two-photon laser scanning system (Prairie Technologies) were used to simultaneously image neurons (at 880 or 920 nm) and to focally uncage MNI-caged-L-glutamate (Tocris; 9-10 mM applied *via* pressure ejection through a 20-30 μm diameter pipette above the slice) at individual dendritic spines (at 720 nm)[22]. Laser beam intensity was independently controlled with electro-optical modulators (Model 350-50, Conoptics). All images shown are collapsed Z stacks of multiple images. Uncaging dwell time

was 0.2 ms; galvo move time was 0.1 or 200 ms. (see text). Linescan imaging was performed at 150-500 Hz.

***Data analysis*.** Analysis was performed using custom-written macros in IgorPro (WaveMetrics). Ca^{2+} and voltage signals were analyzed offline using averaged traces of 3-5 trials. Morphological and distance measurements were performed using ImageJ (NIH) on two-dimensional maximal intensity projections of 2 µm z-series collected at the end of the experiment. Only data obtained in experiments meeting the standard technical criteria for successful recordings (GOhm seal resistance, <30 MOhm access resistance) were included

***In vivo* experiments**

***Surgical procedures*.** All *in vivo* mouse experiments were approved by the Animal Care Committee of the University of Geneva. Adult (2-5 months old) C57/Bl6 wild type, VGAT-ChR2 (YFP-Channelrhodopsin-2 expressing neurons under the control of the locus of the vesicular γ-aminobutyric acid (GABA) transporter, VGAT) or Tg(Thy1-EGFP)MJrs/J (EGFP expressing neurons under the control of a modified Thy1 promoter region) mice of both sexes were used. All surgeries were conducted under isofluorane anesthesia (1.5%) in a custom made stereotactic apparatus equipped with a thermic plate (37°C). Prior to the surgery, toe-pinch nociceptive responses were assessed and mice received anti-inflammatory (2.5 mg/kg dexamethasone i.m; 5 mg/kg carprofen s.c.), analgesic (0.1 mg/kg buprenorphine i.m.) and local anesthetic (1% lidocaine s.c. under the scalp) drugs.

***Stereotactic injections of GCaMP6*.** Two to 4 weeks prior to the electrophysiological experiments, layer 2/3 cortical neurons of C57/Bl6 mice were labeled with the genetically encoded calcium indicator GCaMP6 using a viral vector. The scalp was shaved and sterilized with ethanol 70% and a betadine solution. A small skin incision was performed over the motor cortex (1 mm anterior and 0.8 mm lateral to Bregma) and a small craniotomy was performed with a dental drill to allow for virus injection. Glass capillaries (Drummond) were pulled (Sutter Instrument P-97) and beveled to attain thin and sharp pipettes (outer diameter <30 µm). A pipette was loaded with a suspension of the adeno-associated virus AAV1-Syn-GCaMP6f

(UPenn, 2.96e12 GC) and lowered into the motor cortex (250 μm deep). A 50 nL injection (10-20 nL/min) was performed using a piston-based injection system (Narishige). After the injection, the scalp was sutured and mice were left to recover for at least two weeks.

Craniotomy for in vivo recordings. The day of the electrophysiological recordings, the scalp was removed. The exposed skull was cleaned and dried. The periostium was removed with a scalpel and a custom-made titanium head bar was cemented to the bone with a thin layer of cyanoacrylate glue and covered with dental cement. In order to create a well for the water immersion objective of the microscope, 150 uL of 1% agarose (w/v) were dripped on the skull and left to jellify. The border of the agarose drop was covered with dental cement to create a 1 mm deep recording chamber and the dental cement was allowed to cure for 10 minutes. A round craniotomy of 1-1.5 mm diameter was performed over the motor cortex taking care of not damaging the dura. The exposed dura was thoroughly rinsed with sterile saline to prevent bleeding and to remove bone debris and kept moistened throughout the experiment. Once set, the mouse was placed under the two-photon microscope and held by the titanium head bar with a custom-built holder.

In vivo imaging. Two-photon imaging was performed using a scanimage r4.1 controlled microscope equipped with a resonant scanner head (Thorlabs), two GaAsP photomultiplier tubes (Hamamatsu 10770PB-40; filters: red and green channel) and a 16x 0.8 NA water immersion objective (Nikon)[7]. The laser beam was tuned at 940 nm (Ti-Sapphire Coherent Ultra II Chameleon) and light pulse (140 fs) dispersion was corrected with a group velocity dispersion compressor (Chameleon PreComp). Maximal power used (measured in the air at the focal plane) was <50 mW. To stimulate ChR-2 expressing neurons, the microscope was also equipped with a 470 nm LED illumination source controlled by ephus. Maximal power at the focal plane was 500 uW. During the pipette approximation to the targeted cell, images (256 x 256 pixels) were acquired at 60 fps and online averaged (10 frames rolling window average).

In vivo electrophysiology and single cell electroporation. 4-6 MΩ (for electrophysiology) or 12-15 MΩ (for single cell electroporation) borosilicate pipettes pipettes (Science Products Gmbh)

were pulled with a two-step vertical puller (Narishige) and coated with QDs as described above. Electrophysiological recordings were performed using an Axoclamp 200B amplifier (Molecular Devices) controlled by Ephus. Pipettes were held a 30°- 40° angle with the cortical surface and the tip of the pipette was positioned on the surface of the dura diagonally aligned to the targeted cell. For the dura penetration, the pressure of the pipette was set to 150 mbar and reduced to 50 mbar once it was inside the brain. The pipette was diagonally advanced up to the targeted cell and minor lateral or vertical adjustments were made to avoid blood vessels. Pipette resistance was continuously monitored to check for clogging. GCamp6-, VGAT-ChR2- and Thy1-GFP-expressing neurons were simultaneously visualized with the fluorescent pipette (red or green QDs) and the tip of the pipette was carefully advanced to the center of the neuron and the positive pressure was released after a 50% increase in the pipette resistance. Targeted single cell electroporation was performed as previously described[9] using an Axoporator 800A (Molecular Devices). Borosilicate pipettes were filled with internal solution and 50 µg/µl of DsRed plasmid. After seal formation, a single electroporation train was applied (1 s, 50 Hz, 500 µs pulse duration, -7 V). To prevent brain damage, a maximum of 3 penetrations were performed at the same brain location. Noticeably, QDs are readily adsorbed to the dura, therefore the fluorescence of the pipette that pierced the dura was dimmer - on average - than the following ones. In spite of this, QDs were never adsorbed to the brain parenchyma even after repeated pipette penetrations or long recordings.

Data analysis. In statistical comparisons, differences were considered significant when $P < 0.05$. Statistical analysis was performed using two-tailed unpaired t-test or one-way ANOVA. All data were tested and met the assumption for normal distribution. . In all figures, symbols and error bars represent mean ± S.D. Experiments were not randomized or blind.

Methods-Only References

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