Quantum dot–based multiphoton fluorescent pipettes for targeted neuronal electrophysiology

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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 quantum dot–coated glass pipettes that provide strong two-photon contrast at deeper penetration depths than those achievable with current methods. We demonstrated the pipettes’ utility in targeted patch-clamp recording experiments and single-cell electroporation of 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 activity1–2 and will be critical for the success of brain-mapping initiatives3–4. Advances in genetic labeling of specific cell types open the possibility of targeted patch-clamp recordings from individually identified fluorescent neurons in living brain tissue5,6. However, direct access to neurons, both labeled and unlabeled, is hampered by a lack of methods for visualizing thin pipette 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 fluorophores5–7 that are continuously expelled from the pipette during the approach, thereby creating a ‘shadow’ around a 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 high laser powers, by susceptibility to photobleaching, and by dye photobleaching, 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 that allows for their 2P visualization far deeper within brain tissue than current methods permit.

From a photophysical perspective, the unique properties of semiconductor quantum dots (QDs) make them 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 photobleaching, and symmetrical PL emission (full-width at half-maximum = 25–35 nm), broad absorption spectra coupled to large one-photon (extinction coefficient ε = 104–107 M−1 cm−1) and some of the highest two-photon absorption cross-sections (σ2 = 103–104 Goeppert-Mayer, or GM, units) available8,9. QD utility for 2P imaging in tissue has been repeatedly confirmed8–10. Here, we show that 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 CdSe core–ZnS shell QDs emitting green (φ = 19%; 530 nm), yellow (φ = 33%; 550 nm) or red fluorescence (φ = 45%; 625 nm) (Fig. 1a). These QDs were cap-exchanged with poly(ethylene glycol)-modified or zwitterionic-terminated dihydrolipoic acid ligands for optical characterization11 (Supplementary Fig. 1) or were diluted in hexane with native hydrophobic ligands still present on their surface for pipette coating. We determined QD 2P excitation action cross-sections (φσ) spectra using a 2P spectrometer12. QD φσ values were measured and compared to those of Alexa Fluor 488 (φ = 92%), Alexa Fluor 546 (φ = 79%) and Alexa Fluor 594 (φ = 66%) dyes (Fig. 1b–d). Comparative φσ values at an excitation wavelength of 880 nm were ~400 GM for QD 530 versus 8 GM for Alexa Fluor 488 (φ = 92%), Alexa Fluor 546 (φ = 79%) and Alexa Fluor 594 (φ = 66%) dyes (Fig. 1b–d). Comparative φσ values at an excitation wavelength of 880 nm were ~400 GM for QD 530 versus 8 GM for Alexa Fluor 488, 752 GM for QD 550 versus 6 GM for Alexa Fluor 546 and 16,470 GM for QD 625 versus 12 GM for Alexa Fluor 594. Assuming uniform pipette coating with equal amounts of QD 625 or Alexa Fluor 594 dye, and using a simplistic extrapolation of (φσ)QD/(φσ)dye at equal 880-nm 2P excitation, we calculated that the QD 625 probe should be ~900× brighter.

To coat pipette tips with QDs, we first washed native QDs in organic solvent several times to remove the excess synthetic ligands, and then we dried the QDs and resolubilized them 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. Because 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 our method with the QD-coated pipette, we found substantial intensity differences in the area of the pipette tip (Fig. 1e,f). When Alexa Fluor 488 was ejected from the pipette, measured fluorescence intensity was lowest at the tip, whereas QD-coated pipettes showed the brightest fluorescence at the tip. The latter is ideal for accurately determining pipette tip location in brain tissue, especially because the tip has the first contact with neuronal membranes. To determine the detection limits of coated pipettes in deep brain tissue, we used 2P imaging to compare both methods in anesthetized mice, measuring the intensity of fluorescence signals down to 500-µm depth at various laser powers (Fig. 1g–i). Whereas 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, and using 77% less laser power (Fig. 1i–l). Even at the maximum-excitation wavelength (800 nm), the Alexa Fluor 594 signal was still lower than that of the QD coated pipettes.
Figure 2 | Electrical properties of QD-coated patch pipettes. (a–c) Comparison of uncoated and QD-coated patch pipettes with respect to pipette resistance (two-tailed unpaired t-test, \( P = 0.979 \)) (a), capacitance (two-tailed unpaired t-test, \( P = 0.020 \)) (b) and access resistance (one-way ANOVA, \( n = 6 \) animals, \( P = 0.454 \)) (c). Mean ± s.d. are shown in black. OGB-1, Oregon Green BAPTA-1. (d) 2P monitoring of QD-coated pipettes during patching of hippocampal neurons (red) in acute brain slices from a BAC-CKK-DsRed mouse. Data are representative of 13 cells in 5 animals. Left and center, 2P images; right, voltage responses to positive and negative current injections (±200 pA) in the same cell. (e) Rat hippocampal CA1 pyramidal neuron loaded with Ca\(^{2+}\) sensor OGB-1 (green) through a QD 625–coated patch pipette (red) in acute brain slice. Circles indicate dendritic regions used for recording back-propagation action potential–evoked Ca\(^{2+}\) signals induced by +50- to 150-pA current injections. Right, Ca\(^{2+}\) signals for each location (\( n = 1 \)). \( \Delta F / F \), relative change in fluorescence. (f) Rat hippocampal CA1 pyramidal neuron loaded with Alexa Fluor 594 (red) through a QD 550–coated patch pipette in acute slice. Inset, dendritic region and 12 spines selected for 2P glutamate uncaging. Center, uncaging-evoked excitatory postsynaptic potentials (gluEPSPs) at indicated spines with an interspine stimulation interval (IsSI) of 200 ms. Bottom, simultaneous glutamate uncaging at all 12 spines (IsSI = 0.3 ms) evokes a dendritic spike (arrow, \( n = 8 \) out of 9 tested dendrites in 4 neurons from 2 animals, patched with various QD-coated pipettes). Black, voltage trace; red: dV/dt trace.

( 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 that of uncoated control pipettes, whereas the capacitance was slightly decreased (Fig. 2a–c). QD-coated pipettes formed gigaseal contacts similar to that of uncoated patch pipettes\(^5\)–\(^7\),\(^12\) when we used the 'blow-and-seal' technique. We patched fluorescently labeled cell types in brain slices, including hippocampal DsRed-labeled cholecystokinin-positive and GFP-labeled parvalbumin-positive interneurons (Fig. 2d and Supplementary Fig. 3a). The fluorescence intensity of QD-coated pipettes was consistently higher than the intensity of endogenously expressed fluorescent markers. Indeed, the 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 (i.e., somatic firing and voltage responses to a series of positive and negative current injections), confirming that the QD coating neither interfered 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 back-propagating action potentials (Fig. 2e) as well as to direct synaptic stimulation by 2P glutamate uncaging\(^13\) (Fig. 2f and Supplementary Fig. 3b).

Under in vivo conditions, we recorded with QD-coated patch pipettes from cortical layer 2/3 (L2/3) pyramidal neurons of anesthetized mice expressing the 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\(^6\),\(^14\) (Fig. 3b). QD-coated pipettes also successfully electroporated\(^7\) Alexa Fluor 594 dye and a DsRed-encoding plasmid into individually identified L2/3 pyramidal neurons at ~300-µm depth.
Figure 3 | Neuronal manipulations with QD-coated pipettes. (a) Left, GCaMP6f-expressing cortical L2/3 pyramidal neuron (green) patched with QD 625–coated pipette (red) in vivo at 207-µm depth. Right, GCaMP6f Ca\textsuperscript{2+} signals (top) during spontaneous spiking activity (bottom). Data are representative of \( n = 5 \) cells. \( \Delta F/F \), relative change in fluorescence. (b) Left, mouse cortical interneuron (green) expressing ChR2-YFP under the control of the vesicular GABA (\( \gamma \)-aminobutyric acid) transporter (VGAT) promoter, patched with a QD 530–coated pipette (green) \textit{in vivo}. After spiking activity was recorded in cell-attached mode, the cell was loaded with Alexa Fluor 594 (red). Right, 40-Hz sine wave–modulated 470-nm LED light stimulation (blue; delivered through 2P microscope optical path) and electrical activity of the same patched neuron (black, single trial trace; red, raster plot of light-evoked action potentials, 10 trials). (c–e) Deep-layer targeting in a Thy1–EGFP mouse. (c) Top, \( \times \) projection (80 µm) of targeted neuron soma (arrowhead) at 760-µm depth pre-electroporation. Bottom, three-dimensional reconstructed orthogonal view, corresponding to \( \sim 800 \) µm. Green, EGFP fluorescence; arrowhead, site of pipette contact with the neuron. (d) Targeted neuron during electroporation. Red, QD 625. 40-mW laser power was applied at 940 nm. Ten frames were averaged. (e) Targeted neuron expressing DsRed (red) and EGFP (green) 2 d later.

(c–e) Deep-layer targeting in a Thy1–EGFP mouse. (c) Top, \( \times \) projection (80 µm) of targeted neuron soma (arrowhead) at 760-µm depth pre-electroporation. Bottom, three-dimensional reconstructed orthogonal view, corresponding to \( \sim 800 \) µm. Green, EGFP fluorescence; arrowhead, site of pipette contact with the neuron. (d) Targeted neuron during electroporation. Red, QD 625. 40-mW laser power was applied at 940 nm. Ten frames were averaged. (e) Targeted neuron expressing DsRed (red) and EGFP (green) 2 d later.

In summary, we have introduced a simple technique to fabricate permanently labeled fluorescent glass pipettes to facilitate visually targeted recordings from individual neurons at great depth and with high precision both \textit{in vitro} and \textit{in vivo}. Pipettes have been labeled previously with fluorophores; however, the dyes used did not provide the required 2P properties for deep tissue imaging\textsuperscript{15,16}. Our approach is an alternative or complement to the current ‘gold-standard’ method\textsuperscript{5–7} and removes the need to perfuse dye into the extracellular space continuously, which would reduce visibility and contrast. High-quality imaging with QD-coated pipettes was possible even at depths of \( \sim 500–800 \) µm within \textit{in vivo} brain tissue (Figs. 1 and 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, both visualization modalities may be used simultaneously for specific applications (for example, for monitoring pipette clogging or cell loading). Furthermore, narrow, size-tunable QD PL provides access to coatings of various colors across the spectrum as experimentally required\textsuperscript{8}. Critically, QD-coated pipettes did not interfere with physiological functions monitored throughout our experiments for \( \leq 3 \) h, suggesting they can be used for a wide array of biological experiments. Although 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 deep tissue is needed.

**METHODS**

Methods and any associated references are available in the online version of the paper.
Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS
The authors acknowledge the Defense Advanced Research Projects Agency, Naval Research Laboratory Nanosciences Institute, Defense Threat Reduction Agency Joint Science and Technology Office MIPR B112582M and Invitrogen for providing the 625-nm QDs. We thank G. Szabó and Z. Máté (Institute of Experimental Medicine) for providing the CCK/DsRed3 BAC and PV/GFP BAC transgenic mice. We thank J. Veres, Zs. Kohus, Z. Péterfy, N. Lenkey and E. Papp (Institute of Experimental Medicine) for providing brain slices with fluorescently labeled neurons; A. Holtmaat (University of Geneva) for providing the Thy-1 EGFP-M mice; and J. Weber, A. Ráksai-Maár, M. Prsa and M. Cane for technical assistance. This work was supported in part by the Wellcome Trust (grant 090915/Z/09/Z, J.K.M. and B.K.A.), Hungarian Academy of Sciences (Lendület LP-2011-012, J.K.M.), Howard Hughes Medical Institute and Swiss National Science Foundation (D.H.).

AUTHOR CONTRIBUTIONS
B.K.A., M.B., J.J.M. and I.L.M. conceived of 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
The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Materials and methods. Preparation of hydrophobic QDs. Native organic QDs\textsuperscript{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 20-ml glass vial. The QDs were then centrifuged (2,000g) to a pellet and the supernatant decanted and discarded. The QDs were again resuspended in hexane or toluene. This was followed by another round of washing and precipitation. The QD pellets were resuspended in hexane for probe coating.

Two-photon excitation 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\textsuperscript{12}. Briefly, QD or dye solutions at micromolar Fluor dyes. The QD pellets were resuspended in hexane for probe coating. This was followed by another round of washing and precipitation. QD samples in toluene or decane were precipitated by the addition of several milliliters of an acetone: methanol 50:50 mixture in a 20-ml glass vial. The QDs were then precipitated by the addition of several milliliters of an acetone: methanol 50:50 mixture in a 20-ml glass vial. The QDs were then centrifuged (2,000\textsuperscript{g}) at 33–35°C in aCSF containing (in mM) NaCl 125, KCl 3, NaHCO\textsubscript{3} 25, NaH\textsubscript{2}PO\textsubscript{4} 1.25, CaCl\textsubscript{2} 1.3, MgCl\textsubscript{2} 1, glucose 25, Na-pyruvate 3 and ascorbic acid 1, saturated with 95% O\textsubscript{2} and 5% CO\textsubscript{2}.

Pipette property measurements. Pairs of pipettes were pulled from the same borosilicate glass. QD-coated and uncoated pipettes were filled with internal solution, leaving a blocking bubble at the tip of the pipette, and were 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 Axio Examiner epifluorescence microscope equipped with infrared Dott optics and a water-immersion lens (63x, 0.9 numerical aperture (NA), Zeiss). Current-clamp whole-cell patch-clamp recordings were performed with a Dagan BVC-700. This 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-glucuronate 134, KCl 6, HEPES 10, NaCl 4, Mg\textsubscript{2+}-ATP 4, Tris\textsubscript{2+}-GTP 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\textsuperscript{2+} measurements; all dyes were from Invitrogen). Series resistance was <30 MΩ. For Figure 2a–c, the hypothesis and preliminary observations suggested no major changes in electrical properties of the pipettes by QD coating. We think that a 50–100% change in resistance, capacitance or access resistance by the pipettes would be important for their use. The sample size of 4–8 in the experiments where these values were specifically compared appeared large enough to exclude a major change in electrical properties. This was also consistent with our impression from the rest of the experiments reported in the paper.

Two-photon imaging and uncaging. Two ultrafast pulsed laser beams (Chameleon Ultra II, Coherent) and a dual galvanometer-based 2P laser-scanning system (Prairie Technologies) were used to simultaneously image neurons in the hippocampus (at 880 or 920 nm) and to focally uncage MNI-caged-1-glutamate (Tocris; 9–10 mM applied via pressure ejection through a 20- to 30-µm-diameter pipette above the slice) at individual dendritic spines (at 720 nm)\textsuperscript{22}. Laser beam intensity was independently controlled with electro-optical modulators (Model 350-50, Conoptics). All images shown are stacks of multiple images. Uncaging dwell time was 0.2 ms; galvo move time was 0.1 or 200 ms (see text). Line-scan imaging was performed at 150–500 Hz.

Data analysis. Analysis was performed using custom-written macros in IgorPro (WaveMetrics). Ca\textsuperscript{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 (gigaohm seal resistance, <30 MΩ 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(Tl-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 isoflurane 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 per kg body weight dexamethasone intramuscular (i.m.); 5 mg/kg Carprofen subcutaneous (s.c.)), analgesic (0.1 mg/kg buprenorphine i.m.) and local anesthetic (1% lidocaine s.c.) drugs.

**Stereotactic injections of GCaMP6.** Two to four weeks before 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 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. The positive pressure was released after a 50% increase in the pipette resistance. Targeted single-cell electroporation was performed as previously described9 using an Axoporator 800 A (Molecular Devices). Borosilicate pipettes were filled with internal solution and 50 µg µl−1 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 three penetrations were performed at the same brain location. Noticeably, QDs were 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 the 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. No data were excluded from the analysis.

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