Supporting Information

Imaging sodium flux during action potentials in neurons with fluorescent nanosensors and transparent microelectrodes

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Methods and Materials

Reagents

Ethylene dioxythiophene (EDOT) monomer and poly(styrene sulfonate) sodium salt (NaPSS), Bis(2-ethylhexyl) sebacate (DOS), sodium ionophore X (NaI-X; 4-tert-Butylcalix[4]arene-tetraacetic acid tetraethyl ester), chromoionophore III (CHIII; 9-(Diethylamino)-5-[(2-octyldecyl) imino] benzo[a] phenoxazine, ETH 5350), dichloromethane (DCM), 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), potassium chloride (KCl), sodium chloride (NaCl), sodium tetrakis-[3,5-bis(trifluoromethyl)phenyl]-borate (NaTFPB), and tetrahydrofuran (THF) were purchased from Sigma-Aldrich. Polystyrene spheres (carboxyl latex bead, 4% w/v, 1.0 μm in diameter), Octadecyl rhodamine B chloride (R18), gramicidin, monensin, CoroNa, and all cell culture media and supplements were obtained from Thermo Fisher. 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-550] (ammonium salt) (DSPE-mPEG550) was obtained from Avanti Polar Lipids, Inc.

Nanosensor Fabrication

Optode-based sodium nanosensor (Na^+ OBN) recipe and preparation procedure are previously reported.[1-2] The sodium-selective optode, comprising sensing components NaI-X (2 μmol, 2 mg), CHIII (878 nmol, 0.5 mg), NaTFPB (1.12 μmol, 1 mg), and R18 (123 nmol, 0.09 mg) was reconstituted in 300 μL THF and 200 μL DOS. Just prior to nanosensor fabrication, DSPE-mPEG550 (250 μg) was dried and rehydrated in HEPES-KCl buffer (145 mM KCl, 10 mM HEPES; pH adjusted to 7.2 with KOH) in a glass scintillation vial. In a typical OBN fabrication, 50 μL of as-prepared optode was added to 70 μL DCM, which was then sonicated with the 4 mL of HEPES-KCl buffer containing DSPE-mPEG550 at 10% intensity for 1 minute (Branson digital sonifier S-450D; 1/8" diameter tip). After sonication, the organic solvents were removed using a rotavap (Buhle) for 15 min at room temperature, and the resulting emulsion was filtered with a 100 nm syringe filter (Millipore).

In-solution sensor calibration

In-solution OBN calibration experiments were done with Spectramax M3 plate reader (Molecular Devices). OBNs were calibrated for fluorescent response to a series of Na^+ concentrations. To characterize the fraction protonated during measurements in Na^+, acid and base standards were used as endpoints for protonated or deprotonated fluorophore conditions, respectively.

The fluorescence intensities for CHIII (λ_{EX}: 630 nm, λ_{EM}: 685 nm) and R18 (λ_{EX}: 555 nm, λ_{EM}: 585 nm) were measured with plate reader in bottom read mode through clear-bottom 96-well. The emission fluorescence intensity ratio, *R*, of two fluorophores (CHIII:R18) was calculated as:

$$R = \frac{Emission\ at\ 685\ nm}{Emission\ at\ 585\ nm} \quad \textbf{(Eq. S-1)}$$

Traditionally, optode data is converted to a normalized value termed α, by ratios of 685 nm/585 nm defined as:

$$\alpha = \frac{R - R_D}{R_P - R_D} \quad \textbf{(Eq. S-2)}$$

where, *R* is the fluorescence ratio measured experimentally, and R_P and R_D are the ratios of protonated and deprotonated state of the chromoionophore, respectively. The effective Na^+ concentration at half-maximal sensor response (defined here as, EC_{50}) was determined according to the dose-response (Hill) equation.[2]

Transparent Microelectrode (TME) Fabrication

Fabrication of Gold Nanomesh. An air/water interface with self-assembly method was used as previously described to deposit PS nanospheres on glass substrate.[3] After forming the PS nanosphere monolayer, inductively coupled plasma-reactive ion etching (ICP-RIE) with O_2 and CHF_3 gases trimmed the spheres size. The etching time was 40 s with 40 sccm of O_2, 2 sccm of CHF_3, pressure of 25 mT, 100 W for radio frequency power 1 (RF1) and 150 W for RF2. 1-nm-thick Cr and 15-nm-thick Au were deposited at the rate of 0.5 and 1 A s^{-1}, respectively, utilizing e-beam evaporation for easier lift-off. Sonication of the samples in chloroform for 2 min produced gold nanomeshes on the glass substrate.

Fabrication of Gold Nanomesh Microelectrodes. Positive photoresist (S1818, Shipley) spin-coated the nanomesh using 4000 rpm for 45 s. Then, microelectrode and interconnect patterns were defined using photolithography with UV exposure and development, followed by wet etching of Au and Cr. Sonication of the samples exposed the Au nanomesh patterns. SU-8 2005 (Microchem), which served as the encapsulation layer, spin-coated the patterned sample using 4000 rpm for 30 s for contact isolation, resulting in a 4-um-thick insulator layer. After soft baking at 95 °C for 2 min, the sample went through UV exposure of 6 s, followed by a post exposure bake of 3 min at 95 °C. Sonication in SU-8 developer for 20 s, followed by rinsing with fresh SU-8 developer and IPA formed clear isolation patterns. After

that, hard baking at 200 °C for 20 min completed the process. The fabricated microelectrode has 10 mm length, a 300 μm width, and a 2 × 3 mm^2 contact pad size.

Electrodeposition of PEDOT: PSS. PEDOT: PSS electroplating bath was prepared by mixing ethylene dioxythiophene (EDOT) monomer (0.01 m) and poly (styrene sulfonate) sodium salt (NaPSS) powder (0.1 m) in deionized water (150 mL) and stirring for 30 min. 0.2 mA/cm^2 constant current was applied using galvanostatic mode of Gamry Reference 600+ potentiostat/galvanostat/ ZRA (Gamry Instruments, Inc) for 50 s. Here typical three-electrode configuration was adopted including Ag/AgCl reference electrode and Pt counter electrode. The Au nanomesh microelectrode with 6400 μm^2 electrode area was immersed into the monomer bath as working electrode.

Dorsal root ganglion (DRG) Dissection and Cell Culture

All procedures were carried out in accordance with the regulations in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee (IACUC) at Northeastern University. Male Sprague Dawley rats (150-200 g, Charles River Laboratories, Wilmington, MA) were euthanized by CO_2 and sprayed thoroughly with 70% (vol/vol) ethanol prior to dissection of DRGs. All following procedures were performed under sterile condition. The DRGs were carefully dissected and isolated from spinal nerve roots based on procedure described elsewhere.[4] Briefly, the euthanized rat was decapitated, and the dorsal skin was cut and removed along its midline from the neck to tail. The spinal column was then horizontally hemisected to reveal DRGs as visible white and partially translucent bulbs along both sides of the spinal column. Fine forceps were used to grip the distal process of the DRGs, beneath the bulbs to remove and collect each ganglion. The DRGs from all spinal levels were dissected aseptically and distal and proximal processes emanating from DRGs were removed using surgical scalpel blades prior to placing DRGs in 15 mL conical tube containing ice-cold Ca^{2+}, Mg^{2+}-free HBSS.

Isolated DRGs were then enzymatically treated twice 20 min each at 37 ^oC on a rotator with trypsin (25 μg/mL) and collagenase IV (200 u/mL) in Ca^{2+}, Mg^{2+}-free HBSS. The contents of the tube were mixed thoroughly by flicking the tube between each trypsin/collagenase incubation steps. After incubation, the DRG neurons were dispersed by trituration (10-15 times) in culture medium (DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine) through a series of fire-polished Pasteur pipettes, each successive pipette with a smaller tip diameter, and filtered-through a 0.1 mm filter. The cells were then plated on TME (transparent microelectrode) substrate pre-treated

with Poly-L-Lysine (PLL) and incubated for 16-24 h at 37 °C under an atmosphere containing 5 % CO_2 for subsequent experiments.

In situ cell calibration and fluorescence imaging

In this study, DRG neurons have been classified based on anatomical criteria: small to medium in cell body size (< 40 μm diameter), round morphology, and distinctive perinuclear features.[5] OBNs were then delivered to DRG neuron intracellular space through microinjection (InjectMen 4, Eppendorf). For in situ cell calibration, OBN loaded cells were first incubated with HEPES-KCl buffer containing 10 μM gramicidin and 100 μM monensin for 15 min to equilibrate Na^+ levels across cell membrane. Na^+ calibration buffer with incremental levels of Na^+ were subsequently added and fluorescence images were acquired after 10 min incubation at each Na^+ concentration for subsequent analysis. There were nominal batch-to-batch differences in sensor response and consequently, in solution and in situ cell calibrations were routinely performed to ensure the sensors maintain responsiveness to the intracellular Na^+ levels.

Data and Image Analysis

Data and image analysis was performed using Matlab and OriginPro, and images were constructed in ImageJ. For cell imaging, fluorescence intensities over time were drawn from same region of interest on two color channels based on the vector established by reference points. The sensor intensity ratio over time was then calculated by average fluorescence intensities of their respective channels.

Supplementary Figures

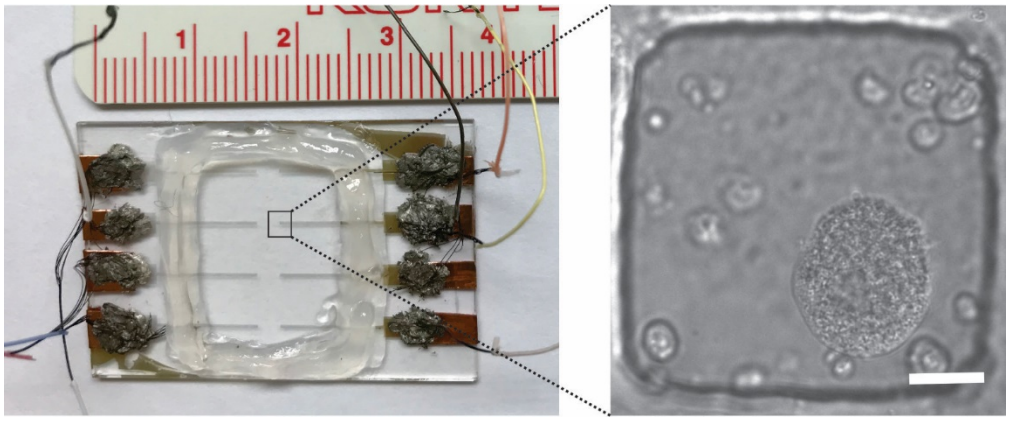

Figure S-1. Au/PEDOT:PSS nanomesh TME array (2×4) imprinted on the glass. The surroundings of the array are corralled by grease to facilitate the cell culturing. Blow-up shows the cell on the active area of individual TME. Scale bar: 20 μm.

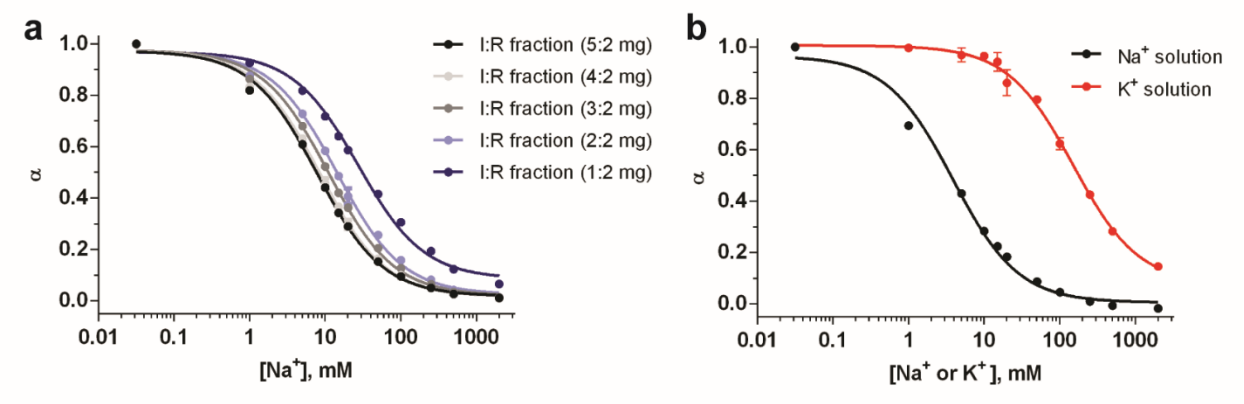

Figure S-2. (**a**) Sensor response can be fine-tuned by altering amounts of sensing components. (**b**) Characterization of nanosensors in response to Na^+ (black) demonstrate 2 orders of magnitude selectivity over K^+ (red).

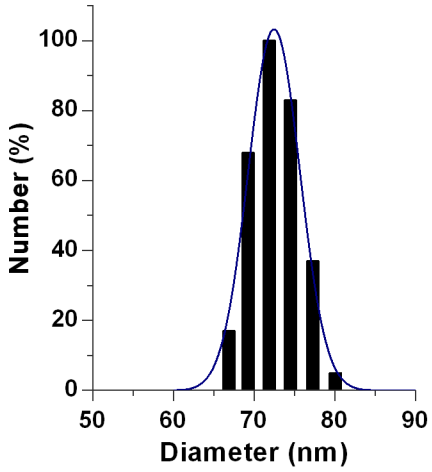

Figure S-3. Size distribution of OBNs as measured by dynamic light scattering show an effective average diameter of 75 nm.

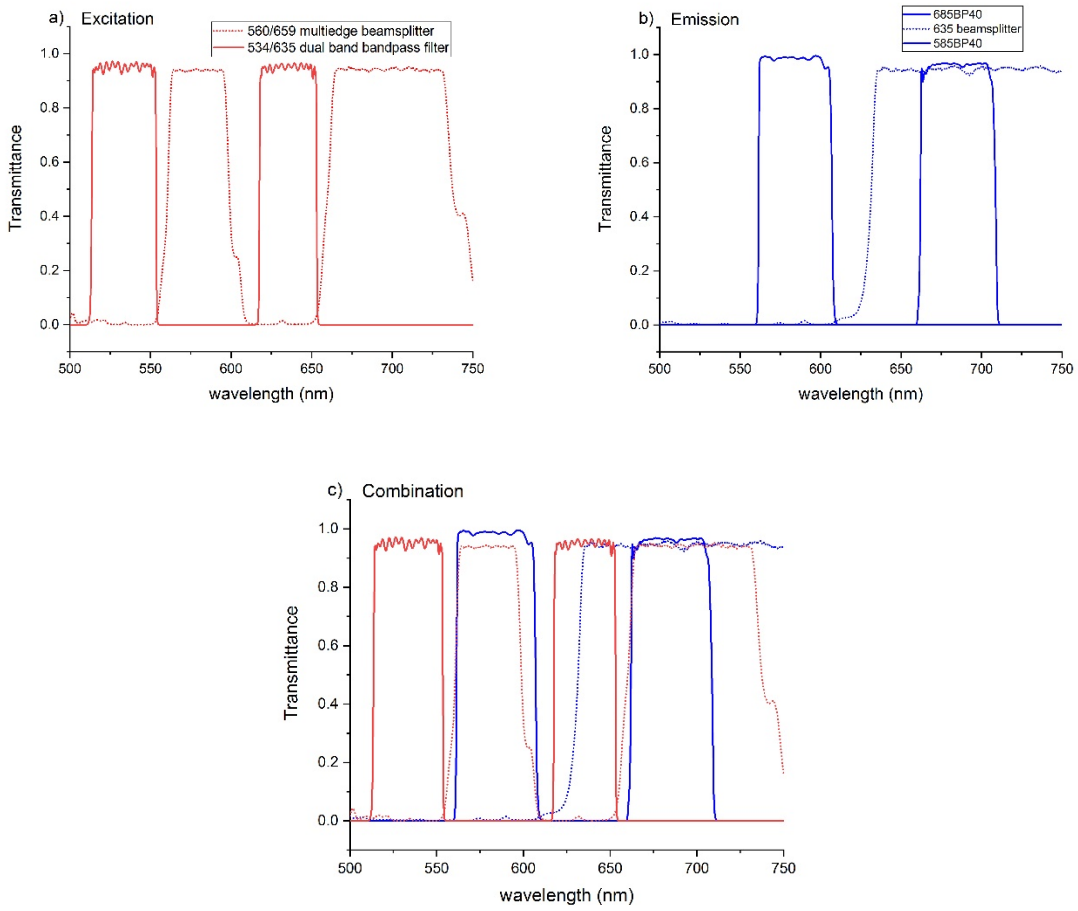

Figure S-4. Spectral profiles of filters and beamsplitters used in the fluorescence ratiometric imaging scheme. Solid line: bandpass filter; dotted line: beamsplitter. (**a**) Spectra of dual band bandpass filter (534/635 nm) and multiedge beamsplitter (560/659 nm) on the excitation end of the optical scheme. (**b**) Spectra of two bandpass filters (685/40 and 585/40) and longpass dichroic (635 nm) on the emission end. (**c**) Spectra of all five optical components. We can see there is minimal spectral overlap over individual optics. Data: courtesy of Semrock, Inc.

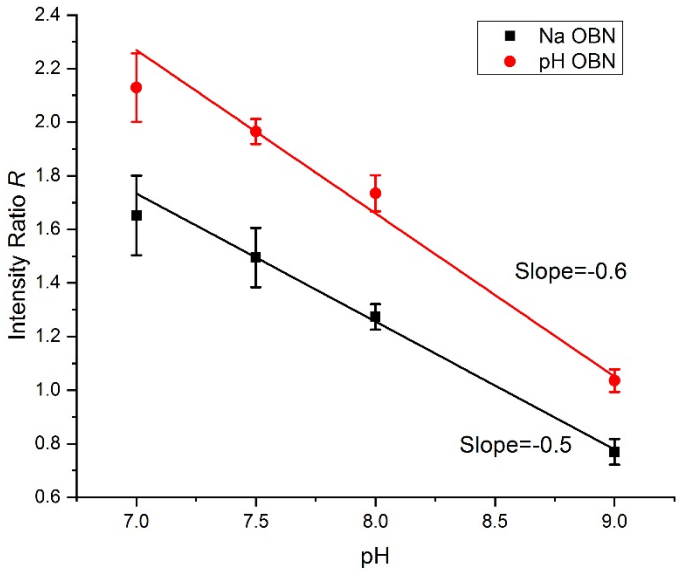

Figure S-5. Na^+ and pH OBN responses to different pH values. Both sensors shared similar proportionality (slope) of responses to pH changes.

Supplementary Videos

Video S-1. OBN response to pulse trains with consistent stimulation intensities on two color channels of the DRG. Left: RhD channel; Right: CH III channel

Video S-2. OBN response to pulse trains with increasing stimulation intensities on two color channels of the DRG. Left: RhD channel; Right: CH III channel

Reference

1. Ruckh, T. T.; Mehta, A. A.; Dubach, J. M.; Clark, H. A., Polymer-Free Optode Nanosensors for Dynamic, Reversible, and Ratiometric Sodium Imaging in the Physiological Range. *Sci Rep* **2013,** *3*, 3366.
2. Rong, G.; Kim, E. H.; Poskanzer, K. E.; Clark, H. A., A Method for Estimating Intracellular Ion Concentration Using Optical Nanosensors and Ratiometric Imaging. *Sci Rep* **2017,** *7*, 10819.
3. Qiang, Y.; Seo, K. J.; Zhao, X. Y.; Artoni, P.; Golshan, N. H.; Culaclii, S.; Wang, P. M.; Liu, W. T.; Ziemer, K. S.; Fagiolini, M.; Fang, H., Bilayer Nanomesh Structures for Transparent Recording and Stimulating Microelectrodes. *Adv Funct Mater* **2017,** *27*, 1704117.
4. Gilbert, D.; Funk, K.; Dekowski, B.; Lechler, R.; Keller, S.; Mohrlen, F.; Frings, S.; Hagen, V., Caged Capsaicins: New Tools for the Examination of Trpv1 Channels in Somatosensory Neurons. *Chembiochem* **2007,** *8*, 89-97.
5. van Dorp, R.; Jalink, K.; Oudega, M.; Marani, E.; Ypey, D. L.; Ravesloot, J. H., Morphological and Functional Properties of Rat Dorsal Root Ganglion Cells Cultured in a Chemically Defined Medium. *Eur J Morphol* **1990,** *28*, 430-444.