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Imaging Sodium Flux during Action Potentials in Neurons with Fluorescent Nanosensors and Transparent Microelectrodes

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Abstract

Sodium flux plays a pivotal role in neurobiological processes including initiation of action potentials and regulation of neuronal cell excitability. However, unlike the wide range of fluorescent calcium indicators used extensively for cellular studies, the choice of sodium probes remains limited. We have previously demonstrated optode-based nanosensors (OBNs) for detecting sodium ions with advantageous modular properties such as tunable physiological sensing range, full reversibility, and superb selectivity against key physiological interfering ion potassium.

¹ Motivated by bridging the gap between the great interest in sodium imaging of neuronal cell activity as an alternative to patch clamp and limited choices of optical sodium indicators, in this Letter we report the application of nanosensors capable of detecting intracellular sodium flux in isolated rat dorsal root ganglion neurons during electrical stimulation using transparent microelectrodes. Taking advantage of the ratiometric detection scheme offered by this fluorescent modular sensing platform, we performed dual color imaging of the sensor to monitor the intracellular sodium currents underlying trains of action potentials in real time. The combination of nanosensors and microelectrodes for monitoring neuronal sodium dynamics is a novel tool for investigating the regulatory role of sodium ions involved during neural activities.

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ASSOCIATED CONTENT

Supporting Information

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Methods and materials; Supplementary figures: TME array imprinted on the glass; sensor characterizations; sensor size distribution; spectral profiles of optics used in the imaging scheme; Na⁺ and pH sensor responses to different pH values (PDF)

Video S-1: OBN response to pulse trains with consistent stimulation intensities on two color channels of the DRG (AVI)

Video S-2: OBN response to pulse trains with increasing stimulation intensities on two color channels of the DRG (AVI)

Graphical Abstract

Keywords

sodium; nanosensor; optode; ion sensing; ratiometric imaging; dorsal root ganglion; transparent microelectrode

The role of sodium (Na^+) ions in intracellular signaling and electrical propagation is primarily to regulate the electrochemical gradient across the plasma membrane. In the case of excitable cells, inwardly directed Na^+ ions are the major charge carrier during action potentials and excitatory postsynaptic currents. Moreover, several studies indicate that Na^+ ions play a major role in activity-dependent synaptic plasticity.²

Many questions concerning the physiological consequences of intracellular sodium ($[\text{Na}^+]_i$) oscillations remain unanswered. In contrast to imaging intracellular calcium (Ca^{2+}) transients, $[\text{Na}^+]_i$ imaging has, to date, progressed at a slower pace. This disparity is in part due to the scarcity of suitable fluorescent indicator dyes with desirable photochemical properties. Na^+ indicator dyes such as Sodium Green,³ CoroNa Green,⁴ and Asante Natrium Green,⁵ which have their absorption maximum around 488–492 nm, and more recently CoroNa Red,⁶ with a longer emission wavelength, have proven useful in a variety of studies.⁷ However, interactions of these dyes with cellular proteins and single wavelength emission can hinder reliable measurements.⁸ Moreover, a recent study has shown some molecular indicators such as CoroNa can rapidly leach out of the cell over the course of a few minutes,⁹ which further constrains the observation time for investigating Na^+ dynamics. Precise analysis of the spatial distribution of Na^+ signals or quantitative measurements has been best achieved using a ratiometric Na^+ dye, sodium-binding benzofuran isophthalate (SBFI),¹⁰ which has been reported for Na^+ imaging in spine and fine dendrites of central neurons.^{2,11} In addition, somatic and dendritic signaling in neurons¹² as well as astrocyte signaling¹³ has been demonstrated with SBFI. However, SBFI need to be excited below 400 nm using UV-lasers, which could limit its use for deep tissue imaging due to high light scattering and potential phototoxic damage.

In recent years, nanoscale sensors have taken promising strides toward a better understanding of dynamic processes in biological systems. Nanosensors have been used to obtain insights into cell membrane organization,¹⁴ identify cancer biomarkers,¹⁵ and monitor concentration changes of analytes of biological significance such as neurotransmitters¹⁶ and ions.¹⁷ In particular, optode-based nanosensors¹⁸ (OBNs) have been established as a promising tool for sensing monovalent and divalent ions. Ion-selective optodes,¹⁹ an optical counterpart of ion-selective electrodes, are composed of three major sensing components: a molecular ion chelator (ionophore); a pH-responsive fluorophore (chromoionophore); and an ion exchanger for maintaining electroneutrality, all of which are hosted in a lipophilic nanoparticle (NP) polymer matrix (Figure 1a). Extraction of the ion of interest by the ionophore leads to a charge imbalance and concomitant deprotonation of the chromoionophore to maintain charge-neutrality. Hence, the changes in fluorescence intensity induced by the fluorophore protonation state within the OBN indicate the concentration of the targeted ion. The modular nature of the OBN allows one to tailor the sensor performance to address these issues collectively: (i) ability to use indicators with high quantum yield and varying emission wavelength; (ii) fine-tune the dynamic range and selectivity by changing the ratio of sensing components; and (iii) minimize photodamage and maximize image penetration depth by incorporating near-infrared (NIR) fluorophores. The OBNs also reach steady-state responses quickly, at least at the time scale of ~160 ms, as indicated in theoretical¹⁸ and experimental²⁰ studies. There has been rapid progress of optode-based sensor optimization in recent years, such as carbon dot incorporation,²¹ discovery of exhaustive²² and nonequilibrium diffusion²³ sensing modes, and advances in paper-based sensing.²⁴ Given that optode-based ion sensing is a mature concept with many advantages, the OBNs should have long since become the method of choice for characterizing spatiotemporal ion dynamics during neuronal signaling, where ion fluxes play prominent regulatory roles.²⁵ However, there have only been a few studies where OBNs are employed to monitor endogenous analyte concentration changes in the cell.^{20,26} One constraint on OBNs for intracellular sensing is its relatively large size (>50 nm) compared to molecular indicators. Unlike acetoxymethyl (AM) esters, nanosensors are cell-impermeant except through endocytic pathways. In addition, if introduced to the cell through conventional patch pipet loading, slow diffusion prevents the sensors from distributing throughout the cytosol.²⁷ We have recently shown the applicability of Ca²⁺-selective OBNs for quantitative imaging of cellular Ca²⁺ dynamics during pharmacological stimulation by delivering the sensors via microinjection.²⁰ However, to our knowledge, OBNs have not been used to monitor Na⁺ ion flux in neuronal cells. One main reason is the loss of viability for microinjected cells caused by further mechanical damage from patch pipet insertion, which is conventionally used to deliver stimulation current during whole-cell ion channel electrophysiological studies.

As such, our approach is to develop a sensing platform that utilizes both highly robust ion-selective OBNs and recently developed gold/poly(3,4-ethylenedioxythiophene)-poly-(styrenesulfonate) (Au/PEDOT:PSS) nanomesh transparent microelectrodes (TMEs).^{28,29} The advantageous optical transparency and electrical properties of TMEs enable simultaneous electrical stimulation and optical measurement. The transparency of the stimulation electrode is crucial for good phase-contrast cell imaging, which is necessary to perform the microinjection procedure. TME arrays can also be imprinted on a glass substrate

to serve as the vessel for cell culturing (Figure S-1). The superb electrical performance of the TME further allows us to skip stimulation via patch pipet which could substantiate further mechanic stress on cells already undergoing invasive microinjection procedures, thereby adversely affecting the cell viability. Moreover, TMEs enable more localized, precise delivery of stimulation current compared with field stimulation, therefore having better control over stimulation parameters.

By employing TMEs as an electrical stimulation media, we have for the first time demonstrated the ratiometric imaging of Na^+ flux in isolated rat dorsal root ganglion (DRG) neurons using OBNs during electrical stimulation. The Na^+ OBN used in this work is based on the recipe and fabrication procedure reported previously (see methods in the Supporting Information).^{1,20} We had successfully fabricated Na^+ OBNs with full characterization of sensor performance, including selectivity, sensitivity, and sensing range (Figure S-2). The nanosensors used in this study have an average hydrodynamic diameter of 75 nm (polydispersity index 0.112) (Figure S-3) with zeta-potential of -23.4 ± 1.3 mV, indicating OBNs are stable in the physiological pH buffer solutions.

We have tailored the ratio of different components within the OBN to ensure its optimal response range is close to the physiological $[\text{Na}^+]_i$, which is typically around 5–15 mM.³⁰ The measured effective concentration at half-maximal sensor performance (EC_{50}) is 7.6 ± 0.4 mM based on the Hill fitting (Figure 2),²² which makes it a promising candidate for intracellular Na^+ monitoring. Here the sensor intensity ratio is normalized using eq S-1 and S-2. This normalized response is termed the deprotonation degree (α , α), a common practice in the optode research community.¹⁸ The sensor calibrations were regularly performed to ensure optimal responsiveness for intracellular studies, given the nominal batch-to-batch differences in sensor response during fabrication. All sensor calibration curves in this study were fitted by Hill equation. In addition, we performed in situ calibration of the sensors (see methods in the SI) and compared the results with that from calibration in buffer alone (Figure 2). In brief, in situ cell calibrations were performed by perfusing nanosensor-loaded cells with varying concentrations of Na^+ using calibration buffer in the presence of 10 μM gramicidin and 100 μM monensin which serve as ion carriers to facilitate the equilibrium of Na^+ levels across the cell membrane.³⁰ We found the response curve of the in situ sensor curve shifted modestly to the right, with EC_{50} of 18.5 ± 2.4 mM, where physiological Na^+ concentration range (5–15 mM) is still within the dynamic range of the sensor (Figure 2). As noted previously, the response of OBNs is subject to various factors such as dye–protein interactions.³¹ Due to these environmental factors, the EC_{50} determined in situ for intracellular OBNs is higher than that determined in cell-free buffer solutions.²⁰ For instance, the dissociation constant (K_d) value of SBFI determined in rat hippocampal neurons (18 mM) was greater than that in cell-free calibrations.³² Fluorescence-based sensors with ratiometric readouts are always preferable compared with single-wavelength alternatives as they can mitigate certain imaging artifacts during the measurement. Our Na^+ OBNs encapsulate two different fluorophores, chromoionophore III (CHIII; Ex/Em: 630/685 nm) and octadecyl rhodamine (Rhd; Ex/Em: 555/585 nm), whose fluorescence emission intensities change in opposite directions during deprotonation. By taking ratiometric measurements our nanosensor has the advantage of

minimizing the experimental artifacts resulting from inhomogeneity in the size or quantity of sensors.

Our aim for the optical setup was to implement an imaging scheme that both covered the entire region of the individual stimulation TME ($80 \times 80 \mu\text{m}^2$) and yielded a ratiometric fluorescence readout from our OBNs in the cell, while maintaining sufficient temporal resolution. To this end, our imaging system (see Figure 1b) is based on a conventional upright wide-field microscope (Olympus BX51) augmented with a dual wavelength detection module (W-View, Hamamatsu) which enables simultaneous recording of two fluorescence images in the same field of view. Whitelight from a 150 W xenon lamp first passes a shutter (Lambda LB-SC) to reduce photobleaching. The light then passes a dual band-pass filter (534/635 nm, Semrock) and multiedge beamsplitter (560/659 nm, Semrock) to obtain one excitation light beam containing two monochromatic wavelengths. The emission light from intracellular sensors is then collected by a 60 $\times$ water immersed objective, and chromatically separated by the dichroic (635 nm, Semrock) and two bandpass filters (685 BP40 and 585 BP40, Semrock) based on the emission spectra²⁰ of the two fluorophores in the OBN. The two emission beams are then finally reimaged on translated areas of the same camera (C11440, Hamamatsu). We carefully chose the above-mentioned filter/beamsplitter combination to match the fluorescence profile of the two fluorophores used in the sensor with minimal spectral overlap (Figure S-4). The imaging experiment was performed with 50 ms exposure time at 1 frame per second. The temporal resolution here is limited by the mechanics of the shutter, and replacement of the Xenon light source with two shuttered monochromatic excitation lasers would boost the photon flux of the sensor and enable higher frame rate. Therefore, the current implementation of the instrument setup is best suited to detect Na^+ transients at longer time scales, such as Na^+ dynamics from trains of action potentials³³ (APs) rather than a single AP which occurs on the time scale of milliseconds.³⁴

Using this 2-color imaging setup, we applied our Na^+ OBNs to image the Na^+ dynamics in DRG neurons during electrical current stimulation by the TMEs. DRG refers to a cluster of sensory neuronal cell bodies isolated from the spinal nerve, and increasing attention has been paid to multiple isoforms of its Na^+ channels.³⁵ Na^+ channel currents play pivotal roles in influencing excitability of DRG neurons which underpin numerous pathological conditions, such as neuropathic pain³⁶ and erythromelalgia.³⁷ However, a detailed mechanism of how Na^+ dynamics regulate these chronic conditions remains unclear.³⁸ Most of the Na^+ studies on DRGs are indirectly investigated through electrical current profiling via patch clamping.³⁵ Here our experimental approach is to perform direct DRG Na^+ chemical imaging by simultaneous electrical stimulation and fluorescence imaging of Na^+ OBNs. Imaging buffer containing (mM) 145 NaCl, 5 KCl, 10 HEPES, 10 glucose (adjusted to pH 7.2) was used for all stimulation studies. Before the imaging experiment, isolated DRGs were first selected from mixed primary cell culture on the TME based on its unique morphology by visual inspection (see methods in the SI), and those that sit on top or in direct contact with the electrode were then microinjected with OBNs. We microinjected 10^2 – 10^3 nanosensors (picomolar) into the individual cell, which we found is optimal by striking a balance between attaining a robust fluorescent signal and damaging the cells through overinjection of fluid.²⁰ Injected DRGs with no visual deformation or blebs were

chosen for subsequent studies. We presented the Na^+ cellular imaging studies as a ratiometric response, rather than the normalized alpha, in order to facilitate comparison to traditional cellular imaging. The Na^+ concentration difference across cell membranes allows us to assess the location of the sensor based on the fluorescence intensity ratio after microinjection. Sensors exposed in the extracellular environment with high NaCl concentration (145 mM) will have a lower fluorescence intensity ratio compared with ones in the cytoplasm ($[\text{Na}]_i$: 5–15 mM). Unlike previous OBNs microinjection into cultured HEK and HeLa cells,^{20,26} the injected OBNs were not dispersed throughout the DRG cytosol, but rather remained close to the injection site (Figure 1b). We postulated that this is in part due to the denser internal structures of the DRGs, such as cytoskeleton³⁹ or endoplasmic reticulum⁴⁰ which may hinder the diffusion of the sensor. The corresponding TME was then wired to the external stimulator (RHS 2000, Intan Technologies).

TMEs made of Au/PEDOT:PSS bilayer nanomesh possess both excellent charge injection properties and high transparency. The fabrication of Au/PEDOT:PSS nanomesh microelectrodes leverages nanosphere lithography and successful templated electroplating of PEDOT:PSS on Au nanomesh to form the bilayer nanomesh structure (Figure 3a). As shown in the SEM image, the lateral growth during the electroplating of PEDOT:PSS on Au nanomesh is minimal compared to the vertical growth, which generates a nearly perfect bilayer nanomesh. The detailed fabrication process is available in the Methods section of the SI. The coating of PEDOT:PSS enables a faradaic electrode/electrolyte interface instead of the purely capacitive behavior characteristic of most metal and transparent conductors like graphene and indium tin oxide (ITO).^{41,42} Importantly, the coating is composed of a nanomesh format, which makes it highly transparent in addition to its advantageous electrical properties. The redox reaction at PEDOT:PSS/electrolyte interface significantly improves the electrochemical performance of the microelectrodes, specifically improving both the impedance and charge injection limit. Here the impedance has a large influence on the noise level of recording. The charge injection limit characterizes the maximum amount of charge that can be applied to the microelectrodes while the polarization of electrodes still remains within the water window (−0.6–0.8 V), outside of which the water electrolysis takes place.²⁸ The charge transfer of Au/PEDOT:PSS is not only through the capacitive (double-layer capacitance) processes but also faradaic (redox reactions) processes, which result in a higher charge storage capacity (CSC).⁴³ The microelectrode with higher CSC will be less polarized by charge injection and thus has a larger charge injection limit when constrained by the same water window. As a result, the Au/PEDOT:PSS nanomesh microelectrode adopted in this work achieved ~12 k Ω impedance at 1 kHz and ~0.4 mC/cm² charge injection limit with a 6400- μm^2 electrode area. The stimulation capability of Au/PEDOT:PSS nanomesh microelectrodes was compared to that of a microelectrode made of ITO, which is a well-developed transparent conductor. Charge injection limit was characterized for both cases using 500 μs , biphasic current pulses stimulus. Au/PEDOT:PSS nanomesh microelectrodes demonstrated nearly 20 times larger charge injection limit than ITO ones (~0.02 mC/cm²). Under the same 0.4 mC/cm² stimulus, the potential transient of Au/PEDOT:PSS microelectrode is still within the water window while that of ITO microelectrode has already exceeded the water window drastically (Figure 3b), which creates an unsafe stimulation environment through water hydrolysis with unwanted

byproducts. Transferring sufficient charge through electrodes is essential to exceed the threshold potential for neuronal depolarization.⁴⁴ From previous studies, electrical stimulation with charge density over 0.1 mC/cm² were often required for different applications such as retinal treatment and motor control.^{45,46} By having charge injection limit around 0.4 mC/cm², the Au/PEDOT:PSS microelectrode is promising as a transparent stimulating microelectrode for a broad range of biological and clinical applications.

We first stimulated the sensor-loaded DRG by repetitive trains of short depolarizing pulses through TMEs. The time interval between two consecutive electrical stimuli was 100 s. Repetitive depolarizing pulse-induced Na⁺ action currents (ACs) were accompanied by simultaneous transient decreases in Na⁺ OBN intensity ratios. We observed pronounced differences in sensor response to the accumulated Na⁺ levels that were associated with incremental changes in action currents (ACs). Although each stimulation pulse train contains multiple pulses within one second, we found optical signal from Na⁺ OBNs at the injection site to last around 30 s (Figure 4a and Video S-1), which is similar to documented studies on somatic Na⁺ dynamics³³ of neurons by trains of action potentials and astrocytes induced by glutamate where the delay was attributed to different Na⁺ diffusion rates.^{33,47} We also observed consistent sensor response under stimulations of consecutive identical pulse trains, with optical signal variations within single trace less than 5%. We further tested the intracellular sensor response to stepwise stimulus intensity (current) changes and found a proportional increase in sensor responses (Figure 4b and Video S-2). Based on the linear extrapolation, we estimate 130 μ A/mm² of stimulus current increase would result in a maximum sensor intensity ratio change (R) of 0.1 at the injection site (Figure 4c). Here the measured sensor responses are more indicative of local Na⁺ transient dynamics near the membrane, where Na⁺ transient amplitude is more pronounced. The response of regionalized sensors could be different from the dynamics measured by indicators dispersed throughout the cytosol in terms of magnitude of fluorescence change and/or temporal responses due to the spatial difference in Na⁺ buffering capabilities and existence of Na⁺ microdomains,⁴⁸ as well as the slow diffusion of Na⁺.⁴⁷ For comparison, we found only small fluorescence changes using CoroNa dye under the same stimulation protocol (Figure 4d), which can be attributed to weak signal resulting from measurement of global Na⁺ transients⁴⁷ and/or leaching from the cell cytosol.⁹ To the best of our knowledge, this is the first time OBNs have been applied to the direct imaging of neuronal Na⁺ transients during electrical stimulation.

Since the sensing mechanism of the OBN is dependent on a chromoionophore, one natural concern is the pH interference from the surrounding environment. Indeed, this is a common issue also faced by the conventional indicators, such as SBFI. It has been reported that fluorescence of SBFI is sensitive to changes in pH between 6.5 and 7.5.³² To assess the impact of cellular pH changes when stimulated with electrodes, our controls used the same stimulation conditions and the same components of the Na⁺ OBN with the exclusion of the Na⁺ ionophore (termed pH OBN). The responses of both OBNs during pH titration are similar (Figure S-5), therefore pH OBN can serve as the proxy to discern whether the corresponding Na⁺ OBN responses are produced as a result of Na⁺ flux or endogenous pH changes in the cell. We observed minimal fluctuation in fluorescence response from the pH

OBNs during DRG stimulation using the same stimulation protocols (Figure 4e), which is indicative of the well-buffered intracellular environment, and the effect of pH is minimized.

In our current study, the Na^+ currents underlying single action potential generation in the soma generated small fluorescence changes and single spikes could not be detected. This is similar to what was observed with SBFI.⁴⁷ Accordingly, we will direct future studies toward investigating Na^+ flux in other neuronal subdomains such as the axon initial segment where Na^+ changes are substantial, but injection will be more challenging, as it requires targeted delivery of Na^+ OBNs by a larger bore pipet tip into a small subcellular region. Smaller sensors would have the advantage of better intracellular diffusion, but the emulsion-based fabrication process limits the size that can be achieved. In the future, other fabrication processes could be explored in order to further reduce the size and increase the likelihood of diffusion.

Overall, the modular nature of OBNs enables sensors to be designed to fine-tune the dynamic sensing range, sensitivity, selectivity, and choice of fluorophores for different needs in biological ion sensing. Microinjection techniques were used for OBN delivery to the cell cytosol, which is a more time-consuming method but also prevents the intracellular compartmentalization sometimes seen with AM esters.^{10,49} Given the invasive nature of microinjection, TME array is employed as a means of electrical stimulation of neuronal cells without secondary damage done by the mechanical intrusion of a patch pipet. Taken together, we believe this study takes a concrete step toward applying OBNs for monitoring ion dynamics during neuronal activity. The synergy created by the OBN-microinjection-TME trio is a valuable complement to the existing toolkit of ion indicators.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Sensor mechanism and optical setup: (a) Chromoionophore (C), Sodium Ionophore (I), Rhodamine (RhD), and Ion Exchanger (R) are all encapsulated in the PEG-lipid coated polymer matrix. The selective recruitment of sodium ion to the sensor results in deprotonations of two fluorophores, C and RhD, to maintain the charge neutrality. This leads to ratiometric fluorescence intensity change in signal readout. (b) OBNs (red) are first microinjected to the DRG on one of the 2×4 stimulation TMEs (expanded image, $80 \times 80 \mu\text{m}^2$, superimposed with schematic stimulation current pulse sequences). The dimensions of the TME schematic drawing are not to scale. The emission light collected from the OBN during stimulation is chromatically separated (585 BP40 and 685 BP40) and recaptured on two translated areas of the camera. Scale bar: $20 \mu\text{m}$.

Figure 2.

In solution, physiological buffer pH 7.2 (blue) and in situ cell (red) calibrations of the Na^+ OBNs. For intracellular applications, the sensor response (EC_{50}) derived from the Hill fit is tuned to the physiological Na^+ concentration between 5 and 15 mM. EC_{50} of the in solution sensor response is 7.6 ± 0.4 mM. In situ cell calibration (EC_{50} : 18.5 ± 2.4 mM) was performed by superfusing different concentrations of Na^+ in the presence of gramicidin and monensin to equilibrate Na^+ concentration across cell membrane. Ratiometric sensor responses are normalized to deprotonation degree (α) on the calibration curve.

Figure 3.

Transparent stimulation microelectrodes. (a) Optical image of transparent microelectrodes (left). Scale bar: 300 μm . Inset: microscope image of nanomesh microelectrode. Scale bar: 20 μm . SEM image of nanomesh structure (right). Scale bar: 500 nm. (b) Voltage transient of Au/ PEDOT:PSS nanomesh microelectrode and ITO microelectrode under 0.4 mC/cm^2 current pulse stimulus.

Figure 4.

Time-lapse measurements (exposure time: 50 ms, frame rate: 1 Hz) of sensor response to intracellular Na⁺ dynamics induced by electrical stimulation. (a) Single-trial sensor response to Na⁺ transients at the DRG injection site to stimulation pulse trains (2 μA , 2 ms duration, 50 Hz, repetition rate 1 Hz, stimulation time 5 s, 100 s between each stimulus). (b) Sensor response to increasing stimulation currents. Numbers above arrowheads indicate magnitude of stimulation current (μA). (c) Average sensor intensity ratio change ΔR plotted against stimulus current indicate sensor response is proportional to the stimulus intensity (Redline: linear fit, $R^2 = 0.95$, $n = 3$). (d) Fluorescence transients of CoroNa response to the stimulus under the same experiment protocol in (a). (e) pH sensor response to the stimulus under the same experiment protocol in (a). Arrowheads above traces indicate starting points in the stimulus sequences.