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A small-molecule photoactivatable optical sensor of transmembrane potential

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Abstract

This paper discloses the design, synthesis, and imaging applications of the first member of a new class of SPOTs, small-molecule photoactivatable optical sensors of transmembrane potential. SPOT2.1.Cl features an established voltage-sensitive dye, VoltageFluor2.1.Cl—or—VF capped with a dimethoxy-*o*-nitrobenzyl (DMNB) caging group to effectively eliminate fluorescence of the VF dye prior to uncaging. SPOT2.1.Cl localizes to cell membranes and displays weak fluorescence until photoactivated. Illumination generates the parent VF dye which then optically reports on changes in the membrane voltage. After photoactivation with spatially restricted light, SPOT2.1.Cl-loaded cells display bright, voltage-sensitive fluorescence associated with the plasma membrane, while neighboring cells remain dark. Activated SPOT reports on action potentials in single trials. SPOT can be activated in neuron cell bodies or uncaged in dendrites to enable structural tracing via “backfilling” of the dye to the soma, followed by functional imaging in the labeled cell. The combination of cellular specificity achieved through spatially-defined patterns of illumination, coupled with the fast, sensitive, and non-capacitive voltage sensing characteristics of VF dyes makes SPOT2.1.Cl a useful tool for interrogating both structure and function of neuronal systems.

Changes in neuronal membrane potential encode the vast range of thoughts, feelings, and behaviors that comprise the human experience. Despite the central importance of the brain to human health and disease, the molecular and cellular mechanisms underlying brain and neuronal function remain incompletely characterized, prompting efforts at the national and international level to develop more comprehensive maps of neuronal activity.^{1–3} Much of modern neurobiology stands upon the electrophysiological recordings of the activity of single neurons embedded within a network context. While this approach has proven incredibly powerful, real limitations exist. Specifically, the invasive requirement of impaling cells with electrodes in biological samples severely disrupts overlying tissue and restricts recordings to the cellular soma, making multi-site recording challenging or impossible.

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ASSOCIATED CONTENT

Supporting Information

Synthetic methods, imaging parameters, cell culture conditions. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Fluorescence imaging uniquely addresses this problem because the technique is non-invasive, can provide spatial information on membrane potential changes in multiple cells or in sub-cellular regions distant from the soma, and can be high-throughput. Voltage imaging with autofluorescent proteins,^{4–7} small molecules,^{8–13} or a combination of both,^{14–16} remains an attractive solution because it can enable the direct measurement of membrane potential while providing the spatial resolution, throughput, and parallel recording capabilities of Ca²⁺ imaging approaches. Recently, voltage-sensitive small molecules based on molecular wires emerged as an intriguing class of fluorophores for voltage sensing.^{17,18}

VoltageFluors, or VF dyes, sense changes in transmembrane potential by a photoinduced electron transfer (PeT) mechanism. PeT from an electron-rich aniline through a molecular wire to a fluorescent reporter is controlled by the electric field across the plasma membrane of an excitable cell, such as a neuron. At rest, where typical mammalian neuronal membrane potentials are approximately –60 mV inside the cell, PeT is enhanced, resulting in diminished fluorescence. As the membrane depolarizes—during an action potential or upon integration of excitatory inputs from connected neurons—PeT decreases, resulting in enhanced fluorescence.¹⁹ Consequently, VF dyes display large voltage-sensitive change in fluorescence while maintaining the fast response time needed for resolving single action potential spikes. Because PeT within the VF dye scaffold is fast compared to the biological event of interest, VF dyes add no capacitive load, making them ideal candidates for non-disruptive sensors of neuronal activity.

Despite these important characteristics, the amphipathic nature of VF dyes results in non-specific uptake into all plasma membranes within a biological sample, obscuring the boundary between adjacent stained cells and making it difficult to detect voltage-induced fluorescence changes against a high background of non-excitable cells stained with the dye. The ability to sparsely label neurons with VF dyes would address this problem. Restricted labeling of a sub-set of neurons within a neuronal network would improve signal-to-noise by lowering background fluorescence and pave the way for optical interrogation of local neuronal circuits both in culture and in more complex preparations. To achieve selective labeling of only a fraction of defined neurons, we envisioned quenching VF fluorescence with a photolabile protecting group (Scheme 1).

Photolabile protecting groups have found broad utility in both organic synthesis and chemical biology.^{20,21} Originally developed as a protecting group for amino acids that could be removed under mild conditions,²² the *o*-nitrobenzyl protection group and its derivatives have since found broad utility in masking the physiological activity of biologically active molecules, including nucleotides,^{23–25} metal ion ligands^{26–29} and ionophores,³⁰ neurotransmitters,³¹ reactive oxygen species,³² receptor agonists,³³ and amino acid residues.³⁴ Nitrobenzyl motifs have also been applied to fluorophores for superresolution microscopy.^{35,36} We propose to use photoactivatable VF dyes based on DMNB photocages to spatially control the apparent staining of cells in biological samples. The caged VoltageFluor would localize to cell membranes and remain weakly fluorescent until uncaging or photoactivation via illumination liberated the parent VF fluorophores (Scheme 1). Restricting uncaging illumination by scanning a region of interest (ROI) or through spatial light modulation (SLM) technology would provide targeted fluorescent labeling of

defined sub-populations of neurons. Toward this end, we now present the design, synthesis, and application of a small-molecule photoactivatable optical sensor of transmembrane potential, or SPOT (compound **1**), as a first class of voltage sensing fluorophores that enable targeted photoactivation of VF dyes in cells of interest via selective photo-uncaging.

SPOT2.1.Cl is readily available in one step from previously reported VF compounds.^{17,18} Alkylation of VF2.1.Cl with 2-nitro-4,5-dimethoxybenzylbromide in DMF provides SPOT2.1.Cl in 94% yield (Scheme 1). We examined the photophysical behavior and characteristics of SPOT2.1.Cl under simulated physiological conditions (PBS, pH 7.4). As synthesized, SPOT2.1.Cl displays an absorbance profile significantly altered from the parent VF dye (SI Fig. 1a, grey and black traces), with a λ_{max} centered at 400 nm ($\epsilon=44,000 \text{ M}^{-1}\text{cm}^{-1}$) attributed to the molecular wire absorbance and a minor absorbance centered at 500 nm ($\epsilon=21,000 \text{ M}^{-1}\text{cm}^{-1}$) corresponding to the alkylated fluorescein scaffold. At 522 nm, the λ_{max} for VF2.1.Cl, SPOT2.1.Cl shows only weak absorbance ($\epsilon=6,200 \text{ M}^{-1}\text{cm}^{-1}$). In contrast, the free VF dye demonstrates strong absorbance at 522 nm, with a shoulder at 488 nm. Emission from SPOT2.1.Cl is minimal (Fig. 1a, black trace), as reflected by its low fluorescence quantum yield ($\Phi=0.002$), which is 28-fold lower than VF dye ($\Phi=0.057$). UV illumination of SPOT2.1.Cl promptly delivers VF, as measured by complete recovery of absorbance profiles characteristic of VF2.1.Cl (SI Fig. 1a, green and black traces) and a 25-fold increase in fluorescence emission (Fig. 1a, green trace). HPLC comparison against VF2.1.Cl confirmed the photochemical conversion of SPOT2.1.Cl into VF2.1.Cl (SI Fig. 2). Identical UV irradiation had no effect on VF2.1.Cl absorbance or emission (SI Fig. 1b and c). The photochemical quantum yield was determined by actinometry to be 0.007. SPOT has an ϵ_{365} of $33,000 \text{ M}^{-1}\text{cm}^{-1}$ giving an overall efficiency ($\Phi \times \epsilon_{365 \text{ nm}}$) of 230, comparable to reported values for *o*-nitro photocages.²⁰

We next turned our attention to photoactivation of SPOT2.1.Cl in living cells. Bath application of SPOT2.1.Cl to human embryonic kidney cells (HEK 293) resulted in very little cellular fluorescence staining, due to the low intrinsic brightness of SPOT2.1.Cl (Fig. 1c). Illumination with 390 nm (30 x 1 s) light results in an immediate increase in cell membrane-associated fluorescence characteristic of VF staining (Fig. 1d) over the entire field, indicating that SPOT2.1.Cl had localized to cell membranes and remained optically silent until photoactivation. Quantification of the mean cellular fluorescence intensity pre- and post-UV indicates that SPOT2.1.Cl provides a 12 ± 1.2 -fold ($n=3$ separate experiments) increase in fluorescence intensity following irradiation. Membrane-associated fluorescence depends on prior UV illumination, as examination of fields of view not exposed to light show little fluorescence (SI Fig. 3a, c, e). However, subsequent photoactivation of these regions (390 nm, 30 s) results in a similar fluorescence increase (SI Fig. 3b, d, f), demonstrating that patterns of spatially restricted light can selectively photoactivate distinct cell populations.

Having established the ability of SPOT2.1.Cl photoactivation to control apparent staining at a region-specific level, we next examined the ability of SPOT2.1.Cl to label individual cells, using confocal microscopy. By defining a photoactivation ROI around several cells, SPOT2.1.Cl could be selectively activated in the cells of interest (SI Fig. 4a and b), giving an approximately 11-fold increase (± 0.8 , $n=5$ cells, SI Fig. 4d) in fluorescence intensity in

cells illuminated with 405 nm light (25 x 1 s illumination, SI Fig. 4a–c). Upon release by light, SPOT2.1.Cl remained localized in single cells; the dye did not migrate away from the originally uncaged cells (SI Fig. 4b). Uncaged SPOT2.1.Cl, however, freely diffuses within cell membranes. Confocal imaging of HEK cells loaded with SPOT2.1.Cl show minimal fluorescence prior to photoactivation, as before (Fig. 2a). Photoactivation of one half of a HEK cell (yellow area, Fig. 2a) delivered a prompt increase in VF2.1.Cl fluorescence in the illuminated half of the cell (Fig. 2b). After 10 minutes, the rest of the cell “fills” with dye, as photoactivated SPOT2.1.Cl diffuses laterally through the plasma membrane (Fig. 2c). The neighboring cell (Fig. 2d) remains darker (Fig. 2b and c), and shows minimal fluorescence increase. Directly uncaged regions increase fluorescence by 520% ($\pm 60\%$) while neighboring regions increase fluorescence by only 40% ($\pm 17\%$, SEM for $n = 4$ experiments, SI Fig. 5). Diffusion of SPOT away from the uncaging site in confluent monolayers is minimal (SI Fig. 6), and VF2.1.Cl behaves nearly identically to the canonical lipophilic tracer dye, DiO, in FRAP experiments in confluent monolayers of HEK cells (SI Fig. 7). Taken together, these experiments establish that uncaged SPOT diffuses rapidly within cells, but much more slowly across plasma membranes, in a manner similar to DiO.

Following illumination, cells stained with SPOT2.1.Cl become bright and voltage-sensitive. Whole-cell patch clamp electrophysiological measurements in HEK cells reveal that post-illumination, activated SPOT2.1.Cl has a voltage sensitivity of 17% F/F per 100 mV (Fig. 1b and SI Fig. 8d), approximately 77% of the sensitivity achieved by the parent VF2.1.Cl under similar recording conditions (SI Fig. 8d and e). This decrease in voltage sensitivity may arise from improper membrane orientation of SPOT prior to uncaging^{17–19} or photochemically-initiated side reactions not observed *in vitro*, and experiments are underway to determine the genesis of this effect. The small decrease in voltage sensitivity does not prevent optical recording of physiologically relevant voltage changes. Whole-field uncaging of cultured neurons loaded with SPOT, followed by field stimulation, demonstrated that activated SPOT can optically record action potentials in single trials (SI Fig. 9). Photolysis of SPOT is well-tolerated by both HEK cells and neurons, as judged by analysis of MTT viability assays (SI Fig. 10) and electrophysiological parameters of HEK cells treated with SPOT2.1.Cl+UV light (SI Fig. 11a–e). Neurons loaded with either SPOT2.1.Cl+UV or VF2.1.Cl show no difference in action potential duration (SI Fig. 11f).

We envisioned that SPOT2.1.Cl could find utility in complex neuronal contexts wherein optically orthogonal fluorescent proteins targeted to genetic sub-sets of neurons guide spatially-resolved photoactivation of SPOT2.1.Cl and enable sparse labeling and optical recording in neurons. We cultured rat hippocampal neurons (E18) for 14 days *in vitro* (DIV) and transfected them with mCherry localized to the inner leaflet of the cell membrane via a CAAX-mediated farnesylation sequence.³⁷ A small percentage of neurons displayed good expression of farnesylated mCherry (Fig. 3a and b). These neurons were targeted for uncaging by restricting the uncaging region to a $<20\ \mu\text{m}$ diameter photoactivation spot centered within the soma defined by mCherry fluorescence (Fig. 3b and d). Photoactivation yielded bright membrane-localized fluorescence (Fig. 3c, e, and f). Photoactivated SPOT in neurons reports on action potentials evoked by field stimulation (Fig. 3g) with a response of

9% F/F per action potential ($\pm 0.2\%$, $n=29$ APs from 3 cells) and a SNR of 14 ± 4 to 1 ($n=29$ APs from 3 cells).

Precise identification of neuronal pathways and function are critical for dissecting connectivity and information processing in the brain. Current neuronal tracing methods rely on diffusion of lipophilic carbocyanine fluorescent tracers³⁸ and typically require invasive local delivery of dye via pipette. These tracers, while providing a structural description of neuronal projections, cannot report on the activity of labeled neurons. We envisioned that SPOT, as a voltage-sensitive dye that diffuses within membranes, could be exploited to map local circuitry. By uncaging SPOT in cellular processes and allowing diffusion of the dye to the soma, cells which were “wired” into the photoactivated region would become bright, allowing imaging of neuronal activity in the labelled cells.

To explore this, we first ascertained the ability of photoactivated SPOT to diffuse within neuronal membranes. Indeed, neurons loaded with SPOT and uncaged in the soma showed substantial diffusion of activated SPOT away from the soma into distal processes when the neurons were maintained at 37 °C (SI Fig. 12 and 13). We then loaded cultured hippocampal neurons expressing cell membrane-localized mCherry (Fig. 4a and b) with SPOT and photoactivated a region (Fig. 4c, yellow circle) lacking cells bodies, but containing numerous processes, including those of an mCherry-positive cell. Following uncaging, we maintained the neurons at 37 °C to allow activated SPOT to diffuse back into cell bodies that projected processes into the uncaging region. After 30 minutes, several cell bodies were highlighted (Fig. 4e), including the mCherry-positive cell, indicating that SPOT can be used to map local connectivity in living neurons. Upon field stimulation, uncaged SPOT clearly distinguishes action potentials in single trials from neurons labeled by retrograde tracing (Fig. 4g and h), demonstrating that SPOT methodology can be used to report both structural and functional connectivity and dynamics within local circuits, which cannot be achieved with VF or lipophilic tracer dyes in isolation.

In summary, we present the design, synthesis, and biological evaluation of the first member of a new class of voltage sensitive dyes—SPOTs, **s**mall **p**hotoactivatable **o**ptical sensors of **t**rans-membrane potential. In particular, SPOT2.1.Cl, based on the Volt-ageFluor2.1.Cl scaffold, exhibits a large fluorescence dynamic range following near-UV photoactivation, good voltage sensitivity, staining defined by spatially-restricted illumination, and enables functional imaging of genetically specified neurons through an optically orthogonal marker—either through direct photoactivation of cells of interest, or via “back-filling” in which neuronal processes are uncaged to provide labeling in cell bodies. Current efforts are underway in our lab to apply SPOT2.1.Cl to brain tissue, as well as to explore new photocaging scaffolds with increased two-photon uncaging cross-section, improved water solubility, and enhanced targeting and retention in membranes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Characterization of SPOT2.1.Cl. a) Emission spectra of SPOT2.1.Cl in PBS, pH 7.4, before (black trace) and after (green trace) illumination at 365 nm ($\lambda_{\text{ex}}=522$ nm). b) Plot of $\Delta F/F$ vs mV for SPOT2.1.Cl after photoactivation in HEK cells. Error bars are $\pm$ S.E.M. for $n = 3$ experiments. c) HEK cells loaded with 500 nM SPOT2.1.Cl prior to illumination. d) Cells from panel (c) after illumination with 390 nm light for 30 seconds. Scale bar is 20 μm .

Figure 2.

Photoactivation of SPOT2.1.Cl within a single cell. HEK cells loaded with 500 nM SPOT2.1.Cl a) prior to and b) immediately after uncaging in an area over the left side of the cell, as indicated by the yellow region of interest in panel (a). Initially, fluorescence fills only the uncaged portion of the cell (b), but after 10 min, c) the entire cell membrane appears fluorescent, while the neighboring cell remains dim. d) Transmitted light image of cells in previous panels. Scale bar is 20 μm .

Figure 3.

SPOT2.1.Cl uncaging in single neurons. Dissociated, cultured rat neurons (a) were transiently transfected with CAAX-mCherry for use as a fiducial marker for illumination. Panels (a) and (b) show DIC and mCherry fluorescence images, respectively. Cells were loaded with 500 nM SPOT2.1.Cl and then photoactivated (390 nm, 10 s, 22.3 W/cm²) over a region defined by the somatic staining of the mCherry signal (white arrow). (c) Following photoactivation, VF fluorescence appears to be membrane-localized. d–f) EMCCD image of indicated cell, showing mCherry fluorescence. VF dye fluorescence from the mCherry-positive neuron, e) prior to SPOT photoactivation and f) immediately after. g) Field stimulation of the SPOT-stained neuron at 5 Hz produced a train of optically recorded action potentials, which were captured in a single trial at 500 Hz with an EMCCD camera. Scale bars are 20 μm for panels a–c and 10 μm for panels d–f.

Figure 4.

Voltage sensing in neurons identified by SPOT retrograde tracing. Neurons were stained with 2.5 μM SPOT2.1.Cl. a) DIC image of neurons stained with SPOT2.1.Cl. b) Widefield epifluorescence image of neurons in panel (a), demonstrating labeling with mCherry-CAAX. c) Green fluorescence image of same cells, prior to SPOT2.1.Cl photoactivation. Area of photoactivation is indicated by yellow dotted circle. d) Cells in panel (c) immediately after photoactivation. e) Diffusion of photoactivated SPOT2.1.Cl away from uncaging area after 30 min. at 37 °C. f) mCherry-positive neuron from panel (b) within the region in dotted white box. g) Image of SPOT-traced neuron used to generate functional imaging data, after photoactivation and diffusion. h) Action potentials evoked by field stimulation of neurons at 5 Hz and recorded optically in the indicated cell—white arrow—in a single trial at 500 Hz. Scale bar is 20 μm (a–e) and 10 μm (f and g).

Scheme 1.
Synthesis and Photoactivation of SPOT