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Fluorogenic targeting of voltage sensitive dyes to neurons

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Abstract

We present a method to target voltage-sensitive fluorescent dyes to specified cells using an enzyme-catalyzed fluorogenic reaction on cell surfaces. The dye/enzyme hybrids are composed of a photoinduced electron transfer (PeT)-based fluorescent voltage indicator and complementary enzyme expressed on the cell surface. Action of the exogenous enzyme on the dye results in fluorogenic activation of the dye, enabling fast voltage imaging in defined neurons, with sensitivity surpassing purely genetically-encoded approaches. We employ a bulky cyclopropylmethyl acetoxymethyl ester to diminish the fluorescence of a PeT-based voltage-sensitive dye, or VoltageFluor. The hydrolytically stable ester can be removed by the action of porcine liver esterase (PLE) to reveal the bright, unmodified VoltageFluor. We established that the chemically modified VoltageFluor is a substrate for PLE *in vitro* and in live cells. When PLE is targeted to the external face of cell membranes, it controls the apparent staining of cells. Use of neuron-specific promoters can direct staining to mammalian neurons to provide clear detection of neuronal action potentials in single trials. All of the new VoltageFluors targeted by esterase expression report single spikes in cultured mammalian neurons. The best, VF-EX2, does so with a signal-to-noise ratio (SNR) nearly double that of comparable genetically-encoded voltage reporters. By targeting PLE to neurons, VF-EX2 can interrogate the neuromodulatory effects of serotonin in cultured hippocampal neurons. Together, we show that a combination of synthetic chemistry and biochemistry enables bright and fast voltage imaging from genetically-defined neurons in culture.

Graphical abstract

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Supporting Information. Synthetic, spectroscopic, and imaging data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Introduction

Direct visualization of changes in membrane potential using fluorescent indicators offers a powerful method for probing voltage dynamics in neurons with unprecedented spatial and temporal resolution.¹ Despite the promise of voltage imaging, key challenges of speed, sensitivity, brightness and localization remain. Purely genetically-encoded approaches based on fluorescent proteins or opsins afford the opportunity to target neuronal cell types of interest in complex brain contexts, providing exquisite fluorescence contrast between the cell of interest and its surroundings.²⁻⁶ However, brightness, speed, or sensitivity often limit the wide-spread application of these powerful new genetically-encoded methods. To address these limitations, we have been developing fluorescent voltage indicators that rely on photoinduced electron transfer (PeT) as a membrane potential-dependent trigger to control the emission of a fluorescent dye.⁷ PeT-based dyes, or VoltageFluors (VF dyes), display voltage-sensitive fluorescence, owing to modulation of the rate of PeT within the VF scaffold by the transmembrane electric field.⁸ VF dyes therefore possess the requisite speed and sensitivity to report on action potentials in neurons in single trials. Although VF dyes localize to the outer leaflet of cell membranes to sense voltage,⁹⁻¹⁰ one current limitation of chemically-synthesized dyes is their inability to discriminate between membranes of different cells. This precludes cell-specific targeting, which significantly erodes signal to noise and cellular resolution compared to genetically encoded approaches.⁹⁻¹⁰

To marry the speed, sensitivity, and brightness of synthetic VF dyes with the cell-type specificity enabled by genetically encoded approaches, we combine VF dyes with a genetically encoded component to enable high-contrast imaging in defined neurons. We achieve this with a fluorogenic approach, in which the parent VF dye is chemically modified to be minimally fluorescent and must be enzymatically activated prior to imaging. The dim VF dye localizes to all cellular membranes where it remains weakly fluorescent until enzymatic cleavage of the chemical modification by an exogenously expressed enzyme (Scheme 1a). This results in bright, localized fluorescence only in the cells which express the cognate enzyme. Based on our previous photoactivation studies,¹¹ we hypothesized that a hydrolytically stable, bulky acetoxymethyl ester¹² appended to the phenolic oxygen of VoltageFluor2.1.Cl (VF2.1.Cl)¹⁰ would diminish VF fluorescence. Previous studies showed that intracellular porcine liver esterase (PLE) is capable of unmasking bulky acetoxymethyl esters inside living cells.¹²

Re-engineering PLE to express on cell surfaces would unmask the chemically-caged VF dye and enable apparent staining only in cells expressing PLE. We now report the design, synthesis, evaluation, and application of VoltageFluors targeted by esterase expression, or VoltageFluor-EX, a hybrid system based on a novel, modified VF dye and a new, cell-surface localized PLE for targeting PeT-based voltage indicators to genetically-defined cells.

Results

VF-EX1 and VF-EX2 (Scheme 1b) are synthesized in a single step from VF2.1.Cl. Addition of iodo-1-methylcyclopropane-carboxylate to VF2.1.Cl in N,N-dimethylformamide (DMF) in the presence of N,N-diisopropylethylamine (DIPEA) gave VF-EX1 (stoichiometric iodoalkane) or VF-EX2 (excess iodoalkane) (Scheme 1b). The two different products were characterized by NMR, HPLC, and mass spectrometry (see SI Spectra in the Supporting Information). Both VF-EX1 and VF-EX2 display optical properties consistent with *O*-alkylated fluoresceins¹³—a weak absorbance at $\lambda = 500$ nm (Figure 1a/d, see Figure S1 for excitation spectra). VF-EX1 and VF-EX2 are minimally fluorescent, with quantum yields of 0.004 and 0.013, respectively (HBSS, pH 7.4). Compared to VF2.1.Cl, which has a quantum yield of 0.08 under identical conditions, this represents a 19- and 6.7- fold decrease in fluorescence intensity (Figure 1b/e). VF-EX0, in which only the sulfonic acid is esterified, has a quantum yield of 0.24, consistent with a 3-fold increase in quantum efficiency for sulfonate esters (VF-EX0, VF-EX2) compared to their cognate acids (VF2.1.Cl, VF-EX1, Table S1 and Scheme S1).

We confirmed that purified porcine liver esterase (PLE, Sigma, E2884) hydrolyzes VF-EX1 and VF-EX2 *in vitro*. The action of PLE on VF-EX1 or VF-EX2 results in a 53- and 11-fold increase in fluorescence after 3 hr. The fluorescence increase is accompanied by a restoration of a fluorescein-like absorption and emission profile (Figure 1a/b and d/e). HPLC analysis revealed VF2.1.Cl as the product of PLE-mediated hydrolysis reactions (Figure S2). Both substrates show saturation-type enzymatic kinetics with PLE. The Michaelis constant, K_m , for VF-EX1 is 1.2 ± 0.5 μM and for VF-EX2 is 0.12 ± 0.03 μM , (Figure 1c/f and Table S1) comparable to the reported values of 0.5 μM for biscyclopropylacetoxyethylfluorescein.¹² VF-EX2 is a better substrate than VF-EX1, as evidenced by the larger k_{cat}/K_M for VF-EX2 ($1.3 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$) vs. VF-EX1 ($2.1 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$) (Table S1). We hypothesize that masking of the anionic sulfonate charge in the case of VF-EX2 makes it a better substrate for PLE.

Having confirmed VF-EX1 and VF-EX2 as substrates for PLE, we next set out to target and express PLE to the surface of eukaryotic cells. Although trafficking to the endoplasmic reticulum (ER) is required for proper cellular PLE activity,¹² we found that removal of the PLE-associated ER retention signal, addition of a transmembrane domain (platelet-derived growth factor receptor, PDGFR, from pDisplay)¹⁴ or glycosylphosphatidylinositol anchor signal (GPI, from decay accelerating factor, DAF),¹⁵ and inclusion of a secretion signal derived from immunoglobulin K (IgK) results in clear membrane localization on HEK cells as determined by immunostaining fixed cells (Figure S3 and S4). Bath application of VF-EX1 or VF-EX2 (500 nM, 30 minutes) to HEK cells transiently transfected with PLE-DAF

or PLE-pDisplay results in clear membrane-associated fluorescence in transfected cells (indicated by nuclear-localized mCherry, Figure 2a-d, g-j).

Cells in the same culture that do not express mCherry (and therefore do not express PLE) show negligible levels of membrane-associated fluorescence (Figure 2a/c, g/i, Scheme 1a). Membrane targeting of VF-EX dyes with DAF gave the brightest cells and the largest contrast between PLE-expressing and PLE(-) cells (Figure S5, see Figure S6 for quantification). DAF-mediated targeting was therefore used for subsequent studies. We examined a number of flexible linker lengths (GGGS repeats) between PLE and the DAF sequence and found no correlation between linker length and contrast between transfected and control HEK cells treated with VF-EX2 (Figure S7). We therefore used the smallest linker length for future studies. Quantification of the difference in fluorescence intensity for PLE-expressing cells vs. controls indicates a contrast of 7-fold between PLE-expressing cells and control cells for VF-EX1 and 17 for VF-EX2, which we attribute to the higher k_{cat}/K_M value for VF-EX2 (Figure 1f, Table S1). Longer incubation times with VF-EX2 at 500 nM (up to 2 hours) increased overall fluorescence, but lowered contrast between transfected and untransfected cells on account of increasing fluorescence in untransfected cells (Figure S8a,b). This increase in fluorescence in untransfected cells is due to accumulation of weakly fluorescent VF-EX2 in membranes and not to uncatalyzed hydrolysis, since cells that had been loaded with VF-EX2 for 30 minutes (500 nM), washed, and then monitored over time showed no increase in fluorescence from untransfected cells (Figure S8c,d).

The fluorescence associated with PLE-expressing HEK cells was voltage-sensitive; VF-EX1 and VF-EX2 displayed high, linear voltage sensitivities of 19 ± 0.3 and $21 \pm 0.3\%$ F/F per 100 mV, comparable to 27% measured for VF2.1.Cl (Figure 2e/f, k/l). We additionally confirmed that cell-surface PLE uncages VF-EX2 only within the cell membrane of the cell which expresses PLE (in *cis*) and not on neighboring cells (in *trans*). HEK cells expressing PLE on the cell surface and mCherry in the nucleus were plated on top of a monolayer of HEK cells that did *not* express PLE and had previously been loaded with VF-EX2. If PLE acted in *trans*, we predicted that addition of PLE-expressing HEK cells to the non-expressing, dye-loaded cells would result in an increase in fluorescence, similar to what we observe when we add purified PLE to HEK cells loaded with VF-EX2 (Figure S9a-d). However, if PLE only acts in *cis*, then addition of PLE-expressing HEK cells will not result in an increase in fluorescence. We observe no increase in fluorescence upon addition of PLE expressing HEK cells to cells loaded with VF-EX (Figure S9e-j), confirming both that cell-surface localized PLE cannot act in *trans*, and that VF-EX2 does not diffuse out of membranes to cells expressing PLE. Importantly, a positive control experiment demonstrates that the harvested PLE-expressing HEK cells can turn-over VF-EX1 if the VF-EX1 is added directly to these PLE-expressing cells (Figure S9k). Previous studies establish that the product of VF-EX2 uncaging, VF2.1.Cl, diffuses primarily within membranes and not across membranes.¹¹ We also established that VF-EX2 does not cross plasma membranes, as HEK cell expressing ER-targeted PLE do not accumulate internal fluorescence (Figure S10).

VoltageFluor-EX dyes provide enhanced contrast in neurons. Fixed-cell immunofluorescence established efficient cell-surface targeting of PLE in rat hippocampal

neurons transfected with PLE-DAF under the neuron-specific synapsin promoter (Syn, Figure S11) or calmodulin kinase II promoter (CaMKII, Figure S12) (Figure S3). Use of a strong, non-specific promoter construct from pCAGS, results in primarily neuronal expression with some glial expression, as assayed by co-localization with glial fibrillary acid protein, GFAP (Figure S13). Live cell staining with VF-EX1 and VF-EX2 (1 μ M, 1 hour) results in clear enhancement of membrane-associated fluorescence in transfected neurons, with a 4.1- and 4.8-fold increase in fluorescence for Syn-PLE expressing neurons stained with VF-EX1 and VF-EX2 (Figure S14a-d, Figure 3a-d) and a 3.0- and 3.7-fold increase in fluorescence for CaMKII-PLE expressing neurons with VF-EX1 and VF-EX2 (Figure S14e-h, Figure 3e-h), respectively (Table S1, Figure S15, and Scheme 1a). The cell-type specific membrane associated fluorescence with VF-EX1 and VF-EX2 is voltage sensitive. Like the parent VF dyes,^{8, 10, 16} VF-EX1 and 2 give clear responses to field-stimulation electrode-evoked action potentials (Figure 4a/d, Figure S16) and the ability to record spontaneous spiking in hippocampal cultures (Figure S14i, Figure 3i). We selected VF-EX2 for future use in neurons because of its greater brightness in neurons (Figure S15). Longer incubation times with VF-EX2 (1 μ M, up to 120 minutes), increased the relative fluorescence intensity of PLE-expressing cells (Figure S15d). The overall contrast between PLE and wild-type neurons decreased slightly as a result of increased accumulation of VF-EX2 in cell membranes (Figure S15d,e). As with HEK cells (Figure S8), this is due to nonspecific accumulation of VF-EX2 and not hydrolysis, as neurons loaded for 30 minutes (1 μ M VF-EX2) followed by a wash show negligible increases in fluorescence from non-transfected neurons (Figure S15f).

We compared the performance of VF-EX2/Syn-PLE to genetically-encoded fluorescent voltage indicators: ASAP1,² based on circularly permuted green fluorescent protein (cpGFP), or Ace2N-mNeon (here abbreviated Ace2N),⁴ which uses mNeon.¹⁷ VF-EX2/PLE compared favorably to both ASAP1 and Ace2N, displaying improved membrane associated fluorescence relative to ASAP1 and Ace2N (Figure 4a-c, Figure S17), turn-on responses to spontaneous action potentials (both ASAP1 and Ace2N show turn-off responses to action potentials, Figure 4e/f), and an average $7.3 \pm 0.8\%$ F/F per spike (20 ± 2.7 SNR) for evoked action potentials compared to $-6.5 \pm 0.3\%$ for ASAP1 (4.9 ± 0.6 SNR) and $-2.9 \pm 0.1\%$ for Ace2N (11 ± 0.7 SNR) (132, 62, and 106 action potentials from 16, 7, and 13 separate neurons, respectively (Figure 4g/h, Figure S16). Under the identical illumination conditions, VF-EX2 has photostability comparable to that of ASAP1 and Ace2N (Figure S16 and S17).

The improved fluorescence staining on the cell membrane results from the use of the hybrid chemical-genetic approach. Although genetically-encoded fluorescent proteins traffic to the cell surface, a significant portion of the fluorescent protein-based indicator remains in the cellular export pathways and contributes to unresponsive background fluorescence. In contrast, while membrane-targeted PLE is also making its way through the cellular export pathways, it is non-fluorescent.

The ability of the VF-EX2/PLE system to label specific, genetically-defined cells, coupled with its high voltage sensitivity, make it a promising candidate for interrogating membrane potential dynamics in complex neuronal systems. For example, the neuromodulator

serotonin (5-hydroxytryptamine, or 5-HT) exerts diverse effects within the brain and body, depending upon cell type, 5-HT receptor (5-HTR) subtype, and pre- or postsynaptic localization of the receptor. Numerous serotonergic neurons project onto the hippocampus in the rat brain, delivering 5-HT to modulate neuronal activity. The complex expression pattern of the 14 distinct 5-HTR subtypes¹⁸ in the hippocampus complicates the dissection of molecular pathways involved in mediating the effects of 5-HT. Treatment of hippocampal cultures stained with VF-EX2/PLE exclusively in neurons (CaMKII promoter) with 5-HT (10 μ M) shows decreased spiking rates, from 2.5 ± 0.7 Hz to 1.5 ± 0.5 Hz in 5-HT (Figure 5a and Figure S18).¹⁹ Washout of 5-HT results in a recovery of neuronal spiking to 2.4 ± 0.4 Hz (Figure 5a). The decrease in firing rate could be mediated through a number of different pathways: the action of 5-HT₁ receptors coupling to inward rectifying potassium channels (Kir channels) via G-protein coupled receptors (GPCRs), resulting in a direct hyperpolarization; the activation of 5-HT₂ receptors coupling to release of Ca²⁺ stores via phospholipase C (PLC) and activating inhibitory neurons to decrease overall firing; or through the opening of ion channel-type 5-HT₃ receptors causing a depolarization of inhibitory cells. To resolve some of these ambiguities, we again assessed the neuromodulatory effect of 5-HT on the activity of hippocampal neurons using VF-EX2/PLE. Pre-treatment with the 5-HT_{1A}-selective antagonist, WAY 100635 (10 nM; 1.4 nM IC₅₀),²⁰ prior to 5-HT addition causes a complete loss of the serotonin-mediated decrease in activity in neurons (Figure 5b and Figure S18). These results suggest the decrease in firing in hippocampal neurons accompanied by serotonin treatment is mediated primarily through 5-HT_{1A} receptors. In support of this model, pre-treatment with ketanserin (100 nM; 2 nM K_i),²¹ an antagonist with specificity for 5-HT₂ receptors, has no effect on the serotonin-mediated decrease in firing (Figure 5c and Figure S18). Importantly, firing rates for all neurons were uniform prior to and after washout of 5-HT ($p > 0.1$, across all pair-wise comparisons, Figure S18). The use of cell type-specific expression of VoltageFluor-EX system enables a rapid method for dissection of complex neurobiological signaling pathways.

Discussion

In summary, we present the design, synthesis, evaluation, and application of the VoltageFluor-EX hybrid system for fluorogenic targeting of PeT-based fluorescent voltage indicators to specific cells. Fluorogenic targeting using a hybrid small molecule/enzymatic activation approach combines the speed and sensitivity of PeT-based voltage indicators with the cellular specificity of genetic encoding. We show that two VoltageFluor derivatives, VF-EX1 and VF-EX2, with methylcyclopropylacetoxymethyl ester modifications, provide an efficient means of generating high contrast fluorescence in individual neurons. While enzymatic targeting has been employed previously to improve membrane localization of electrochromic dyes,²²⁻²⁴ these strategies have never been deployed in neurons for functional imaging. Alternative hybrid approaches rely on lipophilic fluorescence quenchers²⁵ that introduce prohibitive capacitance to the neurons under observation.²⁶ Here we demonstrate that the VoltageFluor-EX system enables the use of fast, sensitive, non-disruptive VoltageFluor2.1.Cl⁸ in specified cells to report on neuronal action potentials with large, linear turn-on responses surpassing the signal-to-noise and sensitivity achieved with

fluorescent protein and FRET-opsin voltage indicators under identical conditions. We show that targeting VF-EX2 to neurons enables the dissection of the neuromodulatory effects of serotonin in hippocampal neurons.

When compared to their genetically encoded counterparts, VF-EX dyes lag behind in terms of contrast between transfected and untransfected neurons. Low contrast for VF-EX/PLE in neurons relative to HEK cells may be due, in part, to the lower expression levels of PLE achieved under specific, but weaker promoters like synapsin and CaMKII α . This may be partially addressed by coupling strong promoters (i.e. CMV) to genetic methods for obtaining cell-type specificity. Improvements can also be made on the chemical side. The dynamic range between caged and uncaged fluorophore can be tuned by altering the amount of PeT between the fluorophore and pendant aryl ring.²⁷ Larger spectral separation between the excitation maxima of the caged and free dye would also improve contrast. Further improvement in cellular contrast is likely needed for routine usage *in vivo*. Taken together, this methodology sets the stage for employing fast, sensitive, fluorogenic PeT-based voltage indicators in intact brain tissues, and efforts are underway in our lab to apply this method both to new targeting modalities using complementary substrate/enzyme pairs, expand the palette of fluorogenic voltage indicators, and deploy these probes to intact brains *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Spectroscopic characterization of VoltageFluor-EX 1 and 2. **a,d**) UV/vis absorption and **b,e**) fluorescence emission spectra of VF-EX1 (**a,b**) or VF-EX2 (**d,e**) in the absence (black line) or presence (green line) of purified porcine liver esterase (PLE, 0.7 mg/mL, 3 h). Excitation provided at 520 nm. **c,f**) Plot of relative initial rate vs. substrate concentration for the reaction of PLE (200 ng/mL) with **c**) VF-EX1 or **f**) VF-EX2. Data points are mean $\pm$ S.E.M. for n = 4 independent measurements. Solid line is the line of best-fit to the Michaelis-Menten equation (Graphpad).

Figure 2.

Cellular and functional characterization of VoltageFluor-EX 1 and 2. Widefield fluorescence microscopy of HEK cells stained with VF-EX1 (500 nM, 30 min., **a-d**) or VF-EX2 (500 nM 30 min., **g-j**) show membrane-associated fluorescence only in cells expressing cell-surface PLE (indicated by mCherry fluorescence). **a,g** Epifluorescence images showing VoltageFluor-associated fluorescence. **b,h** Transmitted light image of HEK cells from panels a and g. **c,i** Fluorescence image of nuclear-localized mCherry indicating PLE expression. **d,j** Merged imaging showing overlay of both VoltageFluor and mCherry fluorescence. Scale bars are 20 μm . **e,k** Plot of fractional change in fluorescence ($\Delta F/F$) vs. final membrane potential for VF-EX1 (**e**) and VF-EX2 (**k**) in HEK cells under whole-cell voltage-clamp conditions. Data are mean $\pm$ S.E.M. for 6 cells (VF-EX1) and 8 cells (VF-EX2). **f,l** Representative plots of fractional change in fluorescence ($\Delta F/F$) vs. time for a series of hyper- and de-polarizing steps from a holding potential of -60 mV to ± 100 mV in voltage-clamped HEK cells loaded with VF-EX1 (**f**) or VF-EX2 (**l**).

Figure 3.

Functional imaging in hippocampal neurons with VoltageFluor-EX 2. Neuronal targeting with VoltageFluor-EX dyes (**a-i**). Live-cell, widefield fluorescence images of rat hippocampal neurons stained with 1 μ M VF-EX2 (60 min.) (**a-h**) and expressing PLE-DAF under control of the synapsin (Syn, **a-d**) or CaMKII α (CaMKII, **e-h**) promoter show clear membrane-associated fluorescence. **a,e** Widefield fluorescence image of membrane-associated VF fluorescence in PLE-expressing neurons. **b,f** Nuclear-localized mCherry confirms PLE expression in defined neurons. **c,g** Differential interference contrast (DIC) images of neurons. **d,h** Merged images of VF and mCherry fluorescence confirms good association between bright, membrane-associated fluorescence and mCherry/PLE expression. Scale bars are 20 μ m. Asterisks indicate mCherry/PLE-positive neurons while arrowheads indicate untransfected neurons. **i** Representative F/F traces for rat hippocampal neurons stained with VF-EX2 and transfected with Syn-PLE. Traces are F/F from regions of interest at the cell bodies of neurons after background offset and are uncorrected for bleaching. Images are acquired at 500 Hz and represent single trial acquisitions.

Figure 4. Comparison of VoltageFluor-EX2 to genetically encoded voltage indicators. **a-c)** Live-cell, widefield fluorescence imaging of neurons expressing PLE and stained with 1 μ M VF-EX2 (60 min.) (**a**), ASAP 1(**b**), or Ace2N-mNeon (Ace2N) (**c**) under control of the Syn promoter. Scale bar is 20 μ m. **d-f)** F/F vs. time plots for action potentials evoked and optically recorded with either VF-EX2/PLE (**d**), ASAP 1 (**e**), or Ace2N (**f**). **g-h)** Quantification of F/F (**g**) and signal-to-noise (SNR, **h**). Data are mean $\pm$ S.E.M. for $n = 16, 7$, and 13 neurons (each neuron is the average of 9 evoked action potentials).

Figure 5.

Interrogation of the neuromodulatory effects of serotonin on neurons from the rat hippocampus. **a-c)** Hippocampal neurons expressing PLE (CaMKII α -PLE) and stained with 1 μ M VF-EX2 (60 min.) were treated with 5-HT (10 μ M) **a)** alone, **b)** in the presence of WAY 100635 (10 nM), or **c)** in the presence of ketanserin (100 nM). The firing rate of neurons was measured before (left bars), during (middle bars), and after (right bars) washout of 5-HT. Data are mean $\pm$ S.E.M. for the average firing rate of 8-10 different coverslips of neurons. Each coverslip's average firing frequency was measured from between 5-8 individual neurons, for a total of between 46-93 neurons for each condition. Two-tailed Mann-Whitney tests were used for determination of statistical significance. *** indicates $p < 0.005$; **** indicates $p < 0.001$.

Scheme 1.

Design and synthesis of VoltageFluor targeted by esterase expression (VF-EX). a) Scheme of neuron-specific targeting with ester/esterase pairs (components are not to scale). b) Synthesis of VoltageFluor-EX 1 and 2.