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Neuronal Calcium Recording with an Engineered TEV Protease

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Abstract

Technologies for measuring the transient Ca^{2+} spikes that accompany neural signaling have revolutionized our understanding of the brain. Nevertheless, microscopic visualization of Ca^{2+} spikes on the timescale of neural activity across large brain regions or in thick specimens remains a significant challenge. The recent development of stable integrators of Ca^{2+} , instead of transient reporters, provides an avenue to investigate neural signaling in otherwise challenging systems. Here we describe an engineered Ca^{2+} -sensing enzyme consisting of a split Tobacco Etch Virus (TEV) protease with each half tethered to a calmodulin or M13 Ca^{2+} binding domain. This Split TEV, Ca^{2+} Activated Neuron Recorder (SCANR) remains separate and catalytically incompetent until a spike in cellular Ca^{2+} triggers its reconstitution and the subsequent turnover of a caged, genetically-encoded reporter substrate. We report the identification of a successful Ca^{2+} -sensing split TEV from a library of chimeras and deployment of the enzyme in primary rat hippocampal neurons.

Myriad cell types translate external stimuli into intracellular Ca^{2+} fluctuations that modulate signal transduction pathways. Notable among them are the brain's neurons, which, upon receiving an electrical stimulus in the form of an action potential, experience a transient spike in their cellular Ca^{2+} concentrations. This increase in intracellular Ca^{2+} is responsible for mobilizing the signaling pathways that propagate a signal through the brain's neural circuitry and can serve as a convenient proxy for neuronal electrical signaling via action potentials.

Because of the importance of Ca^{2+} in diverse cellular processes and, especially, neuronal systems, fluorescent reporters of Ca^{2+} have been in high demand. Numerous small molecule (*e.g.*, Fluo-4 and Fura¹) and genetically encoded calcium indicators (*e.g.*, GCaMP²) have been developed and are powerful tools for monitoring these Ca^{2+} fluctuations in real time and in living systems. By design, their signal is short-lived to provide the most accurate temporal indicator of Ca^{2+} . Unfortunately, the transient nature of the signal produces an array of unavoidable practical challenges. For example, imaging Ca^{2+} transiently demands that the imaging is completed on the same timescale as the Ca^{2+} spike. While straightforward in simple systems, these imaging requirements begin to require specialized

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AUTHOR CONTRIBUTIONS

B.K.O. performed experiments and analyzed data. S.T.L. conceived the project, analyzed data, and wrote the manuscript.

Supporting Information

The supporting information containing detailed methods and Supplementary Figures is available free of charge via the internet.

instrumentation when attempting to visualize neurons during animal behaviors, even in the small transparent brain of the larval zebrafish. In larger brains, such as in the mouse, application of methods for visualizing Ca^{2+} transiently are limited to analysis of neurons in local brain regions, leaving neuronal activity that spans spatially segregated brain regions largely inaccessible.

These challenges, which are posed by many situations of interest to neuroscientists, have spurred strategies for visualizing a population of active neurons on more convenient timescales, and, therefore, potentially over longer distances. Consider, for example, classic approaches that take advantage of Immediate Early Gene upregulation in response to neural activity in order to mark active populations of neurons^{3,4}. The Immediate Early Gene-based strategies have been widely employed in the absence of alternatives, however, they suffer from the Immediate Early Gene's inconsistent upregulation in different types of neurons. This shortcoming is beautifully illustrated by the difference in upregulation observed between the popular immediate early genes *Arc*, *c-Fos*, and *Egr1* in response to neural activity in the mouse vomeronasal organ⁵.

A more recently developed permanent Ca^{2+} recorder, CaMPARI, is composed of a circularly permuted photoconvertible fluorescent protein (EosFP) that can only be photoconverted in high Ca^{2+} environments⁶. CaMPARI enables visualization of long range anatomical details of active neuron populations in zebrafish, fruit flies, and mice that would be inaccessible with the transient Ca^{2+} imaging methods. However, CaMPARI is limited to only one fluorescent reporter, precluding multicolor comparisons of cell populations activated by different stimuli. Further, CaMPARI cannot direct expression of additional reporter molecules, or other useful tools, in active populations, and its fluorophore photo-switches reversibly between the dim and bright state when imaging in the green channel, requiring consistent, low-intensity illumination with 405 nm light during imaging.

Finally, other long-term recording strategies have employed Ca^{2+} -triggered recruitment of a transcription factor to control expression of a desired construct. For example, TRIC, developed by Luo and coworkers, employs Ca^{2+} -dependent recruitment of a gene activation domain directly to the gene of interest in drosophila⁷. Alternatively, FLARE, recently described by Ting and coworkers, marks active neuron populations by releasing a plasma membrane-tethered transcription activator using an intact TEV protease, freeing it to translocate to the nucleus and activate transcription of the desired reporter protein⁸. Each of these strategies provides powerful capabilities for recording neural activity-evoked Ca^{2+} . However, the components of each of the systems described above work together in a non-modular fashion, requiring new tool development depending on the required application.

Here we describe a system for recording neural activity-evoked Ca^{2+} using a modular reporter system (e.g., caged proteins or transcription activators) that will permit its modification to suit individual experimental requirements or adaptation to future advances in fluorescent proteins or genetically-encoded protease substrates. This system, the Split Tobacco Etch Virus (TEV) protease, Ca^{2+} Activated Neuron Recorder (SCANR), is a modular Ca^{2+} reporter system that can produce fluorescent proteins, or other transgene tools of interest, in cell populations that experience Ca^{2+} concentration spikes, including neurons.

SCANR is composed of two fragments of TEV protease, each fused to one-half of a Ca^{2+} binding module, calmodulin or M13 peptide. Importantly, TEV protease has been successfully employed in eukaryotic systems⁹, including neurons^{8,10,11}, due to its specific cleavage of a peptide sequence that is absent from eukaryotes. This allows for the split enzyme components and substrates to be genetically encoded, which makes possible the application of this strategy to popular animal model systems.

As shown in Figure 1, we designed SCANR such that the split TEV fragments are separate and thus catalytically incompetent at resting cellular Ca^{2+} concentrations. When Ca^{2+} levels increase, for example, in the case of a neuronal action potential, the calmodulin and M13 moieties associate, triggering the formation of active TEV protease. The now catalytically competent enzyme can then cleave its 7 amino acid peptide consensus sequence (ENLYFQS) to liberate the fluorescence of caged substrates previously developed by Rossner and coworkers⁹. For example, the caged DsRed substrate is trapped in the cytoplasm as a dimly fluorescent monomer by virtue of its flanking ERT2 domains linked by TEV's consensus sequence. Using this substrate, Ca^{2+} -activated SCANR would cleave the consensus sequence, freeing the DsRed monomer to oligomerize, form a mature fluorophore, and translocate to the nucleus by virtue of an appended nuclear localization signal.

To begin our search for a suitable orientation of each SCANR component (N-terminal TEV (nTEV), C-terminal TEV (cTEV), calmodulin, and M13), we cloned a panel of chimeras containing each possible orientation of calmodulin and M13 connected by a short glycine-alanine-serine linker to TEV fragments that were based on previously reported split sites⁹ (Figure 2A). Initially, we attempted high-level expression of the constructs in *E. coli* to allow detailed *in vitro* characterization of pairwise combinations of each chimera in the panel. Unfortunately, the M13-containing chimeras localized to insoluble inclusion bodies and resisted our efforts to promote soluble expression (*e.g.*, maltose binding protein fusions and solubilization buffer panels, Supplementary Figure S1). Poor solubility of these constructs, which are small, mostly unstructured in low Ca^{2+} , and non-native to a bacterial host system, was not surprising, but it precluded *in vitro* characterization and forced us to analyze the chimera's properties in more complex, albeit more relevant, biological systems.

Thus, we explored the expression of the candidate SCANR panel in HEK293 cells, a more natural mammalian host for the challenging calmodulin and M13 moieties. Transfection of each fragment into HEK293 cells and expression analysis by immunohistochemistry with antibodies directed against each construct's pendant Myc tag revealed cytosolic expression of all constructs (Supplementary Figure S2). Notably, the constructs containing M13 peptide remained the worst expressers, suggesting expression optimization of these fragments as a likely avenue for optimization in future iterations of this system.

Having confirmed expression in HEK293 cells, we next determined which SCANR chimera combinations could promote TEV reformation at high but not resting Ca^{2+} concentrations. To accomplish this, we submitted binary combinations of the chimeras, as well as the caged DsRed substrate, to an assay in HEK293 cells that exposes them to transiently high Ca^{2+} concentrations. Essentially, we induced a release of intracellular Ca^{2+} in the transfected cells

by bathing the cells in ionomycin, an ionophore that increases intracellular Ca^{2+} in a wide range of cell types, including HEK293 cells^{12–14}. In this assay, Ca^{2+} concentrations rise from resting levels of below 50 nM up to 0.5–1 μM ^{15,16}, which is comparable to the Ca^{2+} concentrations achieved in populations of rat hippocampal neurons evoked by 20 iterations of 40 Hz field stimulations¹⁷. After exposure to ionomycin, we allowed the cells to recover for 0–24 h to permit DsRed translocation to the nucleus, oligomerization, and fluorophore maturation (The $t_{0.5}$ maturation time for DsRed is 10 h¹⁸) prior to imaging by confocal microscopy. Following the exposure to ionomycin, only one pair of chimeras displayed the DsRed positive nuclei that indicate reformation of active TEV protease (Figure 2B, quantified in Figure 2E). We noted lower than expected transfection efficiency, likely due to the large number of distinct plasmids that were co-transfected (As described below, combining multiple SCANR constructs in a multicistronic vector decreased the number of distinct plasmids transfected and improved transfection efficiency). Other chimera combinations, singly transfected constructs, or caged DsRed substrate alone revealed only dim and diffuse cytoplasmic DsRed fluorescence (Figure 2C and Supplementary Figure S3). Finally, comparison to full length TEV plus caged DsRed substrate revealed that C1+M1 produces approximately 50% the activity of full length TEV measured by the abundance of marked nuclei, taking into account the transfection efficiency of each construct (Figure 2D and Supplementary Figure S4). Examination of previously reported crystal structures of calmodulin and M13 association¹⁹ and TEV protease⁹ revealed that the successful C1+M1 chimera combination likely positions the calmodulin and M13 domains anti-parallel (C- to N-terminus) during their association with Ca^{2+} , which allows an ideal alignment of the TEV fragments for reformation.

Quantification of the number of fluorescent nuclei in C1+M1 transfected cells with or without ionomycin treatment revealed low levels of DsRed positive nuclei in cells that were not exposed to ionomycin (Figure 2E, 12 and 24 h time points). Importantly, the observed number of apparent background DsRed nuclei is consistent with previous reports describing intracellular Ca^{2+} release from endoplasmic reticulum stores of HEK293 cells in response to media exchanges²⁰. This suggests that the (–) ionomycin signal derives not from SCANR activity in low Ca^{2+} concentrations, but rather from an inherent Ca^{2+} response in this cell line over the course of the experiment, in particular, during media exchanges associated with ionomycin treatment. Indeed, the absence of DsRed positive nuclei in cells that had not experienced media exchange but have expressed the SCANR constructs for 24 h (time = 0 in Figure 2E) indicates that TEV reformation is not occurring at an observable level at resting cellular Ca^{2+} concentrations. To confirm that the observed activity in the absence of ionomycin treatment is not due to background affinity of the C1 and M1 fragments, we cloned a variant of C1 with the D133E mutation in the calmodulin component, which decreases calmodulin's affinity for Ca^{2+} by over 1000-fold²¹. C1 (D133E) has levels of expression similar to C1 (Supplementary Figure S5), but produced essentially no increase in DsRed substrate turnover upon ionomycin exposure (Figure 2F and Supplementary Figure S6).

Next, we sought to determine if SCANR can serve as a Ca^{2+} integrator by reporting on differences in the duration of exposure to high Ca^{2+} levels. Towards this end, we measured the intensity of DsRed-fluorescent nuclei after exposure of SCANR-expressing HEK cells to

ionomycin in 1 min increments between 1 and 5 min (Ionomycin exposure times of longer than 5 min resulted in dissociation of the cells from the culture plate and could not be accurately tested in this assay). Despite the differing transfection levels in this transiently transfected population and the naturally-occurring Ca^{2+} concentration fluctuations in HEK cells (*e.g.*, Figure 2E, –Ionomycin), we found that SCANR turnover of DsRed substrate increased with increasing ionomycin exposure time (Figure 2G). This result demonstrates that SCANR, in conjunction with this pro-fluorescent reporter substrate, functions as an integrator of Ca^{2+} concentration spikes. Additionally, SCANR functions with as little as a 30 s exposure to ionomycin (Supplementary Figure S7), indicating the lower limit of SCANR's Ca^{2+} response rate. More rigorous control of expression levels through stable expression in cells or organisms, and/or future developments of ratiometric substrate reporter systems, would likely improve on the temporal resolution of Ca^{2+} spike integration.

Having identified successful SCANR constructs using a fluorogenic protein substrate, we next evaluated the system's compatibility with a caged transcription activator substrate that will permit convenient production of a desired transgene in a population of Ca^{2+} activated cells. This substrate employs the Gal4 transcription activator, which is widely used in conjunction with an associated upstream activation sequence (UAS) in model systems like *Drosophila* and zebrafish²². We employed a TEV caged Gal4 substrate designed by Rossner and coworkers that has been rendered inactive by sandwiching it between ERT2 domains linked via a TEV consensus sequence, resulting in exclusion of the transcription activator from the nucleus, and thus inability to promote transcription⁹. In this system, Ca^{2+} -activated SCANR liberates the Gal4 to translocate to the nucleus, interact with UAS, and promote expression of a desired transgene, in our case, GFP (Figure 3A) or an mCherry fused to a nuclear localization signal (Supplementary Figure S8). To evaluate SCANR with this substrate, we transfected the SCANR constructs, the caged Gal4 substrate, and a UAS sequence with a downstream GFP reporter into HEK293 cells, exposed them to resting or high Ca^{2+} by exposure to ionomycin, and imaged the cells by confocal microscopy. Consistent with the caged DsRed substrate results, although with a much lower number of cells transfected with each construct due to the large number of individual plasmids included in the transfection, the caged Gal4 substrate displayed minimal background in control cells (Arrowheads, Figure 3B) and intense green fluorescence similar to that observed in full TEV controls in cells exposed to ionomycin (Figure 3C–D, quantified in Figure 3E).

Finally, we examined the ability of SCANR to record Ca^{2+} concentration spikes that occur during neuronal action potentials. The rat hippocampus is primarily composed of three types of neurons (pyramidal cells, granule cells, and GABAergic interneurons) and is responsible for memory and spatial navigation tasks²³. Because primary rat hippocampal neurons are post-mitotic and prone to low transfection efficiency, and because we observed low efficiency in HEK293 cells due to the large number of individual plasmids transfected, we sought to limit the number of plasmids required for transfection by creating a multi-gene expression SCANR construct, which would enable stoichiometric expression of each construct from a single transfected plasmid. To accomplish this, we designed plasmids with each SCANR construct included on a single plasmid separated by the P2A self-cleaving peptide²⁴. Importantly, the P2A multi-gene expression system, although highly efficient, has a non-zero incidence of failed P2A peptide cleavage²⁴. In our split enzyme system, the result

of a failed P2A cleavage would be a linked split enzyme, which could activate the SCANR fragments and produce significant background TEV activity in low Ca^{2+} environments. To examine this possibility, we directly linked the two SCANR fragments using the P2A peptide. Transfection of this construct and the caged DsRed substrate followed by analysis by confocal microscopy revealed numerous DsRed-fluorescent nuclei in the absence of ionomycin treatment, indicating high levels of TEV activity in the absence of Ca^{2+} from the small amount of uncleaved P2A peptide (Supplementary Figure S9).

In order to avoid background activity in P2A-linked SCANR chimeras, we introduced a GFP between the SCANR fragments, which, in the event of a failed P2A cleavage, would separate each SCANR fragment and prevent TEV reformation in resting Ca^{2+} (Figure 4A). Transfected into HEK293 cells and analyzed with the ionomycin Ca^{2+} -sensitivity assay, the constructs displayed a strong Ca^{2+} response and did not display TEV activity in the absence of Ca^{2+} (Supplementary Figure S10). Additionally, the construct improved transfection efficiency and the GFP served as a convenient marker for transfected cells.

To evaluate its performance in neurons, we transfected cultured rat hippocampal neurons with the P2A-GFP-modified SCANR constructs, and, after allowing for protein expression, induced physiologically-relevant Ca^{2+} spikes by electrical stimulation at 15V in trains of 1 ms on and 33.3 ms off (Figure 4B)^{17,25}. After allowing the liberated DsRed substrate to translocate to the nucleus and form mature fluorophore, the stimulated population contained neurons with bright, DsRed-positive nuclei (Figure 4C). Conversely, the unstimulated control neurons possessed more rare DsRed-positive nuclei (Figure 4D, quantified in Figure 4E). Importantly, the presence of some SCANR-marked cells sans external neuron stimulation was expected due to the hippocampal neuron's tendency for spontaneous firing of action potentials in the 5-days *in vitro* cultures employed in our assay²⁶. Future iterations of this strategy that provide control of SCANR activation using opto- or chemogenetics strategies will minimize recording of such spontaneous neural activity.

In conclusion, we have developed a Split TEV, Ca^{2+} Activated Neuron Recorder (SCANR). Using a fluorogenic TEV substrate or a TEV-caged transcription activator for expression of reporters or transgenic tools, this system reports on artificially applied Ca^{2+} concentration spikes in HEK293 cells or Ca^{2+} spikes during neuronal signaling. SCANR's ability to permanently record neural activity permits its use in systems where activity cannot be accessed in real time, for example, in thick brains over long distances, during behaviors sensitive to concurrent imaging, or *in utero*. SCANR's modularity enables the use of diverse substrate reporters to suit the experiment's requirements. These substrate possibilities include the discussed caged dsRed and Gal4 substrates, other previously-reported protease reporter substrates like the zipGFP and fluorescent protein quenching peptide reporters^{27,28}, and future protease indicators that grant unique capabilities like caged photoconvertible fluorescent proteins to highlight distinct time frames of neural activity in separate colors. Additionally, SCANR has the potential to permit ratiometric integration of Ca^{2+} transients using, for example, a FRET-based TEV protease substrate, to provide more reliable quantification of the measured Ca^{2+} response. Finally, SCANR's modularity makes possible its use with opto- or chemo-genetics modules to provide spatiotemporal control of SCANR activity, for example, by limiting subcellular access to the TEV substrate or including a

steric switch that prevents or enables TEV reformation. Ultimately, application of SCANR in living cells and animals will enable long-range recording and manipulation of active neuron populations that were previously challenging to interrogate.

METHODS

For a detailed description of materials and methods, see the Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

The Split TEV Ca²⁺-Activated Neuron Recorder (SCANR). Cells expressing split TEV protease chimeras cTEV and nTEV linked to Ca²⁺ dimerizing domains calmodulin or M13 peptide remain separate and catalytically dormant at resting cellular concentrations of Ca²⁺. In response to an increase in intracellular Ca²⁺, such as in the case of neuronal signaling, the Ca²⁺ binding domains dimerize, reforming the active TEV protease. Once activated, the TEV protease cleaves the caging groups from a pro-fluorescent cytoplasmic protein, permitting nuclear targeting and fluorophore maturation that marks Ca²⁺ active cell populations with fluorescent nuclei.

Figure 2.

Identification of split TEV, calmodulin, and M13 chimera combinations that enable Ca^{2+} sensing. (A) Schematic of cTEV, nTEV, calmodulin and M13 combinations tested. (B–C) Constructs C1 + M1 (B), or C2 + M3 (C) and a caged DsRed TEV substrate were transfected into HEK293 cells. 24 h post transfection the cells were treated with ionomycin to induce a transient high intracellular Ca^{2+} concentration (+ ionomycin) or vehicle control (- ionomycin). Cells were allowed to recover for 24 h, fixed, probed with α -myc to visualize cells expressing the TEV constructs, and visualized by confocal microscopy. (D) HEK293 cells transfected with full length TEV and a caged DsRed TEV substrate. (E) Quantification of the number of DsRed-positive nuclei in HEK293 cells transfected with C1+M1 and the caged DsRed substrate at 0–24 h post ionomycin or vehicle treatment. (F) Quantification of the number of DsRed-positive nuclei in HEK 293 cells transfected with a calmodulin-mutated D133E variant of C1+M1 +/- ionomycin exposure. (G) Fluorescence intensity of DsRed positive nuclei upon exposure to ionomycin for times between 1 and 5 minutes. All images are maximum intensity z-projections of confocal microscopy images. Error bars represent standard deviation (E, F) or standard error of the mean (G). Scale bar = 20 μm . n.s. = not significant; ** $p < 0.01$; *** $p < 0.001$.

Figure 3.

Ca²⁺ recording using a TEV-caged transcription activator substrate for production of diverse transgenic reporters. (A) Schematic of caged Gal4 substrate function. Caged Gal4 TEV substrate remains dormant in the cytoplasm until active TEV removes tethered ERT2 domains, permitting nuclear localization and triggering transcription of reporter proteins. (B–C) Maximum intensity projections of HEK293 cells transfected with the SCANR constructs, Gal4 TEV substrate and UAS-EGFP and exposed to control or high Ca²⁺ conditions by exposure to vehicle (B) or ionomycin (C), respectively. Arrowheads in panel B denote an instance of faint GFP fluorescence in the absence of ionomycin. (D) Maximum intensity projection of HEK293 cells transfected with full TEV protease, the Gal4 TEV substrate and UAS-EGFP. (E) Quantification of the number of DsRed-positive nuclei in HEK293 cells transfected with C1+M1, the caged Gal4 substrate, and the UAS-GFP reporter construct 24 h post ionomycin or vehicle treatment. Scale bar = 20 μ m. Error bars represent standard error of the mean for n = 8. *** p < 0.001.

Figure 4.

Recording Ca^{2+} associated with neuronal signaling in rat hippocampal neurons. (A) Schematic of P2A construct used to deliver both SCANR fragments and GFP expression control. (B) Cultured rat hippocampal neurons were transfected with the P2A SCANR construct and caged DsRed substrate and stimulated to produce action potentials, and, thus, transient Ca^{2+} concentration spikes, by an applied electric field. (C–D) Maximum intensity z-projection of a confocal microscopy image of SCANR transfected neurons with (C) or without (D) externally supplied electrical stimulation. (E) Quantification of the number of DsRed-positive nuclei per transfected cells in neurons transfected with the SCANR construct described in panel A and the caged DsRed substrate. Scale bar = 20 μ m. Error bars represent standard deviation. *** $p < 0.001$.