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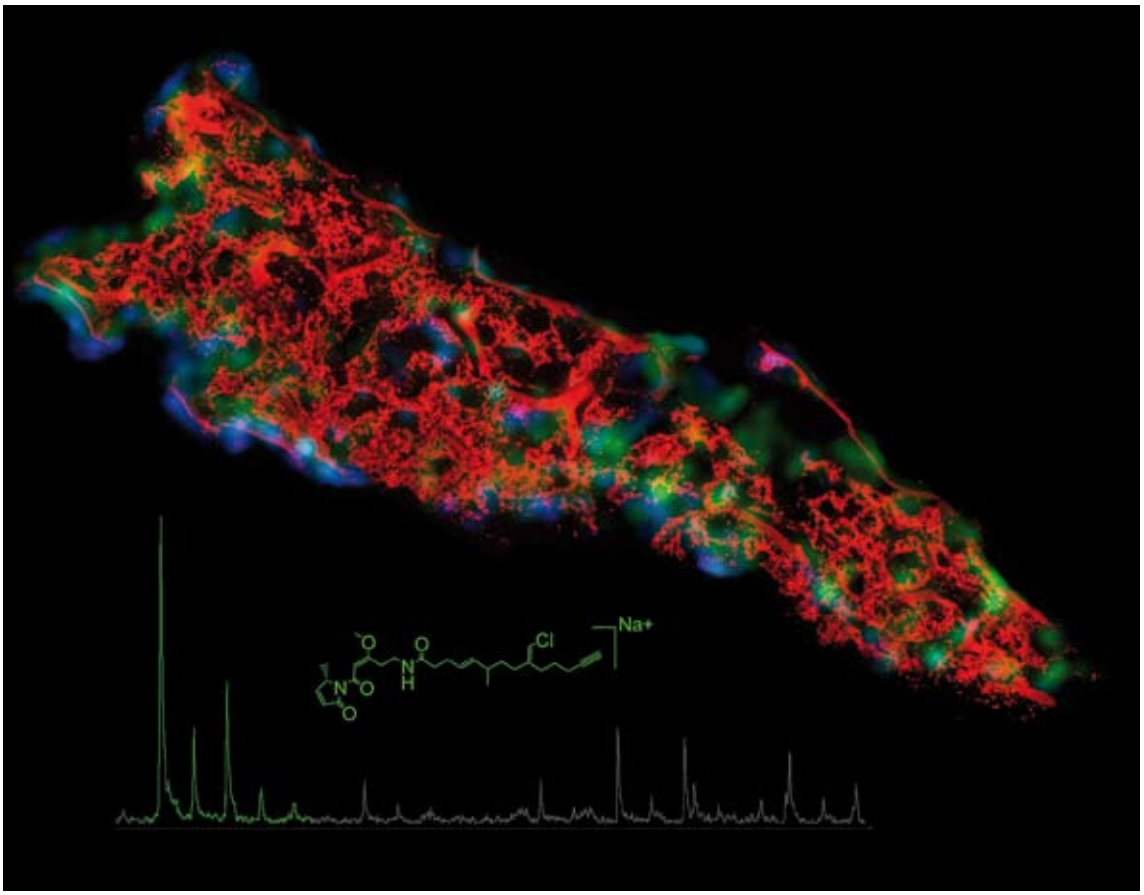

Visualization of phosphatase activity in living cells with a FRET-based calcineurin activity sensor†‡

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Protein kinases and phosphatases are organized into complex intracellular signaling networks designed to coordinate their activities in both space and time. In order to better understand the molecular mechanisms underlying the regulation of signal transduction networks, it is important to define the spatiotemporal dynamics of both protein kinases and phosphatases within their endogenous environment. Herein, we report the development of a genetically-encoded protein biosensor designed to specifically probe the activity of the Ca^{2+}/calmodulin-dependent protein phosphatase, calcineurin. Our reporter design utilizes a phosphatase activity-dependent molecular switch based on the N-terminal regulatory domain of the nuclear factor of activated T-cells as a specific substrate of calcineurin, sandwiched between cyan fluorescent protein and yellow fluorescent protein. Using this reporter, calcineurin activity can be monitored as dephosphorylation-induced increases in fluorescence resonance energy transfer and can be simultaneously imaged with intracellular calcium dynamics. The successful design of a prototype phosphatase activity sensor lays a foundation for studying targeting and compartmentation of phosphatases.

Protein phosphorylation is a reversible post-translational modification utilized by cells to regulate a variety of cellular processes. Inside a cell, a protein's phosphorylation state is dependent upon a delicate balance between the dueling activities of protein kinases and protein phosphatases.[1] As a result, cells have developed intricate mechanisms to coordinate the activities of these families of enzymes in both space and time.[2] To gain insights into the regulation of these important signaling molecules, molecular probes that facilitate the real-time analysis of protein phosphorylation with high spatial and temporal resolution are required. To this end, researchers have recently developed a series of genetically-encoded protein biosensors designed to measure the activity of protein kinases in living cells.[3] However, to date, a general design for phosphatase activity biosensors has not been described. Here we report the development of a genetically-encoded phosphatase activity sensor, based on a generalizable design, which utilizes a molecular switch that is sensitive to the activity of the phosphatase of interest (Fig. 1). This fluorescence resonance energy transfer (FRET) based biosensor is designed to track the activity of the serine/threonine protein phosphatase, calcineurin, within its endogenous environment.

Calcineurin, also known as protein phosphatase 2B, is a ubiquitously-expressed protein phosphatase whose activity is controlled by Ca^{2+}/calmodulin.[4] Calcineurin is unique among the major protein phosphatases in that it is highly sensitive to fluctuations in the concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$).[5] As such, calcineurin plays an important role in coupling transient Ca^{2+} signals to downstream cellular processes. In particular, calcineurin has been shown to modulate neuronal excitability during memory formation,[6,7] to promote cardiac hypertrophy,[8] and to drive T-cell activation.[9,10] During the latter, calcineurin couples receptor-mediated Ca^{2+} fluxes to cytokine production by altering the sub-cellular distribution of members of the nuclear factor of activated T-cells (NFAT) family of transcription factors.[11]

The distribution of NFAT family members depends upon the phosphorylation state of an N-terminal regulatory domain containing, among other regulatory motifs, a nuclear localization signal (NLS) and three highly-conserved serine-rich motifs termed SRR-1, SP2, and SP3 (Fig. 2A).[11] In resting T-cells, multiple serine residues within the SRR-1 and SP motifs are phosphorylated by various kinases, including constitutively-active casein kinase 1α (CK1α).[12] Phosphorylation of the serine-rich regions is believed to promote electrostatic interactions between negatively-charged phosphate groups and positively-charged residues located within the NLS.[13] These interactions effectively mask the NLS and prevent NFAT from translocating into the nucleus. In response to elevated $[Ca^{2+}]_i$, calcineurin catalyzes dephosphorylation of the regulatory domain, inducing a conformational change that unmasks the NLS.[13,14] Once its NLS is exposed, NFAT is able to migrate into the nucleus where it promotes the expression of several genes involved in the production of cytokines (Fig. 2A).

To develop a genetically-encoded biosensor capable of monitoring the spatiotemporal dynamics of calcineurin activity within the cellular environment, we took advantage of the ability of calcineurin to specifically and efficiently dephosphorylate the regulatory domain of NFAT in response to increases in $[Ca^{2+}]_i$.[13] To this end, truncated forms of the regulatory domain of the NFAT isoform, NFAT1,[15] were sandwiched between the enhanced cyan fluorescent protein (ECFP) and a

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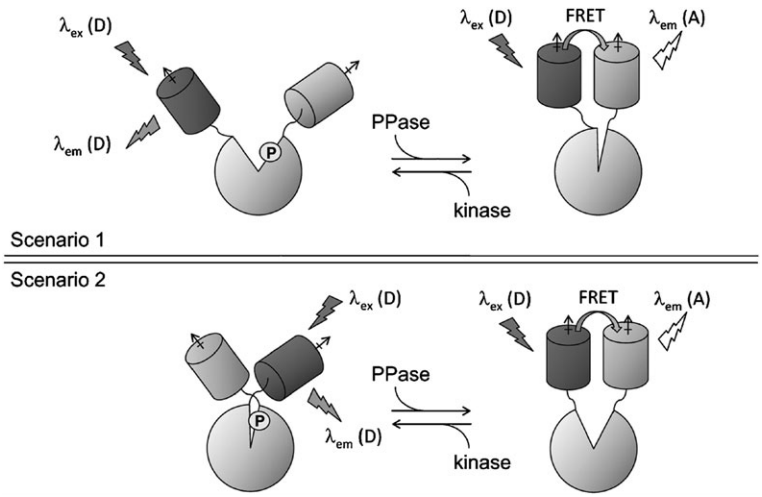

Fig. 1 General design of a FRET-based phosphatase activity reporter. Two possible scenarios, each leading to increased FRET, are shown. The basic biosensor design consists of a phosphatase activity-dependent molecular switch (light gray pacman) sandwiched between a FRET donor (D; dark gray cylinder) and a FRET acceptor (A; light gray cylinder). Dephosphorylation-dependent conformational changes in the substrate region cause a change in interfluorophore distance and/or reorientation of the fluorophore dipoles (↕) resulting in an increase in FRET (curved arrow). PPase: protein phosphatase; λ_{ex}(D): excitation wavelength of the donor fluorophore; λ_{em}(D): emission wavelength of the donor fluorophore; λ_{em}(A): emission wavelength of the acceptor fluorophore.

circularly-permuted version of the yellow fluorescent protein, Venus, termed cpV(L194) (Fig. 2B).[16–18] In this reporter design, NFAT1 serves as a molecular switch whose conformational state is regulated by calcineurin-mediated dephosphorylation. Meanwhile, ECFP and cpV(L194) act as a FRET pair to rapidly detect conformational changes in NFAT1.

In resting cells, the regulatory region of NFAT1 is expected to be hyperphosphorylated due to the action of cellular kinases such as CK1α and the mitogen activated protein kinase, p38.[12,19] As a consequence, the reporter does not require the activation of additional kinases to put it into a "dephosphorylation-competent" state. This feature ensures that the cellular environment remains relatively unperturbed prior to Ca^{2+} stimulation, reducing the potential for crosstalk with other signaling pathways which may otherwise complicate data interpretation. To test whether the fusion of GFP-like proteins to the N- and C-termini of the regulatory domain of NFAT1

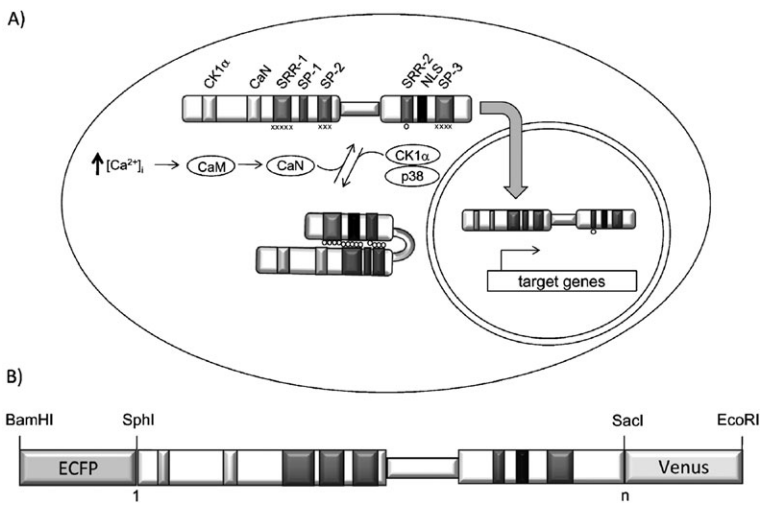

Fig. 2 Calcineurin activity reporter based on NFAT. (A) The subcellular distribution of NFAT1 is regulated by the phosphorylation state of its N-terminal regulatory domain. A rise in $[Ca^{2+}]_i$ activates calmodulin (CaM) thereby stimulating calcineurin (CaN) activity. Once activated, calcineurin catalyzes dephosphorylation of the regulatory domain of NFAT1 leading to its translocation into the nucleus where it drives the expression of target genes. For simplicity, only the regulatory domain is shown. Several regulatory elements contained within the regulatory domain of NFAT1 are also highlighted. Light gray boxes: CK1α and calcineurin docking sites; black box: NLS composed of basic amino acid residues; dark gray boxes: highly-conserved serine-rich motifs found in all canonical NFAT family members (SRR, serine-rich region; SP, SPxx motif). Sites of phosphorylation are depicted as circles while those dephosphorylated by calcineurin are marked by an "x".[13] (B) Domain structure of the calcineurin activity reporter. A region of the regulatory domain of NFAT1, ranging from amino acid residue 1 to *n*, is sandwiched between the FRET pair, ECFP and cpV(L194). Restriction sites used for cloning are shown (see supporting information for details‡).

impedes calcineurin-mediated dephosphorylation, a calcium-induced nuclear translocation assay was used to track the subcellular distribution of a protein chimera composed of ECFP fused to the N-terminus of the regulatory region of NFAT1.[15] This construct, termed ECFP-NFAT(1-415), undergoes nuclear import following the addition of the Ca^{2+} ionophore, ionomycin (Fig. S1‡). These results are consistent with previous data showing that the nuclear import of the regulatory domain proceeds in a manner similar to that of endogenous NFAT1, regardless of whether a fusion tag is attached to the N- or C-terminus of the protein.[15,20]

To generate a FRET-based biosensor capable of monitoring calcineurin activity in live cells, we initially chose to flank NFAT1(1-415) with ECFP and cpV(L194) at the N- and C-termini, respectively. In response to Ca^{2+} stimulation, the resulting construct exhibited an increase in its nuclear fluorescence intensity similar to that observed for ECFP-NFAT-(1-415) (data not shown). However, this construct showed no change in FRET following Ca^{2+} stimulation (Fig. S2‡). One interpretation of these results is that the calcineurin-mediated dephosphorylation induced conformational changes in NFAT1 are not translated into a FRET change because ECFP and cpV(L194) are not positioned appropriately. We therefore generated a series of truncations of the regulatory domain of NFAT1 while keeping the sites of conformational changes—located near the SRR-1 (residues 167–179) and the NLS (residues 249-253)—intact.[13] Since the N-terminus of NFAT1 contains several docking sites known to recruit enzymes involved in the regulation of its phosphorylation state,[12,20] we chose to truncate NFAT1 at its C-terminus, in the vicinity of the NLS. cpV(L194) was thus fused to NFAT1 at residues 254 (abutting the NLS), 265 (just prior to SP-3) and 297 (encompassing the SP-3 region) while ECFP remained fused to the extreme N-terminus of NFAT1 (Fig. 3). The emission ratio of yellow over cyan was then measured over time in HeLa cells expressing each construct, and their signals compared. While the construct containing NFAT1(1-265) responded to Ca^{2+} influx in a manner similar to the original NFAT1(1-415)-containing species, the other two constructs were characterized by clearly detectable increases in their emission ratios following Ca^{2+} stimulation (Fig. 3, Fig. S2‡). Of the four candidate reporters, the construct that contains NFAT1(1-297) showed the largest increase in its emission ratio following Ca^{2+} stimulation (Fig. 4B) and is thus dubbed Calcineurin (*CaN*) *A*ctivity *R*eporter *1* (CaNAR1).

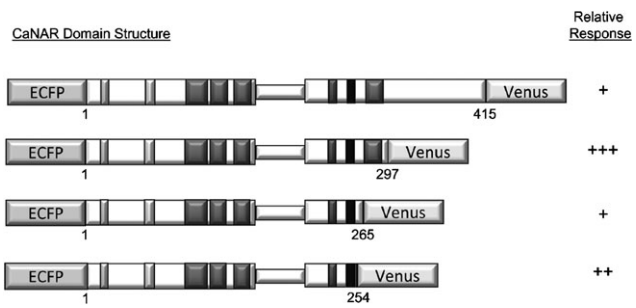

Fig. 3 Relative response of several test constructs. Constructs containing various-sized fragments of the regulatory domain of NFAT1 were transfected into HeLa cells and changes in their emission ratios in response to ionomycin treatment were measured as a function of time. The relative response of each construct is indicated.

When expressed in HeLa cells, CaNAR1 is distributed primarily in the cytosol (Fig. 4A, first panel). After drug addition, this reporter exhibited an immediate increase in its emission ratio that continued to rise steadily for twelve minutes before reaching a maximum value of 6.3 ± 1.5% ($t_{1/2}$ = 3.5 min) (Fig. 4A and 5A). Once it had plateaued, the emission ratio remained constant for several minutes. A further increase in extracellular calcium did not induce a significant change in the emission ratio, suggesting that the maximum response had been achieved during this time. In contrast, removal of ionomycin from the imaging media resulted in a decrease in the emission ratio back to resting levels (data not shown), consistent with previous studies showing that endogenous NFAT1 is rephosphorylated by cellular kinases shortly after ionomycin is removed from the culture medium.[21] Together, these data imply that Ca^{2+} influx elicits a cellular response that converts CaNAR1 from an inactive (low FRET) to an active (high FRET) conformational state and that a new equilibrium between these two states is established shortly thereafter.

Though it is well-known that calcineurin dephosphorylates the regulatory domain of NFAT1 in a highly specific manner, we wanted to ensure that the increased emission ratio observed for CaNAR1 following Ca^{2+} stimulation was indeed due to calcineurin-mediated dephosphorylation. Cyclosporin A (CsA) is a potent pharmacological inhibitor of calcineurin activity.[22] Therefore, we examined the effects of CsA pretreatment on the ability of CaNAR1-expressing HeLa cells to respond to increases in $[Ca^{2+}]_i$. As shown in Fig. 5A, CsA completely abolished the FRET change induced by ionomycin, suggesting that the FRET changes observed for CaNAR1 result from calcineurin activity and not other Ca^{2+}-mediated processes.

Next, to correlate the FRET change with dephosphorylation of CaNAR1, we took advantage of the fact that the electrophoretic mobility of NFAT1 is strongly influenced by its phosphorylation state. For example, previous studies have shown that NFAT1 molecules isolated from unstimulated cells migrate as a single band whose mobility is retarded relative to that of the unphosphorylated species.[21] This behavior has been attributed to hyperphosphorylation of the regulatory domain.[13] In contrast, Ca^{2+}-stimulated cells initially contain a collection of differentially-phosphorylated NFAT1 molecules that converge over time to migrate as a single, lower molecular weight species corresponding to the fully-dephosphorylated form of the protein. Therefore, we examined the electrophoretic mobility of CaNAR1 isolated from transiently-transfected HeLa cell lysates at various times after ionomycin treatment (Fig. 5B). Though its calculated molecular weight is 85 kDa, CaNAR1 migrated almost exclusively as an approximately 125 kDa species in uninduced cell lysates, consistent with hyperphosphorylation of its substrate region. However, five minutes after ionomycin treatment, the distribution of CaNAR1 became broader and less uniform, suggesting that a substantial portion of the reporter molecules had been dephosphorylated during this time period. Longer incubation

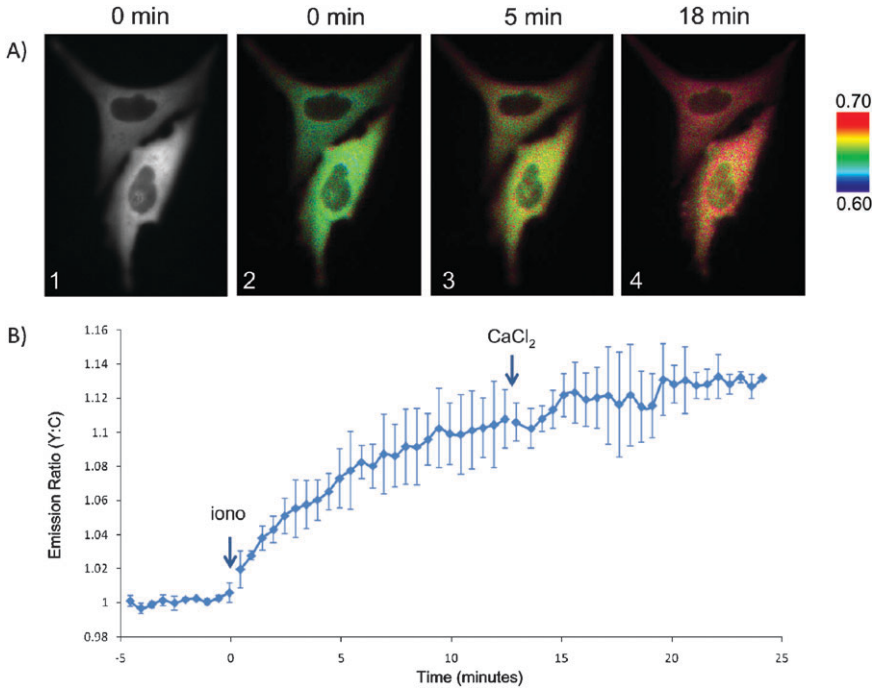

Fig. 4 Characterization of CaNAR1. (A) Panel 1: Fluorescence intensity image showing the subcellular distribution of CaNAR1 at the beginning of the experiment. Panels 2–4: Pseudocolor images of the emission ratio corresponding to CaNAR1 at 0, 5 and 18 minutes after ionomycin addition (1 μM). Blue: low emission ratio; red: high emission ratio. (B) Representative response of the CaNAR1-expressing HeLa cells shown in (A) following ionomycin treatment. Ionomycin and additional $CaCl_2$ (5 mM) were added to the imaging solution at the indicated times (blue arrows). The standard deviation for each time point is shown.

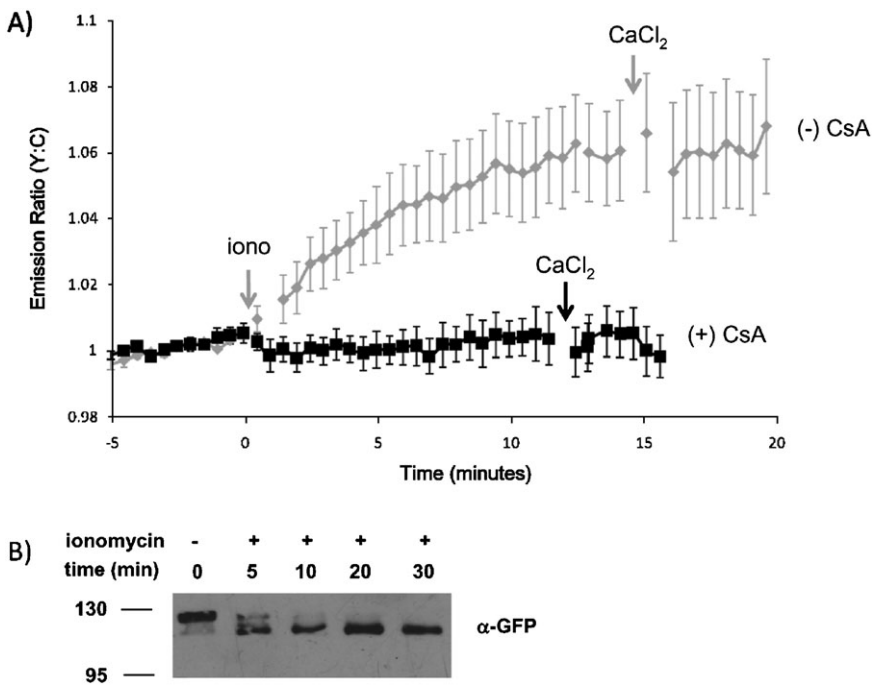

Fig. 5 CaNAR1 is dephosphorylated in a calcineurin-dependent manner. (A) Average response of CaNAR1 in the presence (black squares) or absence (gray diamonds) of CsA (10 μM, 10 min preincubation). Ionomycin and $CaCl_2$ treatments are as described in Fig. 4B. The standard error at each time point is shown (n = 4 and 6 in the presence or absence of CsA, respectively). (B) The electrophoretic mobility of CaNAR1 isolated from HeLa cell lysates was determined at various times after ionomycin addition using standard western blotting techniques. A rabbit anti-GFP antibody (diluted 1 : 4000 in blocking buffer) was used to probe for CaNAR1.

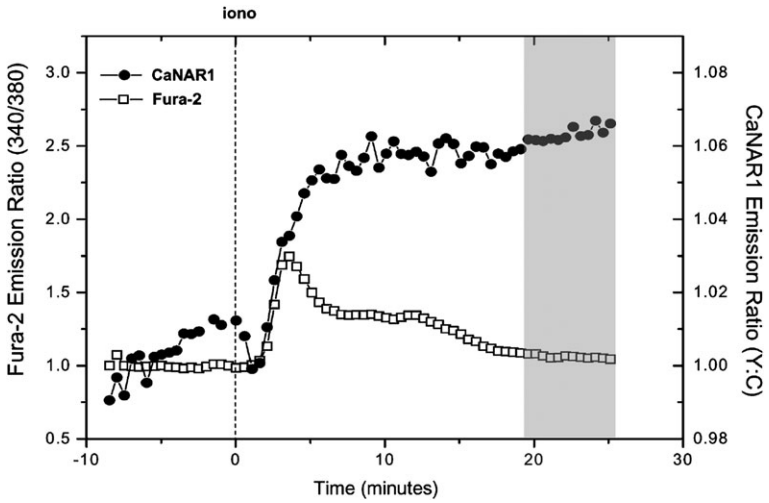

Fig. 6 Simultaneous measurement of $[Ca^{2+}]_i$ and calcineurin activity in a single cell. The emission ratios of CaNAR1 (black diamonds) and Fura-2 (open squares) were measured over time in response to ionomycin addition (1 μM, dotted line) in a single HeLa cell. A gray box indicates the period of time during which the apparent $[Ca^{2+}]_i$ had returned to basal levels following ionomycin-induced Ca^{2+} stimulation (see text for details). A representative response from three cells is shown.

times resulted in an increase in the mobility of nearly all of the CaNAR1 molecules. As expected, when cells were preincubated with CsA prior to ionomycin treatment, no change in the electrophoretic mobility of CaNAR1 was observed during the same period of time (data not shown). Taken together, these data are consistent with the notion that CaNAR1 is progressively dephosphorylated by calcineurin in response to elevated $[Ca^{2+}]_i$. Thus, the observed FRET change is correlated with dephosphorylation of the reporter by calcineurin, providing a real-time readout for calcineurin activity.

To correlate the activation of calcineurin with Ca^{2+} dynamics at the level of single cells, CaNAR1 and the Ca^{2+} indicator, Fura-2, were used to simultaneously monitor changes in calcineurin activity and $[Ca^{2+}]_i$, respectively.[23] As can be seen in Fig. 6, addition of ionomycin induced a rapid increase in $[Ca^{2+}]_i$ which led to the activation of calcineurin. No discernible lag was observed between Ca^{2+} influx and calcineurin activation, suggesting that, in HeLa cells, calcineurin is activated seconds after Ca^{2+} stimulation. Peak Ca^{2+} concentrations were observed approximately 3.5 min after stimulation, at which time the emission ratio of CaNAR1 reached half-maximum (Fig. 6). Following an initial spike, the Ca^{2+} concentration gradually decreased back to baseline levels. Interestingly, the emission ratio of CaNAR1 continued to increase during this period, suggesting that the reporter was being dephosphorylated by calcineurin even as the concentration of Ca^{2+} decreased. Moreover, the emission ratio of CaNAR1 remained constant for several minutes after $[Ca^{2+}]_i$ had returned to near basal levels (Fig. 6, gray box), raising interesting mechanistic questions about the termination of calcineurin activity and the regulation of NFAT1. Future experiments will investigate termination of calcineurin activity under this and other cellular conditions.

Despite the relatively broad substrate specificity of most protein phosphatases, many cellular proteins are dephosphorylated in a highly-specific manner. Cells often achieve this feat by restricting the activity of protein phosphatases to distinct subcellular regions. Inside the cell, calcineurin is targeted to discrete microdomains where it is involved in the regulation of a diverse set of cellular processes.[24–27] Thus, in addition to providing information about the timing of calcineurin activation, CaNAR1 may also be used to answer questions about the spatial regulation of calcineurin activity. Indeed, as a genetically-encoded and subcellularly targetable activity sensor, CaNAR1 represents a powerful tool for probing calcineurin activity in different subcellular regions and for monitoring specific pools of calcineurin within their endogenous environment. Future experiments will thus utilize both targeted and untargeted versions of CaNAR1 to monitor the activity of different pools of calcineurin in various cellular contexts to better understand the spatiotemporal regulation of this important protein phosphatase.

In summary, we have developed and characterized CaNAR1, a genetically-encodable biosensor capable of specifically tracking the activity of the serine/threonine phosphatase, calcineurin, inside living cells with high spatial and temporal resolution. The phosphatase activity sensor design illustrated here should be generally applicable to other protein phosphatases as specific molecular switches are identified or engineered. Thus, as a prototype phosphatase activity sensor, CaNAR1 lays a foundation for studying the targeting and compartmentation of protein phosphatases within the cellular environment.

List of abbreviations

FRET	Fluorescence resonance energy transfer
$[Ca^{2+}]_i$	Intracellular calcium ion concentration
CaN	Calcineurin
NFAT	Nuclear factor of activated T-cells
NLS	Nuclear localization signal
CK1α	Casein kinase 1α
ECFP	Enhanced cyan fluorescent protein

(*continued*)

cpV(L194)	A circularly-permutated version of the yellow fluorescent protein variant, Venus
CaNAR1	CaN Activity Reporter 1
CsA	Cyclosporin A

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