Twitching towards the ideal calcium sensor

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A new family of genetically encoded ratiometric calcium indicators optimized for imaging of calcium signals *in vivo* exhibits near-linear fluorescence dynamics while minimizing artifacts caused by movement.

Calcium is an exquisitely versatile signaling molecule, determining both cell differentiation and death as well as triggering neurotransmission and regulating synaptic plasticity. Consequently, determining the spatial and temporal dynamics of intracellular calcium concentration, [Ca^{2+}], is crucial not only in cell biology but also for understanding immune physiology and brain function. Unsurprisingly, measuring [Ca^{2+}] dynamics is one of the main uses of fluorescence microscopy and has spurred the development of new indicators for many decades. In this issue of *Nature Methods*, Griesbeck and colleagues[1] present a novel family of genetically encoded calcium indicators that exhibit improved properties for *in vivo* calcium imaging experiments.

The advent of genetically encoded calcium indicators (GECIs)[2] has made it possible to target indicators to defined cells and even subcellular compartments[3] and has allowed repeated imaging of the same identified cells over long time periods. During the past 5 years, a flurry of development has improved GECIs to the extent that they have joined the mainstream of cell biology and neuroscience research tools. However, GECIs are currently still outperformed in many areas by small-molecule calcium indicators such as Oregon Green 488 BAPTA-1 (OGB-1), and so further developments are needed. The ideal GECI needs to (i) be bright, allowing clear detection of the imaged cells even at rest; (ii) exhibit a linear relationship between changes in [Ca^{2+}] and fluorescence changes in the [Ca^{2+}] range of interest; (iii) minimize the detrimental effects of specimen movement by using ratiometric detection; (iv) perturb the cells it is expressed in as little as possible, in particular by minimizing buffering of physiological Ca^{2+} signals; and (v) have sufficiently fast kinetics to precisely follow Ca^{2+} signaling.

The novel family of GECIs developed by Griesbeck and colleagues, which they named 'Twitch' sensors, fulfill the majority of these criteria and represent a major improvement over most existing indicators[1]. The name of the indicators originates from the fact that their calcium sensor is derived from troponin C, a calcium-binding protein found in skeletal and cardiac muscle (but not neurons) that regulates muscle twitching. Similarly to most calcium-binding proteins, a single troponin C molecule binds up to four Ca^{2+} ions using four specialized protein domains, the so-called EF hands (**Fig. 1**). The resulting binding dynamics are highly nonlinear owing to cooperativity between the individual binding sites. Griesbeck and colleagues aimed to linearize these binding dynamics as well as minimize the Ca^{2+} buffering capacity of their GECI by reducing troponin C to a minimal binding motif. They took as their starting point a high-affinity troponin C variant not previously used in GECI design. In an impressive feat of protein engineering, they managed to reduce the number of Ca^{2+} binding sites from 4 to 2 (corresponding to a domain of just 67 amino acids) while maintaining a high affinity for Ca^{2+} (**Fig. 1**). The low number of Ca^{2+} binding sites improves the biocompatibility of the sensor during long-term expression because it reduces buffering. After further tuning by introduction of point mutations,

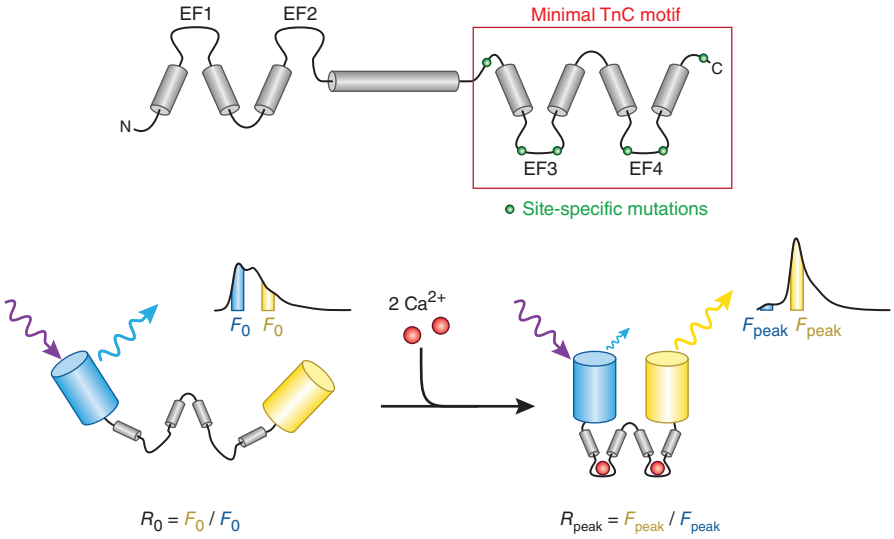

Figure 1 | Twitch, a new family of genetically encoded calcium sensors. Top, troponin C was reduced to a domain consisting of just two EF hands. This minimal calcium-binding motif was fused with cyan and yellow fluorescent proteins to yield Twitch. Bottom, the binding of two calcium molecules leads to a conformational change, which changes the ratio (*R*) of blue and yellow fluorescence intensities (*F*). Using this ratio permits a volume– and indicator concentration–independent readout.

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the resulting construct was fused to a pair of fluorescent proteins capable of fluorescence resonance energy transfer (FRET; **Fig. 1**). The structure of the binding domain and that of the whole prototype indicator, Twitch-1, was determined using nuclear magnetic resonance and small-angle X-ray scattering spectroscopy, respectively. Using insights gained from the structure, the researchers further optimized the prototype in a two-step screening process: constructs exhibiting large FRET changes were identified in a bacterial colony–based assay, after which promising candidates were further tested in a neuronal culture system. Finally, three Twitch variants (1, 2B and 3) were tested *in vitro* and *in vivo* in mouse neurons and T lymphocytes.

Being FRET-based indicators, Twitch sensors intrinsically enable the ratio between the intensities of two separate wavelength regions to be reported rather than the intensity of a single wavelength range (**Fig. 1**). This 'ratiometric detection' corrects for intensity changes resulting not from $[Ca^{2+}]$ fluctuations but from volume changes or movement. This allows for reliable imaging of $[Ca^{2+}]$ in motile cells such as T lymphocytes and in preparations exhibiting considerable movement (such as *in vivo*). Ratiometric indicators are particularly valuable for long-term imaging experiments, as they help mitigate the consequences of fluctuations in excitation light, changes in indicator expression levels, or tissue growth or cell shape changes over time. In addition, cells expressing FRET-based indicators are bright at rest. Owing to the careful selection of fluorescent proteins, Twitch excels here, being substantially brighter than existing FRET-based GECIs and allowing imaging of cell structure in parallel to function while also increasing the signal-to-noise ratio for functional imaging. This represents a notable improvement over previous FRET-based GECIs such as YC3.60 (ref. 4) and the YC-Nano variants[5].

Calibration experiments involving simultaneous imaging and electrophysiological recordings demonstrate that Twitch indicators are sensitive enough to detect single action potentials in cortical neurons imaged *in vitro* using two-photon microscopy, the gold standard for any indicator used for neuroscience applications[6]. Whether Twitch-2B exhibits similar sensitivity *in vivo* still needs to be verified. Fulfilling one of the main development goals, Twitch-2B displays a linear relationship between fluorescence and action potential numbers for bursts of up to 15 action potentials, testifying to the near-linear dynamics of the indicator family. In this crucial range for readout of neural activity, Twitch sensors respond more linearly than the most recent versions of the popular GCaMP family of GECIs[7], though a direct comparison in terms of actual sensitivity to small changes of $[Ca^{2+}]$ remains to be made. When compared with OGB-1, Twitch-2B shows similar sensitivity, but with substantially slower kinetics—which may prove limiting for applications that require resolving fast events occurring at high frequencies.

Over the past 5 years, numerous highly optimized GECIs have been introduced. Many of these have specific advantages: for example, different variants of GCaMP6 show high $[Ca^{2+}]$ sensitivity and have very fast kinetics[7]; RCaMP[8] is red; and Twitch shows near-linear fluorescence dynamics. Under which experimental circumstances each of these indicators is best suited is an important question requiring a direct comparison of the most promising indicators in the same preparation under similar conditions (see, for example, refs. 9,10).

The Twitch family represents a new generation of GECIs that has been designed from scratch, offering fresh opportunities for further optimization. What are the next steps? One of the biggest challenges in GECI design is the thorny trade-off between Ca^{2+} affinity and indicator speed: either a GECI has fast decay kinetics or it has a high affinity for Ca^{2+}. The novel design of Twitch may finally allow this trade-off to be overcome. Because the Twitch binding motif is small, its structure can be solved with unprecedented resolution for both Ca^{2+}-free and Ca^{2+}-bound states. In addition, its compactness and relatively simple structure (just four α-helices) also allows the use of fast time-resolved spectroscopy to elucidate the detailed kinetics and the role of different structural elements in the binding of Ca^{2+}. This information will allow structurally informed engineering of the binding motif, which may permit binding kinetics and affinity to be manipulated independently.

Together with the linear relationship between $[Ca^{2+}]$ and fluorescence, a set of such fast-binding GECIs spanning a range of affinities would finally allow the use of GECIs for quantitative imaging of calcium, a domain currently accessible only with small-molecule calcium indicators. After nearly two decades of development, this would represent the final coming of age of GECIs, enabling them to replace synthetic indicators in virtually all experimental designs.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests

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