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Development of novel indicators and molecular systems for calcium sensing through protein engineering

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Intracellular calcium (Ca^{2+}) is involved in a plethora of cell signalling processes and physiological functions. Increases in Ca^{2+} concentration are *bona fide* biomarkers of neuronal activity, reflecting the spike count, timing, frequency, and the intensity of synaptic input. The development of genetically encoded calcium indicators (GECIs) was a significant advancement in modern neuroscience that enabled real-time visualisation of neuronal activity at single-cell resolution. These indicators leverage the conformational changes induced by calcium-binding proteins, such as calmodulin (CaM) or troponin C (TnC). Harnessing protein engineering approaches such as directed evolution yielded new GECIs with enhanced sensitivity, kinetics, and brightness. Notably, the development of calcium-based integrators, such as scFLARE (single-chain fast light- and activity-regulated expression), convert transient raises in cytosolic Ca^{2+} into a transcriptional readout rather than an optical signal. This review summarises the latest efforts in protein engineering to develop new indicators and molecular systems to sense changes in Ca^{2+} concentrations.

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Introduction

Calcium ions are ubiquitous secondary messengers that serve as key intracellular signals, and their dynamics play a key role in excitable cells, especially in neurons. They regulate diverse neuronal functions, including neurotransmitter release and synaptic plasticity. Calcium is considered a universal proxy of neuronal activity, and the development of genetically engineered indicators to monitor its fluctuations has revolutionised neuroscience.

In nature, various calcium-binding proteins exist to sense Ca^{2+} and mediate biological responses, including the highly conserved signalling protein calmodulin (CaM) and the muscle protein troponin C (TnC). The majority of CaM and TnC consist of two symmetrical globular domains, each containing a pair of EF-hand (helix-loop-helix) structural motifs, which are separated by a flexible peptide. Upon Ca^{2+} binding at the EF-hand sites, with affinities ranging from nanomolar to millimolar, these proteins undergo conformational changes, in which the interacting peptide holds an important role in facilitating structural rearrangements [1].

The calcium-induced conformational changes of CaM and TnC act as molecular switches and have inspired the design of a repertoire of indicators and molecular tools that convert Ca^{2+} transient raises into different readouts [2]. This review highlights the recent advancements in protein engineering regarding calcium sensing, and it is organized into two main sections based on the processing of the signal. The first section focuses on indicators exhibiting rapid and transient changes in their output, such as fluorescence emission depending on fluctuations in Ca^{2+} concentrations. The second section covers integrator systems, which produce accumulative readouts in a variety of responses such as biochemical tagging or gene expression upon calcium recognition.

Genetically encoded calcium indicators

Fluorescent-based genetically encoded calcium indicators

The development of advanced microscopic techniques led to the rapid adoption of calcium imaging for measuring neuronal activity [3]. Fluorescent-based genetically encoded calcium indicators (GECIs) enable real-time visualisation of neuronal activity at single-cell resolution in genetically defined neurons by displaying transient fluorescent signals in response to neuronal firing [4,5]. GECIs represented a significant

breakthrough in neuroscience and can be classified into two categories. The first category consists of a single fluorescent protein (FP) embedded with a Ca^{2+} sensing domain, known as single-FP calcium indicators. The second category, termed dual-FP indicators, comprises two FPs linked by a Ca^{2+} responsive element and operates based on the principle of Förster resonance energy transfer (FRET), where the energy emitted by the donor is transferred to an acceptor. Seminal work by Roger Y. Tsien in calcium imaging consisted of the development of the single FP-based camgaroos [6] and the FRET-based cameleons [7].

Single-FP GECIs

The most widely used single-FP GECIs are the green calcium-modulated protein (GCaMP), which consists of a circularly permutated GFP (cpGFP) flanked by a CaM domain and the M13 or RS20 calmodulin-binding peptides (CaMbp) (Figure 1a) [8]. The increase in intracellular calcium concentration induces a conformational change due to the CaM—CaMbp interaction, altering the chemical environment surrounding the chromophore and its physicochemical properties, leading to a change in fluorescence emission.

The development of GCaMP6 [9] marked a significant advancement in the field of calcium imaging in neuroscience, driven by their enhanced brightness, sensitivity, and dynamic range. Mutagenesis targeting the CaM and both cpGFP-CaM and M13-CaM interfaces resulted in the creation of the GCaMP7 series, which displayed improved properties [10]. Further studies in the CaM complexation motif led to the development of the latest GCaMP8 indicators [11], where the substitution of the RS20-CaMbp with ENOSP-CaMbp, along with additional mutagenesis at the interface sites, resulted also in enhanced kinetics and sensitivity. The GCaMP8 variants exhibited significant improvements across several parameters over previous GECIs (Table 1). This indicates that calcium-dependent interactions between the molecular surfaces of CaM and the CaMbp are crucial in determining the features of the sensor. Significant efforts have been made to expand the colour palette of single-FP-based GECIs, covering the full spectrum from near-infrared to blue, including near-infrared (NIR)-GECO1, *2*, and *2G* [12,13], R-CaMP2 [14], jYCaMP1 [15], XCaMPs [16] and the most recent blue-shifted T-GECO1, which offers spectral advantages and enhanced compatibility for multiplexing in all-optical experiments [17].

In contrast to GCaMPs, other topologies and Ca^{2+} sensing domains, such as troponin C (TnC), have been explored. This approach led to the development of the green NTnC calcium indicator with mNeonGreen as FP and TnC as calcium-sensing domain where the FP is not circularised [18]. NTnC indicator has two high-affinity calcium-binding sites per molecule, resulting in a more linear response to transient changes in Ca^{2+} concentrations. One of the most recent NTnC-based GECIs is YTnC2 [19], where mNeonGreen is replaced by an enhanced EYFP, which is an improved version of the previous YTnC indicator [20,21]. This enhancement was achieved through rounds of directed evolution to address issues such as low brightness, restricted dynamic range, and poor sensitivity to calcium transients observed in previous TnC-based indicators.

Subach et al. also introduced a novel ratiometric green calcium indicator, FNCaMP which was engineered following the NTnC design [22]. This version incorporates the mNeonGreen and the CaM-M13 CaMbp pair derived from *A. niger*, and its properties were enhanced with directed evolution [22]. While FNCaMP can be used for calcium imaging, its performance in terms of contrast, affinity, and sensitivity is inferior to FGCaMP7. Finally, NEMO indicators [23], which share the same topology as NCaMP7 [24], also integrate the CaM-M13 complex in the mNeonGreen. NEMO detects Ca^{2+} signals with rapid kinetics, high dynamic range, and reduced spectral overlapping in the blue channel compared with GCaMPs (Figure 1b), representing a great alternative.

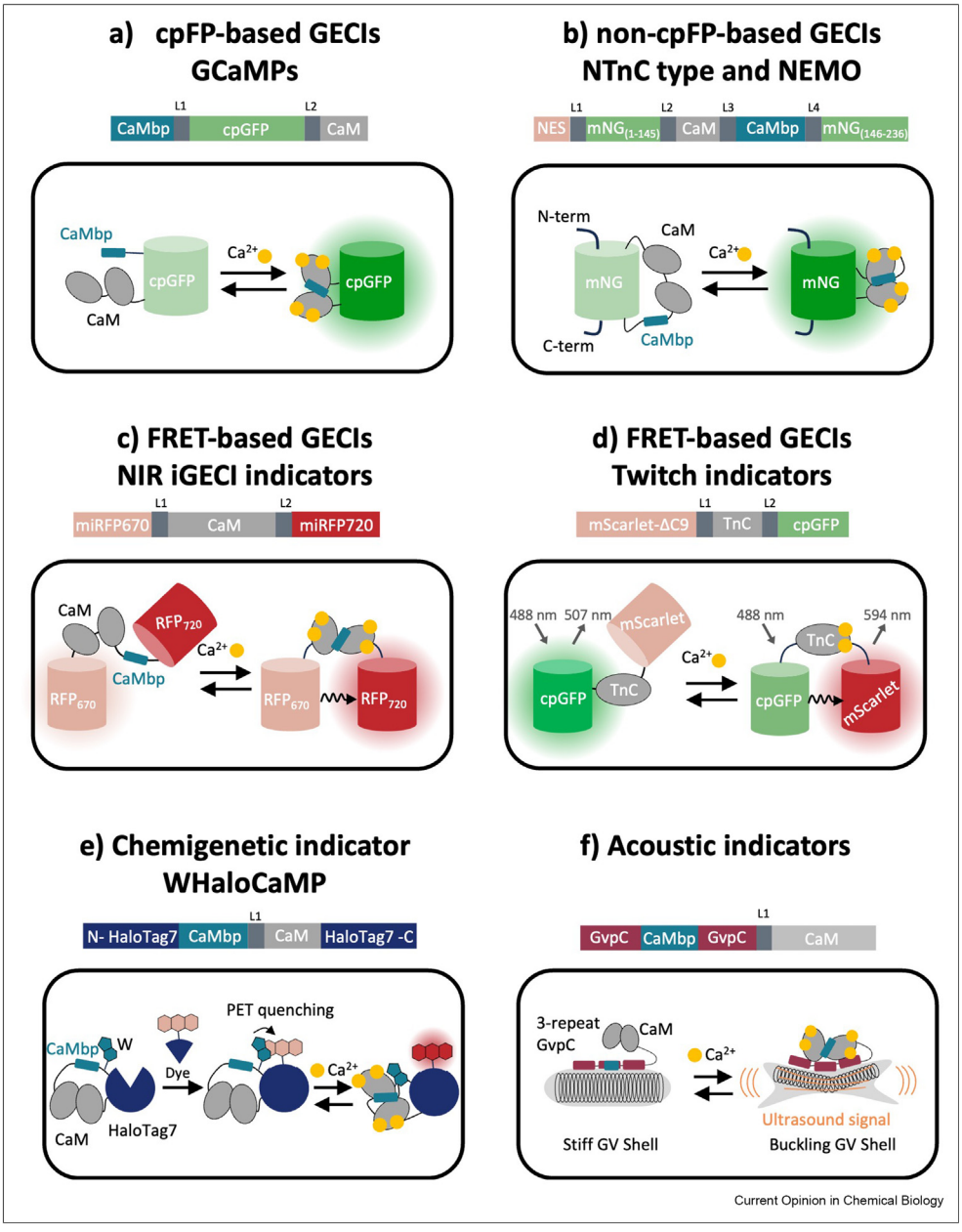
Dual-FP GECIs

The second category of indicators for visualising neuronal activity consists of FRET-based constructs that were also initially developed by Roger Tsien and coworkers. Yellow cameleons (YCs) consisted of a CaM flanked by the Cyan and Yellow FP, serving as the FRET donor and acceptor, respectively.

To address the photophysical limitations associated with cyan-yellow FRET pairs, Shemetov et al. developed iGECI [25] by incorporating two monomeric NIR FP—miRFP670 as the donor and miRFP720 as the acceptor—into the CaM-M13CaMbp (Figure 1c). Recently, iGECInano [26] has been reported; this variant is derived from the Twitch-2B indicator [27] and incorporates a minimal troponin C motif (TnCm) that does not require a binding peptide, emitting in the NIR spectrum. Its activity was enhanced by optimizing the length and composition of the linkers between the elements. This indicator is characterised by its high brightness in cellular environments, exceptional photostability, small size, and rapid response kinetics.

Following this approach, Zhang et al. reported a palette of green-red GECIs, also derived from the Twitch-2B indicator, based on the mScarlet FRET acceptor, and a circularly permuted green fluorescent protein FRET donor (Figure 1d). The resulting indicators, Twitch-GR and Twitch-NR [28], reported brighter fluorescence

Figure 1



Schematic representation of different GECIs. (a) GCaMP comprises a circularly permuted GFP (cpGFP) linked with CaM-M13 and emits a green fluorescence signal upon increases in calcium concentrations. **(b)** GECIs geometry, based on the NTnC indicator, incorporates the CaM-M13 pair into mNeonGreen (mNG), emitting a green fluorescence signal upon increases in Ca^{2+} concentration. **(c)** The structure of FRET-based GECIs, such as Twitch indicators, which utilize TnC and emit green fluorescence at baseline but shift to red fluorescence upon Ca^{2+}binding. **(d)** A schematic representation of WHaloCaMP illustrates the insertion of the Ca^{2+}sensing domain into HaloTag7, along with the tryptophan residue (W). The fluorescence change is due to the reversible quenching of the bound dye, which is facilitated by the strategic placement of the tryptophan. **(e)** A schematic of the ultrasonic calcium reporter illustrates how the structural protein GvpC, which constitutes the shell, incorporates the CaM–CaMbp complex. In the presence of calcium, a reversible conformational change is induced, weakening the structure of GvpC and increasing its flexibility, thereby generating an acoustic response. FRET, Förster resonance energy transfer.

signals in response to calcium changes than the previous indicators. Recently, Matsuda et al. developed the latest versions of the Twitch series, Twitch-GmtT and Twitch-GmRR [29], which utilise the non-circularised Gamillus FP, distinguished by its unique trans-configuration chromophore. Twitch-GmtT encodes tdTomato as the red FP, and Twitch-GmRR presents a higher quantum yield variant known as RRvT. Twitch-GmRR

Table 1

***In vitro* properties of selected GECIs. K_d, apparent equilibrium binding constant in calcium titrations; Hill coefficient, cooperativity; dynamic range, saturating fluorescence increase in calcium titrations.**

GECI	Indicator	Ca^{2+} domain	Kd (nM)	Hill coefficient	Dynamic range	Ref
GCaMP7f	cpGFP	CaM-M13	150	3.10	31.0	[10,11]
GCaMP8s	cpGFP	CaM-ENOSP	46	2.20	49.5	[11]
GCaMP8m	cpGFP	CaM-ENOSP	108	1.92	45.7	[11]
GCaMP8f	cpGFP	CaM-ENOSP	334	2.08	78.8	[11]
YTnC2	EYFP	mTnC	396	1.93	63	[17]
FNCaMP	mNeonGreen	CaM-M13	405ex– 234 488ex–477	405ex– 1.1 488ex–1.6	405ex–27 488ex–7.3	[20]
NEMOc	mNeonGreen	CaM-M13	492.8	3.4	165.3	[21]
NEMOm	mNeonGreen	CaM-M13	198.9	3.3	159.2	[21]
NEMOf	mNeonGreen	CaM-M13	440.1	2.9	141.5	[21]
iGECInano	miRFP670/miRFP720	mTnC	530	1.53	n/a	[24]
Twitch-GmRR	Gamillus-RRvT	TnC	1410	n/a	n/a	[27]
CaBLAM!	OLuc	CaM-RS20	464	3	97	[30]
WHaloCaMP	Rhodamine dyes	CaM-MLCK	JF_{494}-HTL 71 JF_{552}-HTL 87 JF_{669}-HTL 37 JF_{722}-HTL 26	JF_{494}-HTL 1.9 JF_{552}-HTL 3.1 JF_{669}-HTL 2.5 JF_{722}-HTL 2.0	JF_{494}-HTL 10 JF_{552}-HTL 4 JF_{669}-HTL 7 JF_{722}-HTL 16	[32]

CaBLAM!, Ca^{2+} bioluminescence activity monitor; GECIS, genetically encoded calcium indicators.

demonstrated superior performance in sensing dynamic range *in vitro*; however, its fluorescent protein components present certain limitations, including reversible photoswitching or maturation of the red FP moiety.

Bioluminescent-based GECIs

Aequorin is a calcium-activated photoprotein from *Aequorea victoria* that emits blue light upon the oxidation of its chemical substrate coelenterazine. The calcium-dependent emission of light has inspired the development of bioluminescent (BL) GECIs, which offer several advantages over fluorescent GECIs. Since BL GECIs do not require excitation light, they circumvent issues such as tissue autofluorescence, phototoxicity, photobleaching, and spectral overlap. One of the most relevant indicators was CaMBI [30], where the presence of Ca^{2+} restores the catalytic activity of NanoLuc fused to CaM-M13. The subsequent emission of blue photons captured by the CyOFP1 protein results in an orange fluorescence emission. Recently, bioluminescent red indicator for Ca^{2+} (BRIC) [31] implemented a brighter and red-shifted NanoLuc mutant (teLuc) fused to mScarlet-I, enabling the imaging of calcium dynamics within mice brains. This strategy to red-shift the emission enables imaging in deeper tissues by reducing the photon absorption and scattering. The most recent BL GECI, Ca^{2+} bioluminescence activity monitor (CaBLAM!) [32], demonstrates exceptionally high contrast and optimised calcium affinity, making it highly effective for imaging calcium dynamics within the cytosol of cells. CaBLAM! was evolved through error-prone PCR and site-directed mutagenesis, and the final variant displayed higher contrast and dynamic response than CaMBI and BRIC. Although BL GECIs hold significant potential, their primary limitation is the need for continuous supply and cellular uptake of luciferines.

Chemigenetic GECIs

Chemigenetic calcium indicators, such as HaloCaMP, are built on HaloTag technology and consist of a circularly permuted version of haloalkane dehalogenase fused with CaM-CKK CaMbp [33]. After covalent labelling with a fluorophore, the presence of Ca^{2+} induces a rapid conformational change that markedly alters the dye's environment, enhancing its fluorescence emission. A more recent version, known as WHaloCaMP, has been reported, in which the fluorescence signal is modulated through the reversible quenching of the dye by a tryptophan residue from the protein via photoinduced electron transfer (Figure 1e) [34]. Three rounds of directed evolution were performed, utilising single and double site saturation mutagenesis at positions near the insertion site and the dye–protein interface. rHCaMP was engineered based on a CaM-variant HaloTag where the calcium-binding alters the HaloTag7 fluorophore binding site, affecting the equilibrium between the green fluorescent spirocyclic and red fluorescent zwitterionic forms of colour-shifting fluorophores [35].

A split HaloTag indicator for Ca^{2+}-dependent protein labelling, Caprola, has been developed using the same technology [36]. Caprola relies on a silent cp-HaloTag whose activity is restored by a Rosetta-designed decapeptide (Hpep). These two components are linked via CaM-M13 CaMbp to enable calcium-

dependent protein labelling, and it was successfully used to monitor Ca^{2+} dynamics at different wavelengths in flies and zebrafish.

Acoustic indicators

Optical readouts have been the preferred option for calcium sensing, but the *in vivo* applications are limited by autofluorescence, due to tissue scattering, and low penetrability through biological samples. To overcome these limitations, Saphiro et al. have developed an innovative approach by engineering gas vesicles (GVs) to emit ultrasound contrast in response to calcium binding [37]. The structural protein GvpC is tagged with CaM–CaMbp complex and, in the presence of Ca^{2+}, undergoes an allosteric conformational change that modulates the mechanical properties of the GV increasing the ultrasound contrast (Figure 1f). Although their sensitivity and kinetics need improvement to be compared with fluorescent indicators, they have the potential to become an attractive alternative.

Integrator systems

Despite these advancements in calcium imaging, GECIs do not enable further manipulation of active neuronal ensembles, a crucial capability for establishing causal relationships between neuronal activity and behaviours. To address these limitations, integrator molecular systems have been engineered to generate an accumulative fluorescence output, biochemical tagging, or a transcriptional readout in response to increases in cytosolic calcium.

Fluorescence outputs

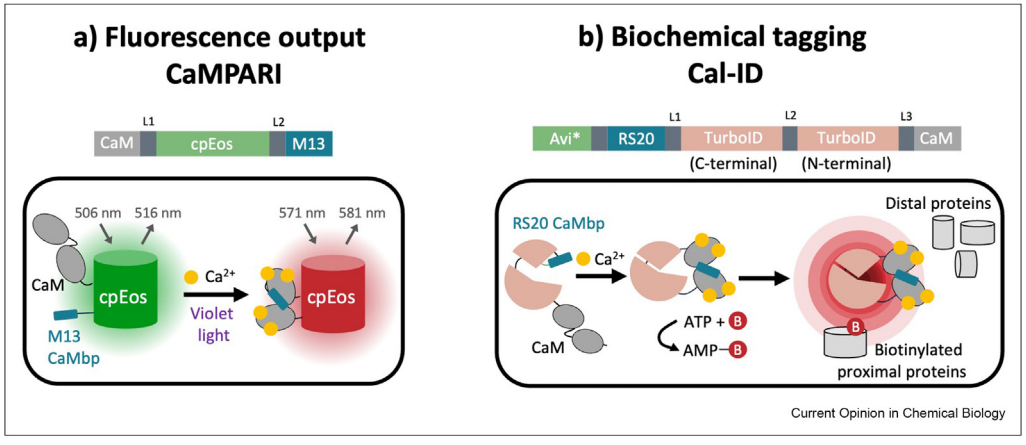
Although single-FP and dual-FP GECIs can detect neuronal activity in real-time within small fields of view, the fluorescence intensity fades away once the calcium concentration decreases, as it is not feasible to permanently label the active neurons. Fosque et al. introduced CaMPARI [38], an engineered calcium-activated photoconvertible fluorescent protein derived from EosFP, which irreversibly converts to a bright red fluorescent state upon exposure to violet light (Figure 2a). This green-to-red conversion consists of a photoinduced reaction that extends the electronic conjugation of the chromophore, and it is dependent on both light exposure and elevated calcium levels. Using site-directed mutagenesis, Moeyaert et al. engineered CaMPARI2 [39], an evolved version which features enhanced green and red fluorescence, faster calcium unbinding kinetics, and reduced photoconversion under low calcium conditions. Despite this, Dan et al. demonstrated that CAMPARI1 is more effective than CAMPARI2 for detecting neuronal activity patterns [40].

Biochemical tagging

In recent years, proximity labelling (PL) has emerged as a complementary method for mapping protein–protein interactions in living cells. PL employs engineered enzymes, such as biotin ligases, that are genetically fused to a target protein. Biotin ligase-based approaches, such as BioID and TurboID, utilise ATP and biotin to catalyse the generation of reactive biotin-5′-AMP. This reactive molecule diffuses from the enzyme and biotinylates nearby proteins, which can subsequently be identified through mass spectrometry [41].

In order to biochemically tag active neurons in response to calcium, two technologies were recently reported: Cal-ID [42] and CaST [43]. The Cal-ID design consists of a circularly permutated engineered biotin ligase

Figure 2



Schematic representation and mechanism of action of CaMPARI and Cal-ID. (a) CaMPARI comprises a calcium-activated photoconvertible fluorescent protein and EosFP, which undergoes an irreversible conversion to a bright red fluorescent state upon exposure to purple light. **(b)** Illustration of Cal-ID, a calcium-dependent proximal labelling enzyme, in which the biotinylation of proximal proteins is contingent upon the presence of calcium.

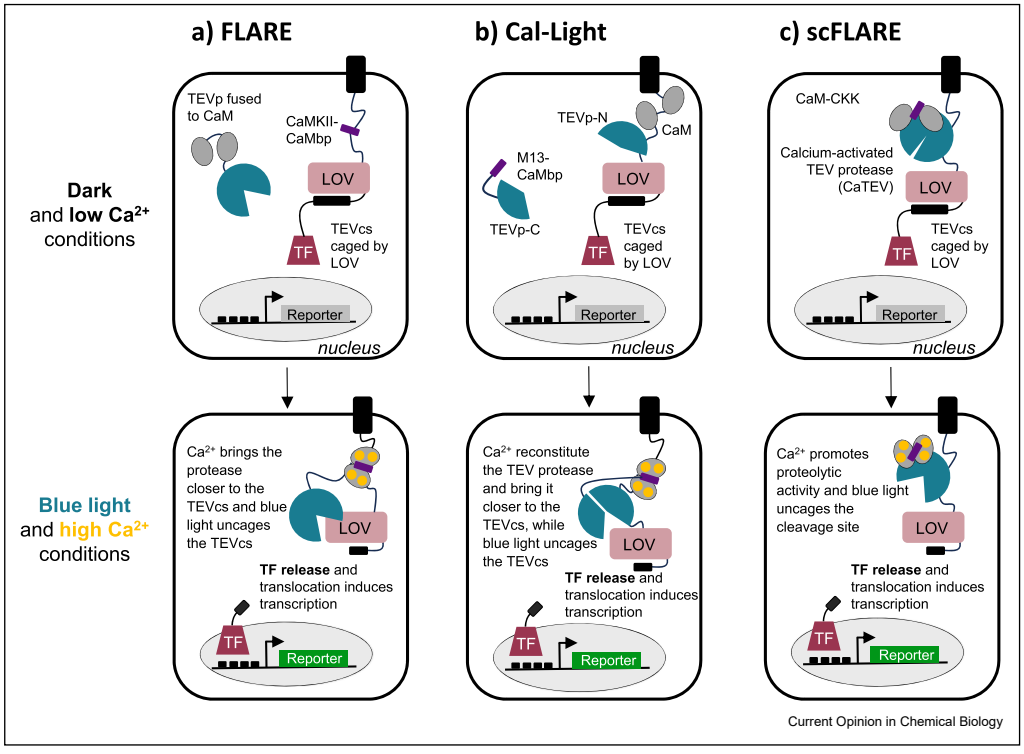
flanked by the CaM-RS20 pair (Figure 2b). In contrast, CaST relies on the reconstitution of the split biotin ligase. Both systems biotinylate nearby proteins within minutes in response to elevated local calcium levels in the brains of mice.

Transcriptional readout

Reijmers et al. combined for the first time immediate-early gene (IEG) promoters with drug-activated transcription factors (TFs) to transform neuronal activity into gene expression [44]. However, IEG activity as a marker for neuron firing has several limitations: IEGs are not present in all model organisms, their activity is dependent on the specific neuron type, and they exhibit low temporal resolution (ranging from 30 min to 6 h). To address these limitations, different molecular systems have been engineered to generate a transcriptional readout in response to increases in cytosolic calcium. One of the original designs, transcriptional reporter of intracellular calcium (TRIC) [45], harnesses the CaM-MKII interaction to reconstitute the activity of a split transcription factor. While TRIC has proven effective in flies, it lacks the capability for external control over the labelling window, limiting its applicability.

The development of activity-based systems such as fast light- and activity-regulated expression (FLARE) [46] and Cal-Light [47] enables the conversion of neuronal activity within a specific time window into a transcriptional readout (Figures 3a and 3b). Both tools use a TF tethered to the plasma membrane and a Tobacco Etch Virus protease (TEVp) fused to a calcium-sensitive domain. The TF is linked to the TEV cleavable site (TEVcs), which is sterically protected from the TEVp by an engineered light-oxygen-voltage (LOV)-sensing domain, anchoring it to the membrane. The presence of cytosolic calcium brings the protease closer to the TF complex, and irradiation with blue light changes the conformational structure of the LOV domain to an open state exposing the TEVcs to the proteolytic activity of the TEVp. This interaction releases the TF, allowing its translocation to the nucleus and driving the expression of a transgene. This reporter gene is typically a fluorescent protein fused to an opsin for dual visualisation and manipulation of the active neuronal circuit. The mechanism of action for calcium gating slightly differs between both tools: FLARE capitalises on the interaction between CaM and the MKII CaMbp to bring in proximity a low-affinity TEV protease to its TEVcs,

Figure 3



Schematic and molecular mechanisms of light- and calcium-dependent expression systems. (a) FLARE, **(b)** Cal-Light, and **(c)** scFLARE. These designs rely on a transcription factor fused to a transmembrane domain through the TEV cleavage site (TEVcs), which is caged in the dark by an engineered light-sensing domain (LOV, light-oxygen-voltage). The LOV domain acts as a molecular switch to mark off the labelling window, and upon blue light illumination, it exposes the TEVcs to proteolytic cleavage. Neuronal activity is sensed through the CaM–CaMbp complexation in the presence of elevated Ca^{2+} concentrations, leading to subsequent proteolysis. In FLARE, Ca^{2+} brings a low-affinity protease into proximity with the TEVcs, whereas in Cal-Light, it mediates the reconstitution of a split TEV protease. In contrast, in scFLARE, as the CaM-CKK pair is embedded within the TEV protease, its proteolytic activity is strictly controlled by the conformational changes triggered by Ca^{2+}. FLARE, fast light- and activity-regulated expression

whereas Cal-Light exploits the CaM-M13 interaction to reconstitute a split TEV protease.

In order to improve FLARE sensitivity, Sanchez et al. developed a directed evolution platform to increase the catalytic activity of the TEV protease (uTEV1) [48]. This mutant, combined with an improved hybrid LOV (hLOV) domain, led to the development of FLiCRE [49]. In parallel, Hyun et al. replace the membrane-anchoring tether of Cal-Light by a soma-targeting sequence (ST-Cal-Light) [50] for relocalising the construct to avoid transient calcium influx at synapses and dendrites independent of action potentials.

FLARE and Cal-Light feature multicomponent designs, conditioning their performance to the relative expression levels of each component. Inspired by calcium-dependent proteases, calpains; Sanchez et al. engineered a calcium-dependent TEVp to develop the single-chain FLARE (scFLARE) [51] (Figure 3c). In contrast to previous molecular tools, TEV proteolytic activity is dependent on intracellular calcium. This design enhances the robustness of the tool and reduces its dependency on protein expression levels. scFLARE2 has been recently used to identify the fasciola cinereum (FC) neurons of the posterior hippocampal tail as an important seizure node in both mice and humans with epilepsy [52]. Furthermore, targeted lesioning of the FC in one patient reduced the seizure burden demonstrating how this technology can transform clinical practice. scFLARE2 has also been used to determine how memories are organised and remembered in the lateral amygdala in the brain of mice in response to auditory threat training [53].

Concluding remarks and future prospects

The calcium-binding proteins CaM and TnC in combination with several target peptides have been fused to a wide repertoire of proteins and enzymes such as FPs, luciferases, ligases, dehalogenases, proteases, and even bacterial gas vesicles, to tie their spectroscopic and enzymatic activities for Ca^{2+} sensing. Calcium imaging through the engineering of GECIs, along with the development of sophisticated microscopes, has produced a deep impact on neuroscience. GCaMPs, a family of GECIs, are the most widely used indicators by researchers and have been engineered over decades, resulting in progressive improvements in brightness, sensitivity, and response kinetics. Despite this continuous effort, GECIs are still the research focus of protein engineers to encode new properties and functions. For example, red-shifted versions are currently being developed to visualise neuronal activity in deeper areas of the brain, and photoconvertible proteins such as CaMPARI have been engineered for permanent tagging of active neuronal circuits in large volumes of tissue.

While imaging neuronal activity is key in understanding neuronal function, the field demands new molecular technology to move forward. Harnessing Ca^{2+} as a universal proxy of neuronal firing, different activity-based tagging systems such as ST-Cal-Light or scFLARE2 were recently developed. These molecular tools consist of a Ca^{2+}- and light-gated TF, where the presence of both inputs is required to drive the expression of a reporter gene. These integrators enable to establish the causal correlation between neuronal activity and the behaviour of interest and have already changed clinical practice in patients suffering from epilepsy.

Protein engineering approaches utilising site-directed mutagenesis and directed evolution have the potential to significantly advance the development of molecular indicators and integrators for detecting and recording neuronal activity. For instance, the outcome of directed evolution campaigns in GCaMPs have identified that the most beneficial mutations often occur at the interfaces between proteins. These findings indicate that conformational changes between CaM−CaMbp and the target proteins are critical to the overall functionality of the system. Additionally, computational design methods utilising artificial intelligence and machine learning hold significant promise for designing these indicators. These strategies could enable the design of molecular tools with broader targeting capabilities that are beyond the reach of traditional experimental approaches. This includes activity-based systems such as FLiCRE or scFLARE2, which remain in the early stages of development to their full potential Currently, these systems exhibit effective responses primarily to acute inputs like opsin or kainate stimulation. Therefore, further development is required to improve their sensitivity and enable them to more accurately labeling of physiological behaviours and cognitive states.

Author contributions

ACC and MIS wrote the manuscript together and approved the submitted version.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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- • of special interest
- •• of outstanding interest

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