cAMP measurements with FRET-based sensors in excitable cells

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Abstract

The development of FRET (fluorescence resonance energy transfer)-based sensors for measuring cAMP has opened the door to sophisticated insights into single-cell cAMP dynamics. cAMP can be measured in distinct cell populations and even in distinct microdomains within cells. However, there is still only limited information on cAMP dynamics in excitable cells, particularly as a function of the activity of voltage-gated Ca2+ channels. A major reason for this is the pH shifts that can occur in excitable cells and their effects on fluorescent proteins.

Measuring cAMP at the single-cell level

For many years, cAMP levels were measured using metabolic labelling with radioisotopes. Although this is an effective method of measuring cAMP, it can only provide gross information from cell populations at single time points. Single-cell measurements allow subpopulations of cells to be identified and continuous spatiotemporal properties of cAMP dynamics to be acquired, e.g. oscillations. Single-cell measurements have been largely based on fluorescent sensors for cAMP that are based on all three of its downstream effectors, namely PKA (protein kinase A), Epac (exchange protein directly activated by cAMP) and CNG (cyclic nucleotide-gated) channels [1–3] (Figure 1).

The first fluorescent sensor for measuring cAMP was named FIChRi. This sensor consists of a fluorescein-labelled PKA catalytic subunit (Cα) and a rhodamine-labelled regulatory subunit (RI) [1]. In the inactive enzyme, the catalytic and regulatory subunits interact and FRET (fluorescence resonance energy transfer) can be measured. Upon binding of cAMP to PKA, the subunits dissociate and FRET is reduced. The development of FIChRi was a breakthrough in measuring cAMP; however, its use is limited by the need to microinject cells with chemically modified PKA subunits.

The first genetically encoded sensors for cAMP were also based on PKA. The regulatory and catalytic subunits were tagged, both at their C-terminus, with GFP (green fluorescent protein) variants [4]. Initially blue fluorescent protein and GFP were used, but these were later replaced with CFP (cyan fluorescent protein) and YFP (yellow fluorescent protein) [5]. PKA-based cAMP sensors require co-expression of two fluorescent proteins. Ambiguities arise if the two proteins are not expressed at roughly equal levels. Subunits from PKA sensors can also associate with non-fluorescent endogenous PKA subunits, causing the signal to decline over time, particularly if PKA is repeatedly stimulated.

A major breakthrough occurred with the introduction of single-polypeptide cAMP sensors, which were based on the structures of Epac1 and Epac2 and the HCN2 (hyperpolarization-activated CNG channel 2) [2,3,6,7]. Epac-based sensors contain either the full Epac1 sequence or an isolated cAMP-binding domain. Both types of sensor use YFP and CFP. As with the PKA-based sensor, an increase in cAMP is detected as a decrease in FRET. The sensor with highest sensitivity to cAMP is based on the second cAMP-binding domain of Epac2 (Epac2-camps, where camps is cAMP sensor) and has an EC50 for cAMP of 0.92 μM [2]. Sensors based on the Epac1 cAMP-binding domain (Epac1-camps) were slightly less sensitive (EC50 = 2.5 μM) [2] and those based on the full-length Epac1 [CFP–Epac–YFP and ICUE (indicator of cAMP using Epac), which contains Citrine rather than EYFP (enhanced YFP) (see below)] were approximately 20-fold less sensitive to cAMP (EC50 = 50 μM) [6,7]. Sensors based on full-length Epac1 are localized in the cytosol and at membranes. Removal of the N-terminal DEP domain [CFP–Epac(ΔDEP)–YFP] prevents membrane localization and slightly increases sensitivity to cAMP [6]. A disadvantage of using sensors based on full-length enzymes (either PKA or Epac1) is the potentially disruptive effects of the enzyme’s catalytic activity on cell signalling. Epac1’s GEF (guanine-nucleotide-exchange factor) activity can be abolished by the introduction of two point mutations in its catalytic domain, removing this potential problem [6].

Most recently, a single peptide sensor has been developed based upon a cytosolic cAMP-binding domain from HCN2 [3]. This domain was fused between YFP and CFP to produce HCN2-camps. The EC50 for this sensor is 5.9 μM, higher than for the sensors based on single cAMP-binding domains from Epac1 and Epac2, but lower than for the sensors based
cAMP sensors can be based upon the structure of PKA or the cyclic-nucleotide-binding domain (CNBD) of Epac1, Epac2 or HCN2. Sensors can be targeted to subcellular locations using targeting sequences or by fusion to proteins of interest. Upon production of cAMP by ACs, the donor and acceptor fluorophores move further apart and there is a decrease in FRET.

Figure 1 | Schematic diagram of FRET-based cAMP sensors

The chromophore of GFP-based fluorophores is formed from three amino acids. In the original GFP, these are Ser⁶⁵, Tyr⁶⁶ and Gly⁶⁷ [18]. The chromophore can exist in neutral or anionic states, which have different absorption spectra. The neutral chromophore absorbs in the UV range, whereas the absorption peak of the anionic form is at 475 nm. The emission peak for both forms of the chromophore is between 500 and 510 nm. Two mutations, F64L and S65T, were introduced to the original GFP sequence to produce the widely used EGFP (enhanced GFP) [19]. These mutations favour the anionic form of the chromophore. The most popular FRET-based sensors for cAMP use the

Effects of pH on GFP-based fluorophores

The authors
GFP variant CFP as the donor and YFP as the acceptor fluorophore. To create CFPs, Tyr66 from the chromophore was mutated to tryptophan. This introduces an indole into the chromophore [20]. YFPs were produced by introducing the T203Y mutation into GFP [21]. The red-shifted excitation and emission wavelengths are due to π-electron stacking interactions between the substituted tyrosine residue and the phenol group of the chromophore. The most commonly used versions of CFPs and YFPs are ECFP (enhanced CFP) and EFYFP. These proteins contain additional mutations that stabilize the chromophore and enhance brightness and solubility [20,21].

Changes in pH can affect the fluorescence of fluorescent proteins by shifting the equilibrium between the two states of the chromophore. EFYFP is reported to have a $pK_a$ of between 6.5 and 7.5, indicating that its fluorescence is particularly sensitive to changes in pH within the physiological range [22,23]. The structure of YFP indicates that the chromophore is less protected from the surrounding buffer than in EGFP and is therefore more likely to exist in the neutral form in low pH. When the chromophore is in this form it is no longer excited by visible light, and so a decrease in pH leads to a quenching of YFP fluorescence [22]. In the context of a FRET-based sensor, this decrease in acceptor fluorescence can cause artefacts, as it mimics a decrease in FRET. ECFP has a much lower $pK_a$ than that of YFP ($pK_a = 4.7$) [23] and its chromophore does not contain a phenol group; however, its fluorescence still varies within the physiological pH range [24]. CFP fluorescence also decreases in the presence of millimolar concentrations of ATP, but not ADP [25]. This effect is thought to be due to an interaction between the negatively charged phosphate groups and a histidine residue on the surface of CFP.

**The value of using ‘dead’ sensors**

One way of illustrating the presence of artefacts in data caused by the quenching of fluorescence is to use a ‘dead’ sensor as a control. A dead sensor is one which does not respond to the molecule being measured. In the case of measuring cAMP using Epac-based sensors, this can be done by mutating the key arginine residue in the cAMP binding domain to glutamic acid so that cAMP no longer binds to the protein [26]. Any apparent changes in the fluorescence must now be due to changes in the environment rather than changes in cAMP levels. Although dead sensors can highlight the presence of an artefact, their use to adjust for artefacts is not ideal.

**Options for decreasing pH-sensitivity**

**Improved yellow fluorophores**

In the last decade, new yellow fluorophores have been developed with improved properties for FRET, including higher quantum yields and molar absorption coefficients, as well as reduced pH-sensitivity (Table 1). Citrine was produced by error-prone PCR and contains a glutamine-to-methionine mutation at position 69 [27]. This mutation changes the environment around the chromophore, favouring the anionic form and decreasing the $pK_a$ to 5.7. Citrine is more photostable than YFP and folds better at 37°C, making it more suitable for both FRET and other types of microscopy. Incorporation of Citrine as an acceptor fluorophore is reported to decrease the sensitivity of a Ca$^{2+}$ sensor to low pH [27].

The yellow fluorophore Venus contains several mutations that were introduced to improve folding and maturation at 37°C (EYFP/F46L/F64L/M153T/V163A/S175G) [28]. These mutations also have the effect of reducing the $pK_a$ to 6.0. Venus has been used as an acceptor for CFP in FRET sensors and is often used in one of its circularly permuted forms [29,30]. These are not reported to alter pH-sensitivity further, but allow the angle of the chromophore to be altered, potentially increasing FRET efficiency or the dynamic range of the sensor.

**Improved cyan fluorophores**

Cerulean was produced by targeted mutagenesis of ECFP, resulting in ECFP/S72A/Y145A/H148D [31]. This fluorescent protein is 2.5-fold brighter than ECFP and has increased photostability. However, the mutations introduced to produce Cerulean did not alter the $pK_a$ (Table 1). Further optimization of Cerulean has led to the development of mCerulean2 and mCerulean3 [32]. Introducing random mutations into the β-barrel and searching for the brightest proteins resulted in mCerulean2, which contains the S147H/D148G/K166G/I167L/R168N/H169C mutations and is 30% brighter than Cerulean. mCerulean3 was produced by converting Thr65 back into serine, the amino acid in the original GFP. This increases both the brightness and the quantum yield of mCerulean3 and also decreases its sensitivity to changes in pH, reducing the $pK_a$ to 3.2. This is the lowest $pK_a$ measured for a CFP and could make mCerulean3 particularly useful for situations in which a pH shift is expected. mCerulean3 was shown to be successful as a FRET donor for Venus [32]. Another recently developed CFP, mTurquoise, is also brighter in vivo and has a slightly lower $pK_a$ than that of Cerulean, but it is not as bright as mCerulean3, nor is its $pK_a$ as low [32,33].

**Teal fluorescent protein as an alternative to CFP**

mTFP1 (monomeric teal fluorescent protein 1) was developed, by directed evolution, from cFP484, a tetrameric CFP from Clavularia coral [24]. A synthetic gene library was initially produced to introduce variety and the brightest fluorescent proteins were identified. The brightest fluorescent protein in this library was a dimer with peak emission at 486 nm which was named teal fluorescent protein. Directed evolution and random mutagenesis were used in the search for a monomer with bright fluorescence and high photostability. The resulting mTFP1 has an excitation peak at 463 nm and an emission peak at 490 nm. These peaks are red-shifted compared with CFPs. mTFP1 was tested as a FRET donor for Citrine in a Ca$^{2+}$ sensor [24]. This pair shows a higher FRET...
efficiency than CFP/Citrine, although there was a decrease in the dynamic range. mTFP1 has a lower $\kappa_\text{a}$ than ECFP (4.3 compared with 4.7) making a Citrine/mTFP1 FRET pair potentially useful for cAMP measurements in excitable cells.

The usefulness of different FRET pairs has been tested in the context of an Epac1-based sensor lacking the N-terminal DEP domain [29]. In this study, a combination of a slightly C-terminally truncated ECFP and circularly permuted Venus DEP domain [29]. In this study, a combination of a slightly C-terminally truncated ECFP and circularly permuted Venus DEP domain 

## Table 1 | Properties of CFPs and YFPs

<table>
<thead>
<tr>
<th>Fluorescent protein</th>
<th>Excitation maximum (nm)</th>
<th>Emission maximum (nm)</th>
<th>$\varepsilon$ ($\text{M}^{-1} \cdot \text{cm}^{-1}$)</th>
<th>Quantum yield</th>
<th>Brightness</th>
<th>$\kappa_\text{a}$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECFP</td>
<td>434</td>
<td>477</td>
<td>26 000</td>
<td>0.40</td>
<td>10</td>
<td>4.7</td>
<td>[23]</td>
</tr>
<tr>
<td>Cerulean</td>
<td>433</td>
<td>475</td>
<td>43 000</td>
<td>0.62</td>
<td>27</td>
<td>4.7</td>
<td>[31]</td>
</tr>
<tr>
<td>mCerulean3</td>
<td>433</td>
<td>475</td>
<td>40 000</td>
<td>0.87</td>
<td>35</td>
<td>3.2</td>
<td>[32]</td>
</tr>
<tr>
<td>mTurquoise</td>
<td>434</td>
<td>474</td>
<td>30 000</td>
<td>0.84</td>
<td>25</td>
<td>4.5</td>
<td>[33]</td>
</tr>
<tr>
<td>mTFP1</td>
<td>462</td>
<td>492</td>
<td>64 000</td>
<td>0.85</td>
<td>54</td>
<td>4.3</td>
<td>[24]</td>
</tr>
<tr>
<td>EYFP</td>
<td>514</td>
<td>527</td>
<td>84 000</td>
<td>0.61</td>
<td>51</td>
<td>6.5</td>
<td>[23]</td>
</tr>
<tr>
<td>Citrine</td>
<td>516</td>
<td>529</td>
<td>77 000</td>
<td>0.76</td>
<td>59</td>
<td>5.7</td>
<td>[27]</td>
</tr>
<tr>
<td>Venus</td>
<td>515</td>
<td>528</td>
<td>92 000</td>
<td>0.57</td>
<td>52</td>
<td>6.0</td>
<td>[28]</td>
</tr>
</tbody>
</table>

## Conclusions

The development of new fluorophores with decreased pH-sensitivity provides options for development of a cAMP sensor that overcomes the difficulties presented by depolarization-induced decreases in pH. The ability to study cAMP levels in excitable cells offers opportunities for breakthroughs in the study of important physiological processes known to involve ACs, including synaptic plasticity, neurotransmitter release and memory formation [8,35].

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## References


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