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Review

New red-fluorescent calcium indicators for optogenetics, photoactivation and multi-color imaging☆

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ABSTRACT

Most chemical and, with only a few exceptions, all genetically encoded fluorimetric calcium (Ca^{2+}) indicators (GECIs) emit green fluorescence. Many of these probes are compatible with red-emitting cell- or organelle markers. But the bulk of available fluorescent-protein constructs and transgenic animals incorporate green or yellow fluorescent protein (GFP and YFP respectively). This is, in part, not only heritage from the tendency to aggregate of early-generation red-emitting FPs, and due to their complicated photochemistry, but also resulting from the compatibility of green-fluorescent probes with standard instrumentation readily available in most laboratories and core imaging facilities. Photochemical constraints like limited water solubility and low quantum yield have contributed to the relative paucity of red-emitting Ca^{2+} probes compared to their green counterparts, too. The increasing use of GFP and GFP-based functional reporters, together with recent developments in optogenetics, photostimulation and super-resolution microscopies, has intensified the quest for red-emitting Ca^{2+} probes. In response to this demand more red-emitting chemical and FP-based Ca^{2+}-sensitive indicators have been developed since 2009 than in the thirty years before.

In this topical review, we survey the physicochemical properties of these red-emitting Ca^{2+} probes and discuss their utility for biological Ca^{2+} imaging. Using the spectral separability index X_{ijk} (Oheim M., 2010. Methods in Molecular Biology 591: 3–16) we evaluate their performance for multi-color excitation/emission experiments, involving the identification of morphological landmarks with GFP/YFP and detecting Ca^{2+}-dependent fluorescence in the red spectral band. We also establish a catalog of criteria for evaluating Ca^{2+} indicators that ideally should be made available for each probe. This article is part of a Special Issue entitled: Calcium signaling in health and disease. Guest Editors: Geert Bultynck, Jacques Haiech, Claus W. Heizmann, Joachim Krebs, and Marc Moreau.

1. Small-molecule chemical Ca^{2+} indicators

Small-molecule fluorescent indicators for Ca^{2+} were first introduced by Tsien and colleagues in the mid-eighties [1] and there is a broad choice among fluorescent probes for measuring the free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) [1–8]. Many of these chemical Ca^{2+} indicators exist in two forms: as membrane-impermeant salts for microinjection or loading *via* a patch pipette and as cell-permeant acetoxymethyl (AM) esters [9] allowing their non-disruptive loading into many cells at the same time. For the same indicator, the Ca^{2+}-dependent fluorescence signal can depend strongly on the method of dye-loading [10].

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According to their photochemistry, fluorimetric Ca^{2+} indicators fall into two categories, dual-excitation or -emission wavelength indicators that allow calibrated $[Ca^{2+}]_i$ measurements through excitation or emission ratiometry, respectively [11–13], and intensometric dyes for which the molar extinction ε and/or fluorescence quantum yield Φ_F varies as a function of Ca^{2+} binding. These non-ratiometric single-wavelength Ca^{2+} indicators report relative fluorescence changes (F/F_0 or $\Delta F/F_0$) only and constitute the largest group. The advantages and disadvantages of each group have been discussed. It is safe to say that precise measurements of basal cytoplasmic $[Ca^{2+}]_i$ values around 100 nM are at best difficult [14–16], and whereas earlier studies often sought to determine absolute $[Ca^{2+}]_i$ levels, the more recent literature is dominated by $\Delta F/F_0$ (and, for some applications, dual-color green (G)/red (R) pseudoratiometric ΔG/R measurements with a red-emitting and Ca^{2+}-insensitive reference dye) measurements of Ca^{2+}-dependent fluorescence transients.

1.1. Working principle of chemical fluorescent Ca^{2+} indicators

Chemical Ca^{2+} indicators consist of a fluorophore moiety determining their photophysical properties and an ionophore moiety complexing Ca^{2+}. Many, but not all indicators have a molecular linker between these two parts (see below). Ca^{2+} binding modifies the fluorescence of the construct. Ca^{2+} sensing is generally based on one of two photophysical principles: photoinduced electron transfer (PET) or photoinduced charge transfer (PCT) [17].

The vast majority of Ca^{2+} indicators are PET-type sensors [18]. PET sensors combine an electron-donating group (D), which is part of the Ca^{2+} chelating moiety (an aniline in the case of BAPTA) that connects to the conjugated aromatic system of the fluorescent dye through a bridge. The donor group quenches fluorescence due to the electron transfer from its highest occupied molecular orbital (HOMO) to the 'half-filled' HOMO of the excited fluorophore, which has a lower energy. In the absence of Ca^{2+}, the HOMO is mostly localized on the lone pair of a heteroatom. Upon Ca^{2+} coordination the sensor becomes fluorescent, because the Ca^{2+} ion, acting as a Lewis acid, binds by way of the lone electron pair of the donor. As a consequence, the energy of the HOMO of the donor decreases and electron-transfer becomes impossible, Fig. 1A. For PET sensors, this fluorescence increase is often high enough to directly quantitate the concentration of free Ca^{2+} ion ($[Ca^{2+}]$),

$$\left[Ca^{2+}\right] = K_D\left(Ca^{2+}\right) \cdot (F - F_{min})/(F_{max} - F), \quad (1)$$

for which it is necessary to measure the fluorescence F and know the indicator's intracellular affinity for Ca^{2+} binding, the fluorescence intensity of the indicator solution in the absence of Ca^{2+} (F_{min}) and the fluorescence intensity of the same solution under Ca^{2+}-saturating conditions (F_{max}). For the $[Ca^{2+}]_i$ to be precise, F, F_{min}, F_{max} and the $K_D(Ca^{2+})$ must be determined at the same dye concentration, ionic strength [19,20], temperature [21] optical path-length and instrument gain and sensitivity, which is not warranted in cells. It is common to report the $K_D(Ca^{2+})$ and dynamic range ($DR = F_{max}/F_{min}$)[5] determined *in vitro* (*i.e.*, in cuvette measurements) using standard conditions (22 °C, 100 KCl, 30 MOPS, pH 7.2).

The second principle — PCT — applies to sensors in which the Ca^{2+}-binding moiety is incorporated into the fluorescent dye so that the Ca^{2+}-sensitive electron-donor (or electron-acceptor) group is conjugated with another electron-acceptor (donor) *via* an aromatic system. Upon excitation, a full charge transfer of the donor to the acceptor occurs. The excited state becomes more polar than the ground state. Therefore, interaction of Ca^{2+} with the acceptor decreases the energy of both the excited and ground states, Fig. 1B. This decrease is more pronounced for the excited than for the ground state. As a consequence, a red shift in the absorption and fluorescence spectra of the Ca^{2+}-bound complex is observed (compared with the Ca^{2+}-free form) and often the molar extinction ε increases, too. Ca^{2+} coordination with the donor moiety produces the inverse effect. Changes of fluorescence quantum yields (Φ_F) and excited-state lifetime (τ) are often associated. The calculation of $[Ca^{2+}]_i$ for PCT sensors is facilitated by compounds having large red- or blue-shifts of the emission or absorption bands. The Ca^{2+} concentration then can be estimated ratiometrically using two different excitation or emission wavelengths; $R = F(\lambda_1)/F(\lambda_2)$ is measured and the ratios for the Ca^{2+}-free and Ca^{2+}-saturated indicator (R_{min}, R_{max}) as well as the $K_D(Ca^{2+})$ must be known,

$$\left[Ca^{2+}\right] = (R - R_{min})/(R_{max} - R) \times F_{min}(\lambda_2)/F_{max}(\lambda_2). \quad (2)$$

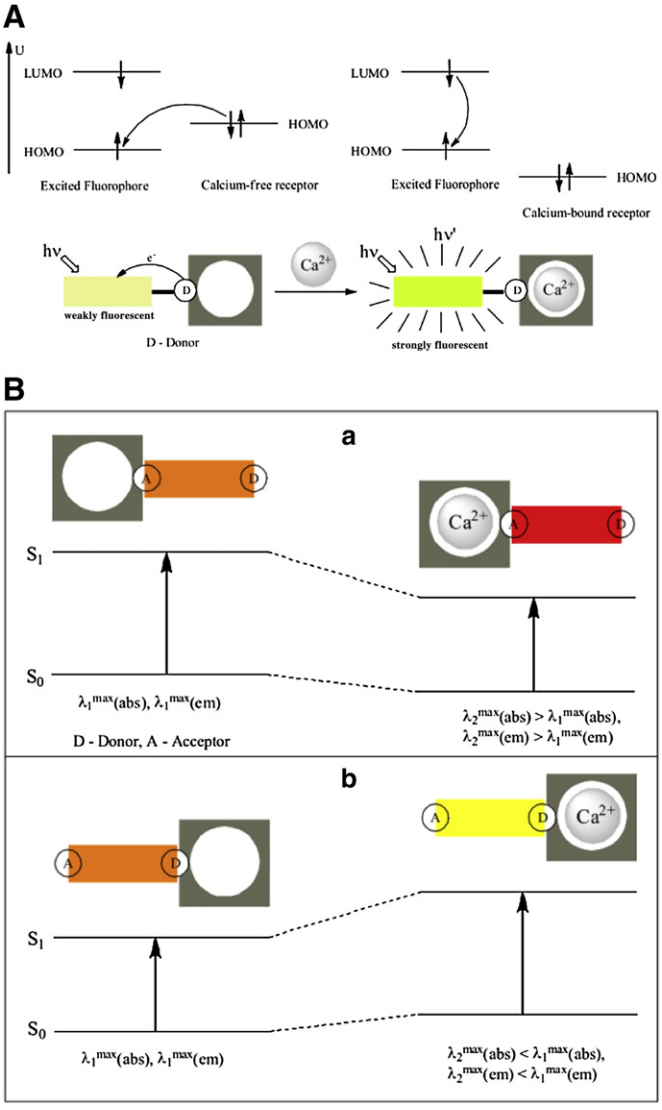

Fig. 1. Ca^{2+} binding modulates indicator fluorescence. (A) Functional principles of PET-based Ca^{2+} sensing. See main text for details. (B) Energy changes of the ground and excited states and the transitions ($S_0 \rightarrow S_1$) governing the spectral bands of the PCT-based Ca^{2+} sensors: (a) the coordination moiety (shown in gray) pulls the Ca^{2+} towards the acceptor group, producing a red-shift in the spectral emission; (b) opposite case, when the coordination moiety pulls the Ca^{2+} ion towards the donor group producing a blue-shift.
Adapted from 'Molecular Fluorescence' (B. Valeur, *ed.*), Wiley-VCH, Weinheim, 2002, see also Yan [36].

[5] The DR as defined here denotes the maximal theoretically attainable dynamic range *in vitro*. The maximal signal expected in a practical experiment (which confusingly is often referred to as the DR as well) will be reduced by modifications of the indicator properties by the local chemical environment inside the cell (this is the DR *in situ*) but also be limited by the baseline fluorescence $F_0 \geq F_{min}$ and the signal amplitude $\Delta F \leq F_{max}$.