Fluorogenic and Genetically-targeted Red-Emitting Molecular Calcium Indicator

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ABSTRACT: We introduce a strategy for the fluorogenic and genetic targeting of a calcium sensor by combining a protein fluorogen with the BAPTA sensing group. The resulting dual-input probe acts like a fluorescent AND logic gate with a Ca^{2+}-sensitive red emission that is activated only upon reaction with the protein self-labeling tag HaloTag with a 18-fold intensity enhancement. The fluorogenic targeting of the calcium probe was evidenced by the selective wash-free imaging of calcium in the nucleus of Hela cells expressing a nuclear HaloTag protein.

To decipher biological processes of increasing complexity using fluorescence imaging, the development of "smart" probes able to report on biological analytes or processes with good selectivity and appropriate spatial and temporal resolutions is crucial. Ca^{2+} imaging is used extensively by biologists as an indirect measure of the electrical activity of cells to follow neuronal communication. It is a complex endeavor since it aims at recording localized and transient concentration spikes and is a typical example where sophisticated probes are required.[1] Calcium probes essentially fall into two categories: (i) Genetically-encoded calcium indicators (GECIs) based on fluorescent proteins[2] and (ii) molecular fluorescent calcium probes.[3–5] Currently, the field is dominated by GECIs, owing to the ability to selectively express them in a chosen cell type or sub-cellular compartment.[2]

Yet, despite a constantly evolving palette, GECIs lack the diversity of available organic fluorophores and they have slower binding kinetics than chemical sensors resulting in lower temporal resolution. The genetic targeting of small chemical indicators using protein self-labeling tags has emerged as a promising strategy to combine the selectivity of GECIs with the diversity of molecular probes.[6–8] The most commonly used tags, SNAP-tag and HaloTag, have for instance been used to control the subcellular localization of Ca^{2+} or Zn^{2+} probes.[9–11] However, even with an efficient targeting reaction, one has to get rid of the unbound probes that may yield an unspecific signal. Metal cations sensors often involve cell-impermeant carboxylate chelating groups masked as ester functions and the excess of dye cannot always be easily washed away after esterase hydrolysis in cells. One way to circumvent this issue is to use fluorogenic targeting where the fluorescence is only activated upon binding to a target protein.[12,13]

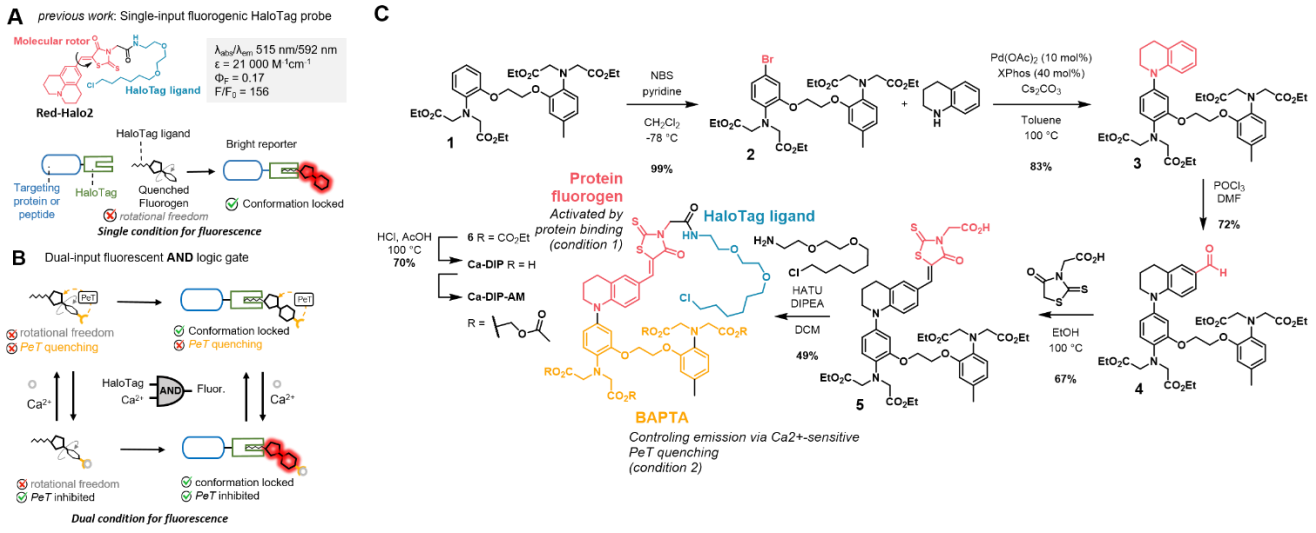


Figure 1. Design of the dual-input calcium probe Ca-DIP. (A) previously reported fluorogenic HaloTag probe Red-Halo2 and general principle of fluorogenic protein targeting. (B) General design principle of the fluorescent AND logic gate dually activated by HaloTag and Ca^{2+}. (C) Synthesis of Ca-DIP.

Considerable effort has been put in the development of single-input fluorogenic reporters for high-contrast protein imaging (Figure 1A).[14–16] Multi-input fluorogenic probes combining several sensing groups for various analytes such as pH and metal cations have also attracted a lot of interest as complex imaging probes. They are sometimes described as fluorescent AND logic gate where the analytes are the inputs and the emission is the output signal. The binding to a protein is however rarely used as one of the fluorescence-activating mechanism in multi-input probes despite its high potential for the targeting of chemical probes. The fluorogenic protein targeting of a functional chemical probe indeed makes for a difficult molecular design, since it requires the combination of two fluorescence activating mechanisms (fluorogenic protein reaction AND analyte sensing ability, Figure 1B) that may work well independently but lose efficiency when nested within the same molecule. Lavis and coworkers coupled a HaloTag-targeted Si-rhodamine probe with the calcium chelator BAPTA.[17] Si-Rhodamines are fluorogenic probes with a polarity-sensitive spirocyclization that switches on the fluorescence upon binding to a hydrophobic protein (*e.g.* HaloTag).[14,16,18] Once coupled to BAPTA, it afforded an excellent far-red targetable calcium indicator, but the probe lost most of its fluorogenic character with only a 2.4-fold increase in intensity upon reaction with HaloTag. To build-up hybrid chemogenetic indicators, researchers have thus so far resorted to alternative strategies where the analyte detection is achieved via the protein moiety[19,20] or using a two-step labeling reaction involving a fluorogenic tetrazine ligation[21] but the combination of fluorogenic protein targeting and analyte sensing within the same molecular probe has only rarely been achieved with satisfying results.[22]

In this work, we have set out to combine a fluorogenic fluorogenic molecular rotor scaffold with the calcium sensing group BAPTA to build the hybrid chemogenetic fluorescent AND logic gate **Ca-DIP** (**Ca**lcium **D**ual-**I**nput **P**robe) with a dual-input emission in the red range activated by reaction with the protein self-labeling tag HaloTag and by binding to Ca^{2+} cations (Figure 1B).[23] The photophysical properties of the probe were studied *in vitro* and it was then applied in wash-free imaging of histamine-induced nuclear calcium fluxes in Hela cells expressing a soluble HaloTag or HaloTag-NLS protein.

Design and synthesis

The structure of **Ca-DIP** is based on **Red-Halo2**, a fluorogenic HaloTag probe that we recently developed (Figure 1A).[15] It is based on dipolar molecular rotor structures with a viscosity-sensitive emission that can be activated in a hydrophobic protein pocket. Upon reaction with HaloTag, its broad orange-red emission is enhanced 156-fold. The polarity and viscosity-sensitive fluorescence emission is an inherent feature of a wide variety of flexible dipolar structures,[24] which made us confident that we could make large structural modifications to transform **Red-Halo2** into a chemical sensor without losing the protein fluorogenicity. To obtain a dual-input probe with calcium sensitivity (**Ca-DIP**), the structure of **Red-Halo2** was redesigned to incorporate a BAPTA moiety (Scheme 1). BAPTA is the common calcium chelator developed by Tsien on which the vast majority of molecular calcium probes such as Fura-2 or Oregon green BAPTA is built.[3–5,25] When non-electronically coupled to a fluorophore, it quenches the fluorescence emission by photoinduced electron transfer (PeT). Calcium binding inhibits the PeT process and thus restores the fluorescence, creating an on/off switch actuated by Ca^{2+} cations. **Red-Halo2** was built on a julolidine electron-rich group, since the locked aniline derivative inhibits the formation of a twisted intramolecular charge transfer (TICT) which results in higher brightness.[15] Since the efficiency of PeT is directly related to the distance between the electron donor and acceptor groups, we seeked to position the BAPTA moiety in close proximity to the **Red-Halo2** scaffold. Replacing the julolidine of **Red-Halo2** by a tetrahydroquinoline had the benefit to maintain the inhibition of TICT while offering a reactive amine function to incorporate the BAPTA moiety.

The synthesis of Ca-DIP is described in Figure 1C. The key step in the synthetic pathway was the Buchwald coupling with the poorly reactive electron-rich aromatic halide. We identified suitable conditions using palladium diacetate and X-Phos to afford the desirable intermediate **3** in good yield (83 %). It is also worth noting that ester hydrolysis in the final step to obtain **Ca-DIP** was performed in acidic conditions since common alkaline saponification conditions resulted in the retro-Knoevenagel reaction and could not be used.

In vitro characterization of the dual-input fluorescence properties

The optical properties of **Ca-DIP** were first characterized *in vitro* to study its fluorescent response to HaloTag and calcium. **Ca-DIP** free in Ca^{2+}-free buffer displays a broad absorption band centered on 509 nm with an absorptivity of 33 000 $M^{-1}{\cdot}cm^{-1}$ and a very weak fluorescence emission (Figure 2A&B and Table S1 for detailed photophysical properties). The addition of calcium to the free or HaloTag bound probe induces a significant hypochromicity, but only a small hypsochromic shift of the absorption wavelength confirming that the BAPTA is only weakly conjugated to the fluorophore, which is a condition for an efficient PeT process. The addition of calcium is expected to inhibit the quenching due to PeT but, thanks to the dual activation, Ca^{2+} alone induces only a minute increase of fluorescence intensity. On the other hand, **Ca-DIP** becomes fluorescent upon reaction with HaloTag with a 18-fold increase in intensity and exhibits a Ca^{2+}-sensitive emission (Figure 2C&D).

In agreement with the rational design, the fluorescence of **Ca-DIP** is thus simultaneously controlled by two external inputs and it operates a fluorescent AND logic gate able to sense calcium only when bound to the HaloTag protein (Figure 2D). The calcium sensing ability was further studied by performing a calcium titration of **Ca-DIP** bound to HaloTag. **Ca-DIP** displays a 4.5-fold increase of fluorescence intensity from 0 μM to 39 μM Ca^{2+} and a dissociation constant K_d of 150 nm (Figure 2C). This affinity ranks it among high affinity calcium indicators and is due to the substitution of the BAPTA with an electron-rich amine. The same substitution pattern and affinity range can be observed in the popular calcium probe Oregon green BAPTA-1.[1]

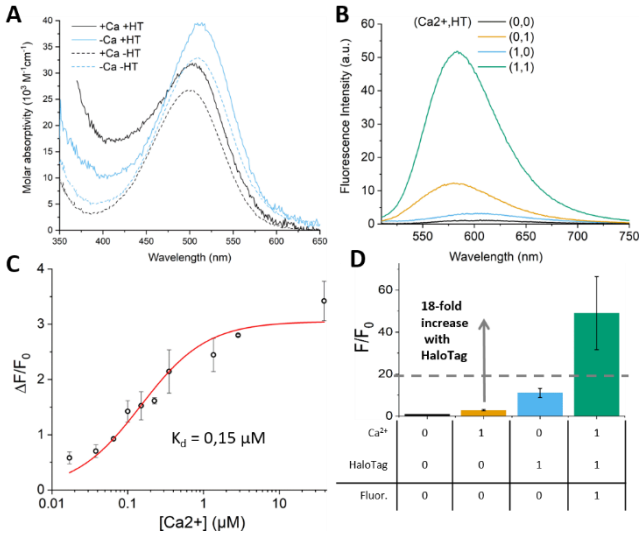
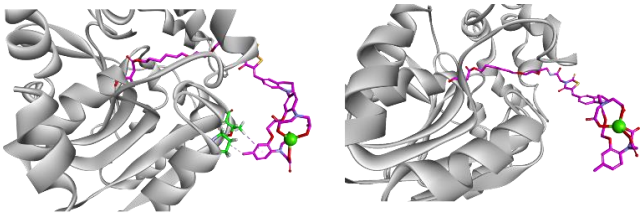


Figure 2. in vitro characterization of Ca-DIP. Absorption (A) and emission (B) spectra of Ca-DIP in the presence (39 µM) or absence (0 µM) of Ca^{2+} in the free and HaloTag bound form. (C) Calcium titration of HaloTag bound Ca-DIP from 0 to 39 µM Ca^{2+}. Signal expressed as $\Delta F/F_0$ where F_0 is the initial fluorescence at 0 µM Ca^{2+} (average of four measurements). To calculate the dissociation constant, the titration was fitted to a hill function (n = 1) shown in red. (D) Logic gate operation and truth table of Ca-DIP as a function of Ca^{2+} and HaloTag. Integrated intensities F normalized to the intensity F_0 of the free probe in the absence of Ca^{2+} and HaloTag (average of two measurements). Input states 0 and 1 correspond to 0 µM Ca^{2+} and 39 µM Ca^{2+} or to the free and HaloTag-bound probe respectively. An arbitrary intensity limit (dashed line) can be set to distinguish between 0 (low) and 1 (high) output.

Despite these very promising properties, the fluorescence brightness of **Ca-DIP** upon reaction with HaloTag and in high calcium conditions is markedly lower than that of the parent compound **Red-Halo2** (Table S1). To try to understand this drop in intensity, we studied the binding of **Ca-DIP** to HaloTag by molecular dynamics. **Ca-DIP** complex with Ca^{2+} was covalently attached to the HaloTag crystal structure and we performed a 2 ns dynamic with a 10 ps step, generating 200 conformations. A cluster analysis underlined the presence of two main structures accounting for 74 % and 26 % of the conformations (Figure 3). In the minor cluster, **Ca-DIP** does not interact with the protein and adopts a planar conformation suitable for fluorescence emission. In the major cluster, the methyl group of BAPTA is found to interact with a hydrophobic pocket formed by Proline 259, Threonine 252 and Alanine 260 on the surface of HaloTag. These hydrophobic interactions successfully minimize the potential energy of the complex but it twists the molecule in a non-planar conformation. Such a poorly conjugated structure is not favorable for fluorescence and may explain the limited brightness of **Ca-DIP** compared to the parent compound **Red-Halo2**.



Cluster 1: 74 % of conformations
E_{pot} = -223 791 kJ.mol^{-1}

Cluster 2: 26 % of conformations
E_{pot} = -223 698 kJ.mol^{-1}

Figure 3 Molecular dynamics of Halo-Tag-bound Ca-DIP. Representative structures of the two clusters of conformations of Ca-DIP bound to Ca^{2+} and HaloTag obtained by molecular dynamics.

Subcellular targeted calcium imaging

To test the probe in cellular imaging, we synthesized an acetoxymethyl (AM) ester analogue **Ca-DIP-AM**, following the classical strategy used for making calcium probes cell permeant (Figure 1C). We assessed the ability of the probes to record localized calcium concentration variations following Histamine stimulation in Hela cells transiently expressing HaloTag fusion proteins. In non-excitable cells, Histamine is known to induce an increase in cytoplasmic calcium concentration with periodic oscillations.[26] We first imaged cells expressing a soluble HaloTag protein. The dye was incubated for 60 min leaving the time for cell permeation and AM ester hydrolysis and time-course imaging over 5 minutes was performed (Figure S1). The addition of Histamine induced an enhancement of the fluorescence intensity consistent with an increase in cytosolic calcium (Figure S1C). The monitoring of non-transfected cells (cell #4 and #5 on Figure S1B) showed no significant fluorescence signal and no variation over the course of the experiment (Figure S1D).

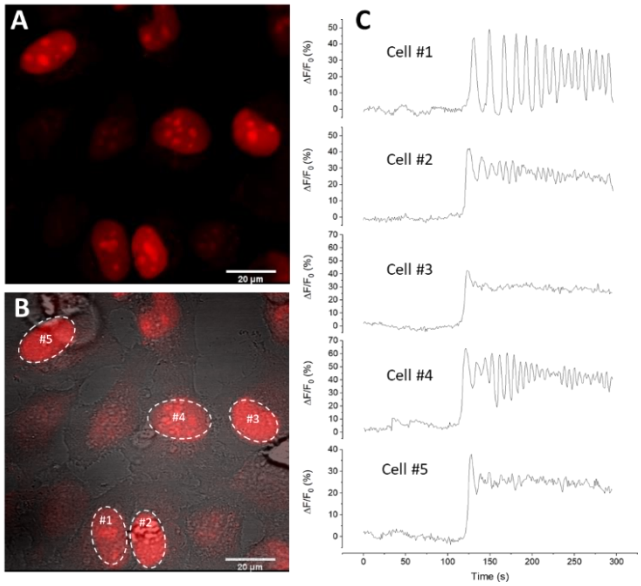


Figure 4. Live imaging of Hela cells transfected with Halo-NLS and incubated with 1.5 µM of Ca-DIP-AM. Time-lapse imaging over 300 s at 1 frame/s. Histamine (50 µM) was added at 100 s. (A) Average intensity projection fluorescence image. (B) Overlay of fluorescence and transmission images. (C) Evolution of the average intensity in the nucleus of the cells numbered on panel B over the course of the experiment, plotted as a percentage of variation relative to the initial intensity $\Delta F/F_0$.

To further evidence the fluorogenic targeting of HaloTag, we performed a similar experiment in cells expressing a nuclear Halo-NLS protein (Figure 4). The average intensity projection image shows a clear selective staining of the nucleus even without a washing protocol (Figure 4A&B). The average intensity measured in the nucleus of 5 different cells shows a clear increase of the fluorescence signal following histamine stimulation (Figure 4C). The calcium oscillations typically expected after histamine stimulation are clearly visible with a good temporal resolution. To control the efficiency of the dual activation, we monitored the intensity in the cytoplasm of two transfected cells. Only a small and stable background signal with no sensitivity to calcium concentration was recorded (Figure S2). Despite having a moderate sensitivity to calcium *in vitro* ($F/F_0 = 4.5$), the cellular performance of the probe is quite satisfactory, with a signal increase up to 100 % with soluble HaloTag and between 40 to 60 % with Halo-NLS that is comparable to the performance of recently reported BAPTA-based red-emitting calcium indicators in similar experiments (Figure 4C).[27,28]

Conclusion

In conclusion, we have successfully used a fluorogenic molecular rotor scaffold targeting HaloTag to develop one of the first fluorescent molecular logic gate for genetically targeted calcium imaging. The modular structure of the probe leaves many possibilities open to tune the optical properties and improve the brightness and the calcium sensitivity. Engineering of HaloTag may also be envisioned to optimize the properties of the fluorogen/protein complex. Despite the great potential of the strategy, very few examples of fluorogenic protein targeting of small chemical indicators (whether for calcium or for other analytes) have been reported. The versatile design strategy using a molecular rotor described herein may be expanded to other analytes and/or to different proteins by selecting the proper sensing moieties and ligands and may open new possibilities for localized functional imaging in genetically-defined cell types or subcellular organelles.

ASSOCIATED CONTENT

Supporting Information

Materials and methods, synthetic methods and additional figures (pdf file). Calcium imaging movies with soluble HaloTag (movie S1) or with nuclear HaloTag (Movie S2).

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Notes

The authors declare no competing financial interests.

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