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Fura-2FF-based calcium indicator for protein labeling†

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We describe the synthesis and fluorescence properties of a Fura-2FF-based fluorescent Ca^{2+} indicator that can be covalently linked to SNAP-tag fusion proteins and retains its Ca^{2+} sensing ability after coupling to protein.

Calcium plays a central role in cellular signaling and participates in processes such as muscle contraction, neurotransmitter release, secretion, fertilization, cell division and cell death.[1] Cytoplasmatic calcium levels are regulated by the release of calcium from the endoplasmatic reticulum (ER), the influx of calcium from the extracellular space and pumping calcium ions over membranes. As calcium is set free near calcium channels, locally different calcium concentrations arise in cells and play a role in the calcium-based signal transduction processes. Specifically, different mechanisms for sensing local and global calcium concentrations have been shown.[2] Calcium concentrations in cells are often measured by fluorescent indicators. One of the most popular indicators for calcium imaging in cells is Fura-2.[3] Its fluorescence excitation maximum shifts from 362 nm to 335 nm upon calcium binding and therefore it can be used for ratiometric measurements.[3] This means that the quotient of the fluorescence excitation intensity at two wavelengths can be used to estimate the calcium concentration. In most studies, this indicator has been employed to measure calcium signals in whole cells or even cell suspensions.[4] However, the determination of sub-cellular calcium signals has also been possible, especially in large nerve cells.[5] Furthermore, some Fura-2 conjugates have been described: for example, Fura-C_{18} and Fura-2FF-C_{18} are hydrophobic derivatives used to detect calcium concentrations near membranes.[6] Fura-2FF (Fig. 1A) is a fluorine-substituted Fura-2 derivative with a lower calcium binding affinity ($K_D = 6\ \mu M$[7]) than Fura-2 ($K_D = 0.14\ \mu M$[3]). Therefore, Fura-2FF measures calcium concentrations in the range of several µM and it displays faster kinetics than Fura-2.[8] In contrast to low-affinity calcium indicators based on the APTRA system, it also retains its high selectivity for calcium over magnesium.[7] However, so far no method to selectively attach Fura-2FF to proteins has been published; such a Fura-2FF derivative would be desirable for local calcium sensing.

One possibility to localize synthetic Ca^{2+} indicators in cells is through specific coupling of the indicator to selected proteins. As

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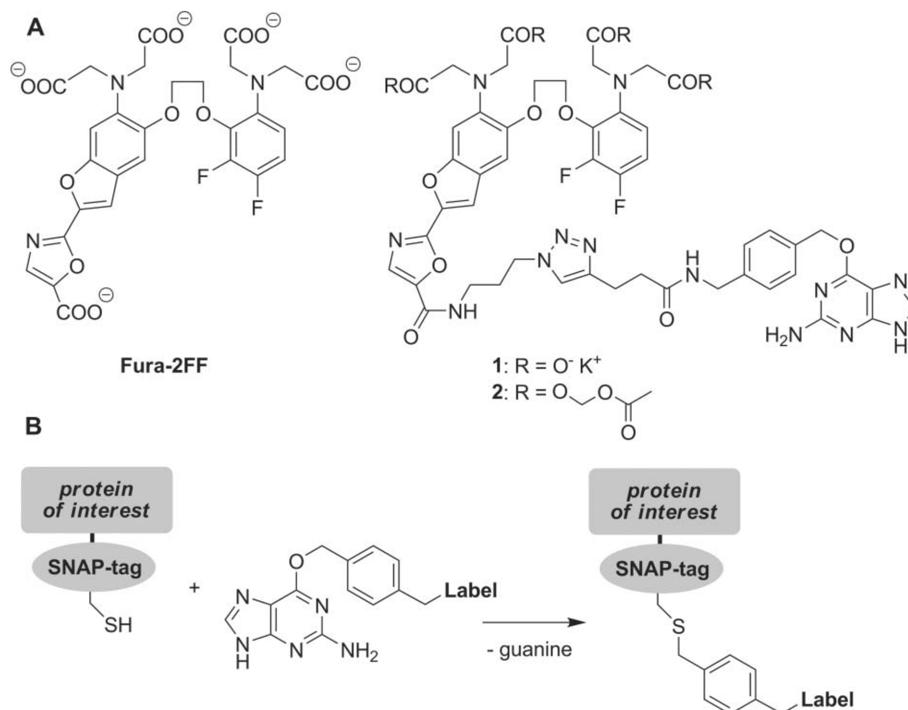

Fig. 1 **A**: Fura-2FF, Fura-2FF-O^6-benzylguanine **1** and its cell permeable acetoxymethylester (AM) derivative **2**. The compounds consist of an indicator linked to benzylguanine, permitting its coupling to SNAP-tag. **B**: Schematic reaction of benzylguanine derivatives with SNAP tag.

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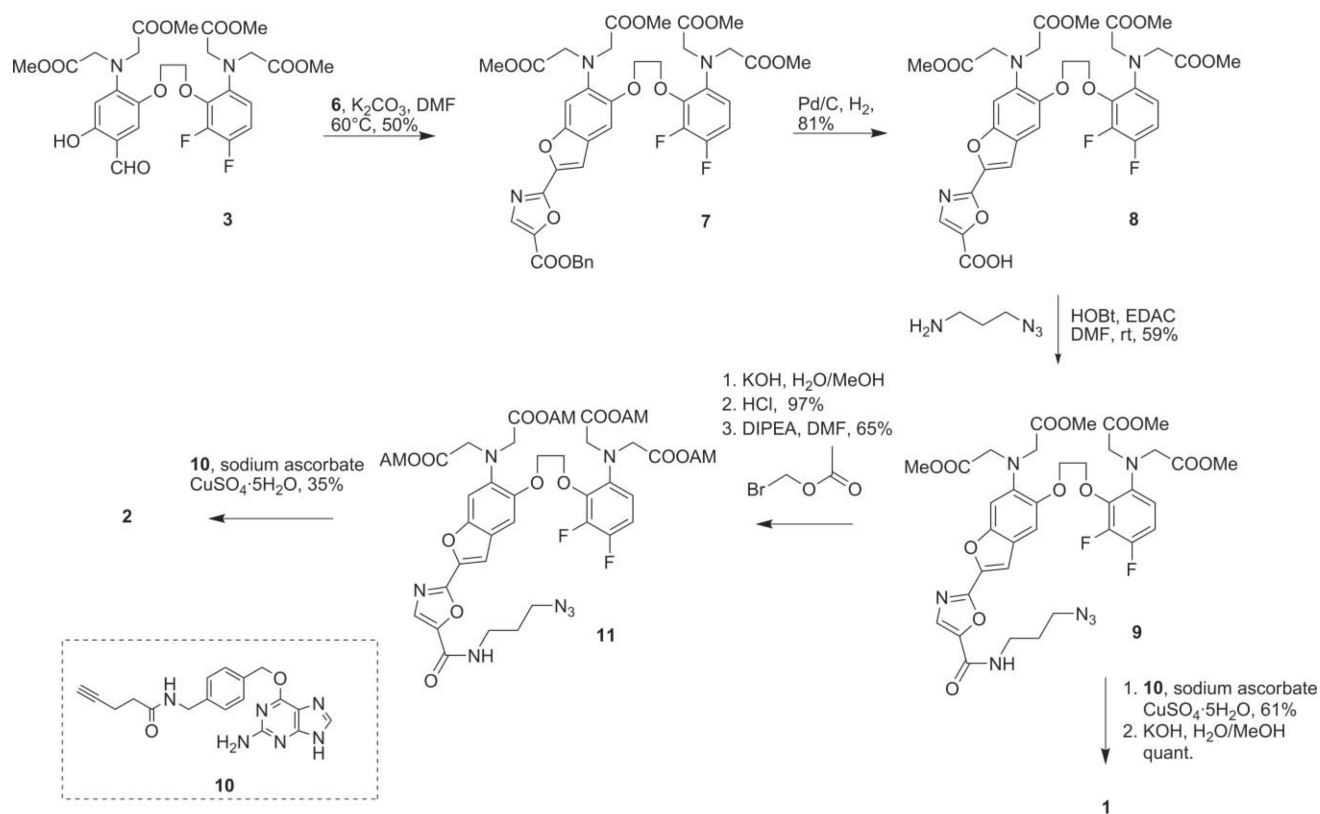


Scheme 1 Synthesis of Fura-2FF O^6-benzylguanine **1** and of Fura-2FF O^6-benzylguanine acetoxymethyl ester **2**

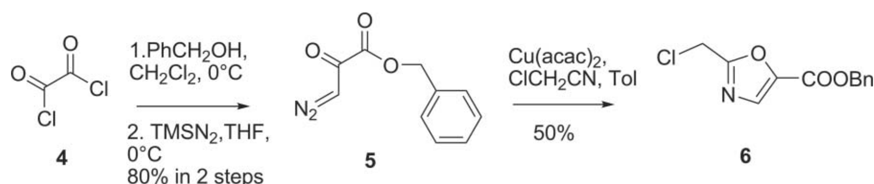
a first example of such an approach, an arsenic derivative of the calcium indicator Calcium Green (Calcium Green FlAsH) was synthesized. Calcium Green FlAsH binds to a short peptide tag containing four cysteines (tetracysteine tag) and was subsequently used for local calcium sensing.[9] However, as the tetracysteine tag generally requires xanthene-based dyes such as fluorescein (*i.e.* FlAsH) and resorufin (*i.e.* ReAsH),[10] it appears less suited for the generation of localizable Fura-2FF indicators. In addition to the tetracysteine tag, there are a number of alternative technologies for protein labeling.[11] A technique that permits selective and covalent labeling inside cells is based on the so-called SNAP-tag.[12] SNAP-tag fusion proteins can be labeled with a large variety of synthetic probes using appropriate O^6-benzylguanine derivatives (Fig. 1B). Furthermore, the SNAP-tag technology was recently used to localize derivatives of the ratiometric fluorescent calcium indicator Indo-1.[13]

Here we describe the synthesis of Fura-2FF-O^6-benzylguanine derivatives **1** and **2** that form covalent SNAP-Fura-2FF-1 conjugates (Fig. 1). The indicator retains its Ca^{2+} sensing ability after coupling to SNAP-tag and should find applications in measuring [Ca^{2+}] with spatiotemporal resolution.

We devised a synthetic strategy to access both Fura-2FF-O^6-benzylguanine **1** as well as its potentially membrane permeable acetoxymethyl ester derivative **2** (Scheme 1). As calcium indicators chelate calcium *via* their negatively charged carboxylate groups, they are not membrane permeable. In contrast, acetoxymethyl (AM) esters are much less polar and thereby can be membrane permeable. This can permit easy loading of calcium indicators into cells where they are rapidly cleaved by nonspecific intracellular esterases.[14]

The synthesis started with salicylaldehyde **3**, which was synthesized following the method reported by London *et al.*[15] The benzofuran was obtained by Rap-Stoermer condensation with a 2-chloromethyl oxazole **6**.[16] The preparation of **6** started from oxalyl dichloride **4** over its monobenzyl ester, which was transformed into the diazomethyl derivative **5** using trimethylsilyl diazomethane[17] (Scheme 2).

Diazopyruvate **5** reacted with chloroacetonitrile in the presence of a catalytic amount of $Cu(acac)_2$ to give the chloromethyl



Scheme 2 Synthesis of oxazole intermediate **6**. Bn: $–CH_2–C_6H_5$

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Table 1 Spectroscopic data of Fura-2FF-O^6-benzylguanine **1** and the SNAP-tag conjugate SNAP-Fura-2FF-1

	Absorption maxima		K_D^{app}/μM	Excitation maxima		Emission maxima		Quantum yield	
	Zero Ca^{2+}	Excess Ca^{2+}		Zero Ca^{2+}	Excess Ca^{2+}	Zero Ca^{2+}	Excess Ca^{2+}	Zero Ca^{2+}	Excess Ca^{2+}
Fura-2FF (commercial)	365 nm	336 nm	3.8 ± 6.0	365 nm	339 nm	514 nm	507 nm	0.34	0.53
Fura-2FF-O^6-benzylguanine **1**	380 nm	347 nm	3.9 ± 2.8	371 nm	352 nm	533 nm	532 nm	0.13	0.13
SNAP-Fura-2FF-1	376 nm	344 nm	4.3 ± 1.5	376 nm	350 nm	525 nm	519 nm	0.26	0.26

oxazole **6** in 50% yield.[18] Hydrogenolysis of the Fura-2FF ester **7** deprotected the benzyl ester and gave the Fura-2FF acid derivative **8** with all BAPTA carboxylates protected as methyl esters. For further coupling of the Fura-2FF to benzylguanine we employed a copper-catalyzed variant of the Huisgen 1,3 dipolar cycloaddition reaction, *i.e.* click chemistry.[19] 3-Amino-1-azidopropane was coupled to acid **8** in DMF using HOBt/EDAC as coupling reagents giving **9** in 59% yield. Hydrolysis of methyl ester **9** with KOH in water–MeOH followed by acidification with 1 M HCl gave the corresponding tetraacid in 97% yield. Alkylation of this tetraacid with bromomethyl acetate in the presence of DIPEA afforded the AM derivative **11** in 65% yield. Reaction of **11** with the benzylguanine alkyne derivative **10** in the presence of sodium ascorbate and copper sulfate gave the AM ester **2** in 35% yield. The non-permeable product **1** was obtained by directly coupling the ester compound **9** with the benzylguanine alkyne **10** in 61% yield and subsequently hydrolysing the methyl esters. To verify that **1** is a substrate of SNAP-tag, **1** was incubated with purified SNAP-tag and the reaction monitored by gel electrophoresis and subsequent in-gel fluorescence scanning (Fig. S2, ESI†). As observed for various other benzylguanine derivatives,[12] **1** reacted readily with SNAP-tag to form the covalent conjugate SNAP-**1**.

UV-Vis absorption spectroscopic properties of **1** and its corresponding SNAP-tag conjugate (abbreviated in the following as SNAP-Fura-2FF-1) were examined. Like all Fura-2 derivatives, the calcium complexes of **1** and SNAP-Fura-2FF-1 absorbed at shorter wavelengths than the free anion form present at zero calcium concentrations (Table 1). **1** and SNAP-Fura-2FF-1 were red-shifted by 11–15 nm in their free anion form and by 9–11 nm in their calcium complex form relative to unmodified Fura-2FF. This difference probably originates from the coupling of BG to Fura-2FF *via* the carboxyl group, *i.e.* changing the carboxylate to a neutral amide.

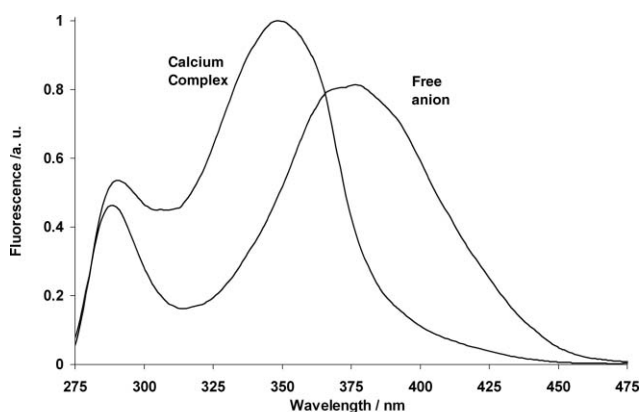

Fig. 2 Fluorescence excitation spectra of SNAP-Fura-2FF-1. Ca^{2+} concentrations are 0 mM (10 mM EGTA) and 1 mM. The K_D^{app} of SNAP-Fura-2FF-1 is 4.3 μM. Emission was recorded at 520 nm. The buffer used was 30 mM MOPS/KOH, pH = 7.2, 100 mM KCl in water.

1 and SNAP-Fura-2FF-1 show a strong shift of at least 19 nm in their fluorescence excitation maxima upon calcium binding, making them useful ratiometric calcium indicators (Fig. 2, Table 1). In comparison to the parent compound Fura-2FF, the fluorescence excitation and (to a lesser extent) the emission maxima of **1** and SNAP-Fura-2FF-1 are red-shifted by 6–25 nm. For example, the excitation maximum of the calcium-bound form is 350 nm for SNAP-Fura-2FF-1, while it is 336 nm for Fura-2FF. Quantum yields were obtained by comparison with the fluorescence of Fura-2, whose quantum yield has been published.[3] SNAP-Fura-2FF-1 has a higher quantum yield than **1**, possibly because guanine acts as fluorescence quencher.[13]

This is advantageous for measurements in cells as it would lead to a lower signal from unreacted indicator. In the free anion form, SNAP-Fura-2FF-1 has a quantum yield similar to Fura-2FF, while the calcium complex of SNAP-Fura-2FF-1 has a lower quantum yield than Fura-2FF (Table S1, ESI†).

Ideally, the excitation wavelengths used to measure calcium concentrations with SNAP-Fura-2FF-1 should not be set at the standard wavelengths for Fura-2FF, 340 nm and 380 nm, but at longer wavelengths corresponding to the fact that their spectra are red-shifted. Calculation of the difference spectra between the free anion and the calcium complex form suggests that the biggest fluorescence changes occur at excitation wavelengths of 337 nm and 387 nm (Table S1, ESI†). Correspondingly, excitation filters around 340 and 390 nm and an emission filter around 530 nm should be best suited for measurements with SNAP-Fura-2FF-1. The wavelength choice and the spectral width of the filters chosen and the background subtraction make a considerable difference in the ratio quotients. The ratio quotient is obtained by dividing the excitation ratios measured for the calcium complex and for the free anion (ESI†). SNAP-Fura-2FF-1 possesses a ratio quotient of 11.7, which is about half the ratio quotient of Fura-2FF of 22.9 (Table S1, ESI†).

Fluorescence spectra were used to determine the apparent dissociation constants for calcium, K_D^{app}. The K_D^{app} for SNAP-Fura-2FF-1 is comparable to that of Fura-2FF (Table 1). This high K_D^{app} should make SNAP-Fura-2FF-1 an appropriate indicator for elevated calcium concentrations as they can occur near calcium influx sites during signaling events.

In summary, we have synthesized and characterized a derivative of the popular ratiometric calcium indicator Fura-2FF that can be specifically coupled to SNAP-tag. As SNAP-tag fusion proteins can be precisely localized within cells, the indicator has the potential for probing changes in calcium concentrations at specific signal sites. Future experiments will focus on the use of the indicator in living cells.

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