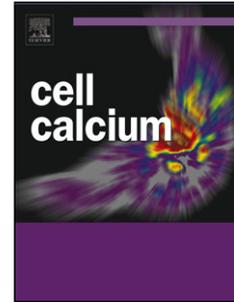# Accepted Manuscript

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Authors: Martin D. Bootman, Sarah Allman, Katja Rietdorf, Geert Bultynck

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Deleterious effects of calcium indicators within cells; an inconvenient truth

Martin D Bootman[1], Sarah Allman[1], Katja Rietdorf[1] and Geert Bultynck[2]

[1]School of Life, Health and Chemical Sciences

Faculty of Science, Technology, Engineering and Mathematics

The Open University

Walton Hall

Milton Keynes

MK7 6AA

[2] KU Leuven, Laboratory of Molecular and Cellular Signaling

Department Cellular and Molecular Medicine

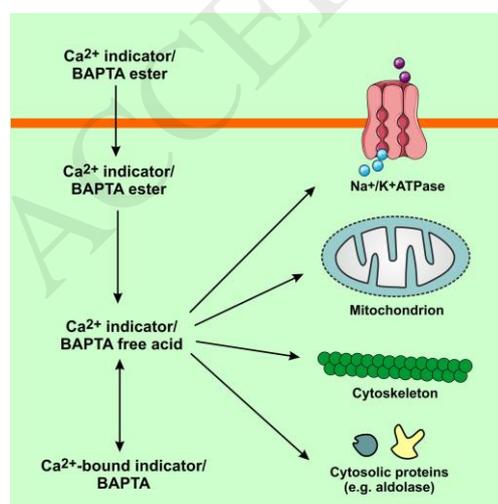Campus Gasthuisberg O/N-I bus 802

Herestraat 49

BE-3000 Leuven

Belgium

Email: martin.bootman@open.ac.uk

Graphical abstract

Highlights

- **Ca²⁺ signalling is widely studied using fluorescent indicators that can be readily loaded into living cells.**

- **Chemical Ca²⁺ indicators provide the means to measure cellular Ca²⁺ changes over long periods of time with sub-second resolution and microscopic precision.**

- **There are known problems with the use of chemical Ca²⁺ indicators, which are largely overlooked.**

- **Genetically-encoded Ca²⁺ indicators overcome some of the issues of using chemical Ca²⁺ indicators, but may introduce problems of their own.**

- **Careful planning and control experiments may overcome the deleterious, off-target effects of chemical Ca²⁺ indicators.**

The study of cellular Ca²⁺ signalling is indebted to Roger Tsien for the invention of fluorescent indicators that can be readily loaded into living cells and provide the means to measure cellular Ca²⁺ changes over long periods of time with sub-second resolution and microscopic precision. However, a recent study [1] reminds us that as useful as these tools are they need to be employed with caution as there can be off-target effects. This article summarises these recent findings within the wider context of confounding issues that can be encountered when using chemical and genetically-encoded Ca²⁺ indicators, and briefly discusses some approaches that may mitigate against misleading outcomes.

Tsien and colleagues invented numerous fluorescent indicators that are used to monitor cellular Ca²⁺ [2, 3]. The fluorescent indicators vary in properties such as affinity, excitation and emission wavelengths, cellular location, and whether they have single wavelength maxima for excitation/emission, or can be used in a ratiometric manner [4]. These chemical Ca²⁺ indicators (as they are sometimes called; hereafter referred to as 'Ca²⁺ indicators') are based on BAPTA, an aminopolycarboxylic acid (Figure 1). Being charged molecules, Ca²⁺ indicators are hydrophilic and therefore cell impermeant. Tsien showed that esterification of the carboxyl groups conferred lipophilicity, allowing the Ca²⁺ indicators to cross cell membranes [5]. Once inside cells, the esters are hydrolysed, and the free acid, Ca²⁺-binding molecule is released. Depending on the loading conditions used, the intracellular accumulation of Ca²⁺ indicators can plausibly span from micromolar to millimolar concentrations. Where loading

of esterified Ca^{2+} indicators is sub-optimal, the free acid forms can be introduced into cells using techniques such as microinjection or via patch pipettes.

The ease with which Ca^{2+} indicators can be loaded and monitored has led to their use in thousands of studies that have characterised the spatial and temporal properties of Ca^{2+} signals in various cell types. Chemical Ca^{2+} indicators are ideal in many ways; they have rapid Ca^{2+}-binding kinetics, are brightly fluorescent, and are functional within cells for long periods of time [6]. Moreover, the output from these Ca^{2+} indicators can usually be readily calibrated into Ca^{2+} concentration [7]. BAPTA is commonly used to test, or negate, the involvement of Ca^{2+} in cellular processes, and is often loaded to large excess to provide stringent buffering of Ca^{2+} concentration. Whilst Ca^{2+} indicators and BAPTA are critical tools for dissecting cellular responses, relatively few studies have included controls for their potential off-target effects.

A recent study from Maiken Nedergaard's lab [1] highlighted an interaction of Ca^{2+} indicators and BAPTA with the Na^+/K^+-ATPase, a ubiquitous cellular enzyme, with consequent deleterious effects on metabolism, signalling and cell survival. Specifically, Smith et al. [1] observed that commonly used Ca^{2+} indicators (Fluo-4, Rhod-2 and Fura-2), as well BAPTA, inhibited the Na^+/K^+-ATPase in several primary cell types independent of Ca^{2+} binding. They found that the Ca^{2+} indicators and BAPTA altered K^+ homeostasis and caused ATP release within the cortex of living animals, as well as reducing spontaneous Ca^{2+} signals within primary astrocytes. An additional effect of Fluo-4, Fura-2 and BAPTA was to decrease the cellular uptake of glucose. Given the critical role of the Na^+/K^+-ATPase in establishing Na^+ and K^+ gradients (which are critical for the membrane potential, secondary active transport, ionic transport and cell volume regulation), it is not surprising that inhibition of this enzyme would influence many downstream processes. For example, the Na^+/K^+-ATPase is the primary mechanism for extracellular K^+ buffering in the brain; the archetypal function of astrocytes, and critical for overall regulation of the excitability of the central nervous system [8]. Moreover, the Na^+/K^+-ATPase can function within cellular microdomains that include other transporters and signalling moieties including Src kinase, phospholipase C-γ and inositol 1,4,5-trisphosphate (IP_3) receptors [9]. Long-term inhibition of the Na^+/K^+-ATPase leads to altered

gene transcription [10] and prevents autophagic cell death (autosis) [11]. Rhod-2 appeared to have a further negative effect on mitochondrial ATP production since it stimulated glucose uptake and glycolysis to compensate for reduced respiration. Although some labs use Rhod-2 to monitor cytosolic Ca^{2+}, due to its delocalised positive charge Rhod-2 accumulates within the mitochondrial matrix and has been used extensively to monitor mitochondrial Ca^{2+} sequestration [12]. Further work is needed to establish how Rhod-2 alters mitochondrial function, but other rhodamine-based compounds have been shown to affect the electron transfer and the F_1F_0-ATPase [13], and the adenine nucleotide translocase [14].

A number of studies have noted that Ca^{2+} indicators and BAPTA have Ca^{2+}-independent off-target actions and may not be freely diffusible within cells (Figure 2 and Table 1) [15]. Although more work is needed to understand how these compounds affect cellular targets, a direct interaction is plausible since they are known to bind to cellular proteins [16, 17]. In fact, it has been estimated that the majority of Fura-2 molecules within a cell may be bound by cytosolic proteins, even if the Ca^{2+} indicator is injected as the free acid [17]. Within muscle cells, typically >80% of Ca^{2+} indicator molecules are bound to cellular components [18], leading to changes in the indicator's fluorescence properties [19, 20]. Non-specific binding of Fura-2 to proteins is enhanced in environments with an acidic pH [21].

Smith et al. [1] observed that the inhibition of Na^+/K^+-ATPase by Ca^{2+} indicators and BAPTA was retained in cell membrane preparations that had been incubated in a lysis buffer for several hours. Under such conditions any freely diffusible molecules would have been lost from the cells. Whilst these data suggest a long-lasting interaction, it is unclear whether the Na^+/K^+-ATPase is directly targeted by Ca^{2+} indicators and BAPTA, or if their effects are mediated by accessory factors. Ouabain, a naturally occurring inhibitor of the Na^+/K^+-ATPase, binds to the enzyme at an extracellular site with nanomolar affinity [22], although other binding sites have been proposed [23]. It remains to be shown if, and where, BAPTA and Ca^{2+} indicators bind to the Na^+/K^+-ATPase. Because BAPTA and Ca^{2+} indicators inhibit the Na^+/K^+-ATPase when loaded into cells it is likely that they access the enzyme from its cytosolic aspect. Given the resemblance of the Na^+/K^+-ATPase to the sarco/endoplasmic reticulum Ca^{2+}-ATPase

(SERCA), responsible for Ca^{2+} uptake into organelles, it is possible that other P-type ATPases may be affected by Ca^{2+} indicators and BAPTA [22, 24].

There is insufficient evidence to draw robust conclusions relating to the structure-activity profile of either BAPTA or chemical Ca^{2+} indicators. The required libraries of structurally diverse analogues have not been systematically tested against these recently identified targets, and it is therefore difficult to make assumptions as to the parts of the molecular framework responsible for their interactions with proteins. The structure of BAPTA (the parent compound) is shown in Figure 1 as the free acid, the AM ester form and, in a complex with Ca^{2+}. BAPTA can be a planar molecule, but it can also rotate around the ether bonds that link its two halves, thereby permitting a degree of flexibility that may allow it to adopt conformations that facilitate its interaction with proteins. The off-target effects of BAPTA are commonly reported to be independent of the Ca^{2+}-binding carboxyl groups, which may indicate other parts of the molecule are involved. For example, analogues of BAPTA stimulated the production of prostacyclin with an inverse relationship to their affinity for Ca^{2+} [25]. However, altering the length of the ether chain linking the aromatic groups, substitution of nitrogen atoms, or altering the position of the benzene rings changed their potency for inducing prostacyclin production.

Reagent	Cell type	Working concentration	Effect	Reference
BAPTA-AM	Rat parotid cells	100 μM	Reduced cellular ATP levels	[26]
BAPTA-AM	3T3-L1 adipocytes	50 μM	Reduced insulin-stimulated translocation of GLUT4	[27]
BAPTA-AM	A6 (Xenopus), RAT2 cells	50 μM	Cytoskeletal disassembly Reduced cellular ATP levels Changes in mitochondrial morphology and distribution	[28]
BAPTA	Murine brown adipocytes	1 - 2 mM	Ca^{2+}-independent effects on Cl^- channel currents	[29]

BAPTA-AM	3T3-L1 adipocytes	12.5 - 50 µM	Decrease tubulin polymerisation	[30]
BAPTA and di-bromo BAPTA	Drosophila photoreceptors	~1 - 10 mM (IC_{50} for BAPTA ~1 mM)	Inhibition of phospholipase C	[31]
BAPTA-AM, di-fluoro BAPTA-AM, and di-bromo BAPTA-AM	Endothelial cells	0.5 – 50 µM	Enhanced prostacyclin release. Concentration- and time-dependent effect of BAPTA; both activation and inhibition were observed.	[32]
BAPTA-AM	HEK293 cells	0.1 – 50 µM (IC_{50} ~1 µM)	Inhibition of heterologously-expressed K^+ channels	[33]
BAPTA-AM	Liver macrophages	10 µM	Inactivation of protein kinase C	[34]
BAPTA	Permeabilised hepatocytes	1 µM – 10 mM (K_d = 1.8 mM)	Inhibition of inositol 1,4,5-trisphosphate-induced Ca^{2+} release	[35]

Table 1. Ca^{2+}-independent cellular actions of BAPTA. The effects of BAPTA, and its analogues, were considered Ca^{2+}-independent via a variety of experimental analyses, such as similar cellular responses being observed when using low-affinity or non-Ca^{2+}-binding BAPTA analogues, or keeping free Ca^{2+} concentration the same but changing BAPTA concentration and having an altered cellular response (i.e. the response was dependent on [BAPTA], but not [Ca^{2+}]).

The Na^+/K^+-ATPase is both upstream and downstream of cellular Ca^{2+} signals. Elevated levels of cytosolic Ca^{2+} have been shown to inhibit the Na^+/K^+-ATPase [36], and conversely inhibition of the enzyme causes Ca^{2+} oscillations [37]. Inhibition of the Na^+/K^+-ATPase within cardiomyocytes by cardiotonic steroids is known to cause increased Ca^{2+} signals, and can

improve cardiac performance during conditions such as congestive heart failure [38]. It is more than a little inconvenient that fluorescent reporters used to monitor cellular Ca^{2+} responses could affect the activity of the Na^+/K^+-ATPase, which is involved in sensing and regulating Ca^{2+} signals. Moreover, Ca^{2+} indicators have been shown to impede Ca^{2+} release and Ca^{2+}-dependent channel inactivation [35, 39, 40], and alter the properties of Ca^{2+} signals [41].

A critical question concerning the continued use of chemical Ca^{2+} indicators is whether it is possible to overcome, or control for, their effects on Na^+/K^+-ATPase inhibition. One of the products resulting from the intracellular hydrolysis of esterified Ca^{2+} indicators is formaldehyde, which is widely used as a tissue fixing agent. However, Smith et al [1] found that the free acid forms of Ca^{2+} indicators and BAPTA could inhibit the Na^+/K^+-ATPase, suggesting that products released via ester hydrolysis were not causative. These observations indicate that introduction of Ca^{2+} indicators and BAPTA via microinjection or patch pipettes cannot solve the issue.

An alternative to the use of chemical Ca^{2+} indicators is genetically-encoded Ca^{2+} indicators (GECIs), which are generally based on the fusion of Ca^{2+}-binding proteins with fluorescent proteins [42]. In their study, Smith et al. [1] demonstrated that the GECI GCaMP3 did not cause inhibition of the Na^+/K^+ATPase, or alter the frequency of astrocytic Ca^{2+} signals. However, whilst GECIs have great utility, particularly for long-term recordings [43], they may not perform as well as chemical Ca^{2+} indicators in terms of their Ca^{2+}-binding kinetics or sensitivity [44], and might be unsuitable in situations where cells (e.g. cardiomyocytes) could functionally change during the time needed for the GECI expression. As with chemical Ca^{2+} indicators, the use of GECIs relies on an adequate expression level, correct localization and the absence of deleterious cellular effects. However, it was found that following viral transduction, the expression level of GCaMP3 was not stable and increased over time, reaching an expression level above the optimum signal-to-noise ratio after 7 weeks [45]. Moreover, following long-term expression, GCaMP3 protein was not only found in the cytoplasm (its intended compartment), but additionally non-functional, N-terminally cleaved

GCaMP3 accumulated in the nuclei of neurons [46, 47], and atypical responses were observed [46]. The presence of non-functional GECI molecules resulted in an under-estimation of the observed Ca^{2+} responses.

Similar to chemical Ca^{2+} indicators, a substantial portion of GECIs can be bound to cellular targets (around 50% in neurons) [47], which could lead to altered properties such as a change of Ca^{2+} sensitivity. Furthermore, long-term expression of GECIs may result in prolonged Ca^{2+} buffering, thereby impacting on Ca^{2+}-regulated processes including transcriptional regulation via NFAT or CREB. Since the expression of several Ca^{2+}-transport systems is controlled through these Ca^{2+}-dependent transcription factors (e.g. inositol 1,4,5-trisphosphate receptors and the mitochondrial uniporter via NFAT2 and CREB, respectively [48, 49]), GECIs might adversely affect the expression of Ca^{2+} channels and transporters. Moreover, GECIs (specifically GCaMPs) change the apparent sensitivity of cells to IP_3 [44]. Most transgenic GECI mice do not show an obviously altered phenotype [47], but profound effects of GECI expression have been observed, e.g. cardiac hypertrophy in a mouse expressing GCaMP2 [50]. It is plausible that mild phenotypic changes, which go undetected, could nevertheless affect the experimental results obtained with specific cell types. For example, a detailed study of transgenic mice expressing the troponin C-based GECI TN-XXL revealed changes in behavioural parameters, cardiac function and gene expression, despite the absence of a strong whole animal phenotype [51].

It is hard to say how much the effects of Ca^{2+} indicators and BAPTA on the Na^+/K^+-ATPase, as reported by Smith et al. [1], impinge on the results of published studies. However, it is a reminder that the loading of Ca^{2+} indicators is best kept minimal and should be controlled for. Whilst substantial loading of Ca^{2+} indicators may improve signal to noise in fluorescence recordings, it can actually lead to diminished Ca^{2+} signals due to buffering. Smith et al. [1] observed that the inhibition of the Na^+/K^+-ATPase was proportional to the concentration of Ca^{2+} indicator loaded. With modest concentrations of Ca^{2+}-indicator loading, there was no significant effect on the Na^+/K^+-ATPase activity. It is possible that empirically establishing minimal Ca^{2+} indicator-loading conditions may avoid deleterious effects on the Na^+/K^+-ATPase

and other yet unknown cellular targets. Some years ago, Erwin Neher pointed out that both very high and very low concentrations of a Ca^{2+} indicator, such as Fura-2, can be useful but for different experimental purposes, and that intermediate concentrations should be avoided as they will not give accurate results. Generally, to measure changes in the cytosolic free Ca^{2+} concentration, a minimal indicator concentration should be used to circumvent extra Ca^{2+} buffering, whilst for measuring Ca^{2+} flux a sufficient amount of Ca^{2+} indicator must be present to overcome the endogenous Ca^{2+} buffers in the cell [52].

In experiments where BAPTA is used to implicate the involvement of Ca^{2+} in a cellular process, it could be important to perform additional controls using low affinity versions of BAPTA such as dibromo-BAPTA and difluoro-BAPTA (K_d values 1.6 and 65 µM, respectively, compared to a K_d of 160 nM for BAPTA) (Figure 1). These low affinity Ca^{2+} chelators should not phenocopy the effect of BAPTA if the cellular response involves Ca^{2+}. Moreover, it would be ideal if the Ca^{2+} buffering of BAPTA was tested after it has been loaded into cells. The buffering of cytosolic Ca^{2+} is generally assumed after BAPTA-AM loading, and is not routinely demonstrated in studies that use BAPTA as a means of blocking cellular Ca^{2+} signalling. The effectiveness of BAPTA could be easily tested by stimulating GECI-expressing or Ca^{2+} indicator-loaded cells with a Ca^{2+}-releasing agonist (e.g. ATP). If this is not suitable, the loading of BAPTA into cells could be verified by measuring absorbance of the compound at ~260 nm. Some of the approaches that may be taken to mitigate off-target effects of Ca^{2+} indicators and BAPTA, and ancillary experimental issues, are described in Table 2.

Approach	Utility	Notes
Application of ouabain	Given that the Na^+/K^+-ATPase has been shown to be affected by Ca^{2+} indicators and BAPTA, ouabain could be used as a control. If ouabain replicates experimental outcomes observed using Ca^{2+} indicators and BAPTA, then the Na^+/K^+-ATPase may be involved.	It would be difficult to know exactly how much ouabain would be needed to replicate putative Na^+/K^+-ATPase inhibition.

Use of low affinity BAPTA analogues	Low affinity BAPTA analogues can be loaded using the same conditions as for BAPTA. Due to their lesser affinity for Ca^{2+}, the analogues should not replicate the effect of BAPTA if a particular cellular process is Ca^{2+} dependent.	This is potentially the simplest control to perform. However, since the low affinity BAPTA analogues may not buffer Ca^{2+} to the same extent as BAPTA, it would be necessary to show that they have loaded into the cells by measuring increased absorption at ~260 nm. One potential caveat in the use of BAPTA analogues is whether they have the same off-target effects. There is evidence that they share the same off-target actions on the Na^+/K^+-ATPase [1], phospholipase C [31] and prostacyclin release [32].
Non-Ca^{2+}-buffering AM ester compounds	To assess the potential effects of de-esterification of Ca^{2+} indicators and BAPTA within cells, it could be helpful to use non-Ca^{2+}-buffering, cell-permeable esters as a reference. For example, BCECF-AM, a cell-permeable pH indicator, could be used as a reference.	Whilst this is an easy control to perform, it might be difficult to match the intracellular concentrations of Ca^{2+} indicators and BAPTA with a reference indicator such as BCECF. In addition, reference indicators may have their own off-target effects.
Use of pharmacological reagents to verify the involvement of Ca^{2+}	An adjunct to the use of BAPTA to investigate the role of Ca^{2+} signals in a cellular response could be pharmacological reagents that target specific Ca^{2+} transport processes. For example, U73122, 2-APB and xestospongin B could be used to implicate IP_3 signaling and/or Ca^{2+} influx [53-55], or mitoxantrone may be used to examine the role of the mitochondrial Ca^{2+} uniporter [56].	Pharmacological tools are sometimes ideal for acute experiments, and when the effective concentrations of the reagents are known. However, it can be difficult to definitively say that there are no off-target effects of a particular reagent, even when used at a low concentration. Some pharmacological tools have genetic correlates that can be used in parallel. For example, xestospongin B and 2-APB can be used in conjunction with the IP_3 5'-phosphatase, or a high-affinity IP_3 sponge, to inhibit IP_3-mediated Ca^{2+} signals [57, 58]. Pharmacological approaches should ideally go hand-in-hand with genetic approaches. Knocking out specific Ca^{2+} transport systems is accessible using techniques such as siRNA and CRISPR/Cas9.
Use of chelators to rule out	Chemical Ca^{2+} indicators all show varying degrees of affinity for heavy metal ions like Mn^{2+} and Zn^{2+} [59]. If	Whether a response is due to changes in Ca^{2+} or heavy metal ions can be tested by the use of heavy metal ion chelators like

effects of heavy metals	changes in the concentration of heavy metal ion concentrations occur, these might be interpreted as changes in the Ca^{2+} concentration without appropriate controls.	TPEN [60], although TPEN itself can affect Ca^{2+} indicators [61]. An alternative compound with a lower K_d for Ca^{2+} is phenanthroline [62-64]).
Effects of pH and experimental reagents on Ca^{2+} indicators and their calibration	The spectral characteristics and affinities of many chemical Ca^{2+} indicators (and GECIs) is pH dependent. The affinity for Ca^{2+} is usually not affected within the physiological cytosolic pH range, but can be reduced at acidic pH [65]. The fluorescence of Ca^{2+} indicators may be affected by experimental reagents such as caffeine [66] and resveratrol [67].	If the cytosolic pH is expected to change substantially during an experiment, or Ca^{2+} measurements in an organelle with a low pH is planned, the K_d of the Ca^{2+} indicators (or GECIs) should be calibrated for the appropriate pH range as described in [68-70]. The effect of experimental reagents on Ca^{2+} indicators can be tested using solutions of the indicator free acid. If an effect is observed, caution will be needed in interpreting experimental results and calibration. Whilst the principles of calibrating the output of Ca^{2+} indicators, and transforming fluorescence recordings into Ca^{2+} concentration, are relatively straightforward there are several potential pitfalls. For example, it may be difficult to obtain maximal and minimal fluorescence signals or the correct K_d values, and there may be Ca^{2+}-insensitive or compartmentalised forms of Ca^{2+} indicators within cells. Some of these issues, and their solutions, have been discussed previously [7, 71-73].
Expression of GECIs and Ca^{2+}-binding proteins	GECIs and Ca^{2+}-binding proteins can be used for the same purposes as chemical Ca^{2+} indicators and BAPTA providing that they can be expressed within the cells of interest.	GECIs are a growing method for monitoring cellular Ca^{2+} signals. They can be targeted to specific cellular regions/structures and are available in different colour variants. GECIs can be expressed in a stable or transient manner. The impact of GECI expression should be monitored, as they may not be entirely benign [44, 51, 74]. Ca^{2+}-binding proteins may need to be very highly expressed to have the same potency as BAPTA in buffering Ca^{2+}-signals.

Mitigating potential pitfalls of chemical Ca^{2+} indicators	There are a number of issues that can arise when performing fluorescence experiments: chelation of heavy metals, indicator bleaching, production of reactive oxygen species (ROS), compartmentalisation/extrusion of fluorescent reporters, and varying affinities of reporters for Ca^{2+} in different cellular environments [73]. An issue that is not widely considered is the induction of autophagy when using simple extracellular solutions that do not have growth factors or amino acids. Autophagy can induce a change in Ca^{2+} transport systems [75].	There are suitable controls and tests for most of these issues. For instance, compartmentalisation and extrusion of fluorescent reporters can often be avoided by loading AM esters at room temperature before warming cells to 37˚C, and by using ABC transporter inhibitors such as sulphinpyrazone [76]. The impact of bleaching and ROS may be minimised by using short excitation exposure times and low incident light energy, or supplementation with ROS scavengers. The induction of autophagy can be tested using a number of markers, and can be avoided by including growth factors and amino acids in the extracellular medium. Complete phenol red-free culture medium can be used for imaging providing that it is supplemented with a pH buffer that can work outside a CO$_2$-gassed incubator. However, long term (>7h) presence of HEPES in the medium has been shown to induce autophagy [77].

Table 2. Assessing, and controlling for, Ca^{2+}-independent cellular actions Ca^{2+} indicators and BAPTA, and other experimental issues arising from fluorescence imaging. Abbreviations: GECI; genetically-encoded Ca^{2+} indicator, IP$_3$; inositol 1,4,5-trisphophate, 2-APB; 2-aminoethoxy diphenyl borate, U73122; 1-[6-[((17β)-3-Methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-1H-pyrrole-2,5-dione.

The study by Smith et al. [1] is a timely reminder that we need to be cautious in our use of chemical Ca^{2+} indicators and BAPTA. Evidence has been presented over many years that these compounds may have deleterious off-target actions (Table 1). However, there are a range of strategies that can be adopted to counteract potential confounding effects (Table 2). Chemical Ca^{2+} indicators and BAPTA remain ideal tools for certain situations, but additional control experiments may be both necessary and prudent.

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Figure legends

Figure 1. Chemical structure of BAPTA and analogues, and complexation of Ca^{2+}. Panel **A**, BAPTA free acid (deprotonated). Panel **B**, stylised representation of the Ca^{2+}:BAPTA complex showing binding by the carboxyl groups, with participation also from the ester oxygen and nitrogen atoms. Panel **C**, BAPTA-AM ester form. Panel **D**, Difluoro- or dibromo-BAPTA-AM analogues.
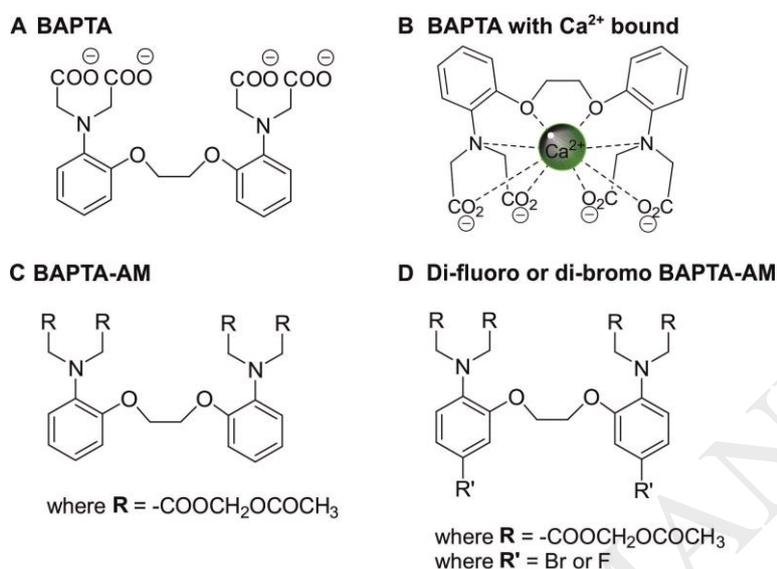

Figure 2. Reported cellular interactions of BAPTA and/or Ca^{2+} indicators. The figure does not show an exhaustive list of the off-target effects of BAPTA/Ca^{2+} indicators, but points out some of the key interactions reported by Smith et al. (1), as well as other reported outcomes.