J Endocrinol Invest (2015) 38:39–45
DOI 10.1007/s40618-014-0178-2

SHORT REVIEW

Spying on organelle Ca^{2+} in living cells: the mitochondrial point of view

D. Pendin · E. Greotti · R. Filadi · T. Pozzan

Received: 15 July 2014 / Accepted: 15 September 2014 / Published online: 23 September 2014

Abstract Over the past years, the use of genetically encoded Ca^{2+} indicators (GECIs), derived from aequorin and green fluorescent protein, has profoundly transformed the study of Ca^{2+} homeostasis in living cells leading to novel insights into functional aspects of Ca^{2+} signalling. Particularly relevant for a deeper understanding of these key aspects of cell pathophysiology has been the possibility of imaging changes in Ca^{2+} concentration not only in the cytoplasm, but also inside organelles. In this review, we will provide an overview of the ongoing developments in the use of GECIs, with particular focus on mitochondrially targeted probes. Indeed, due to recent advances in organelle Ca^{2+} imaging with GECIs, mitochondria are now at the centre of renewed interest: they play key roles both in the physiology of the cell and in multiple pathological conditions relevant to human health.

Keywords Calcium · Mitochondria · Calcium indicators · GECI

D. Pendin · E. Greotti · R. Filadi · T. Pozzan
Department of Biomedical Sciences, University of Padua, viale G. Colombo 3, 35131 Padua, Italy

D. Pendin (✉) · E. Greotti · T. Pozzan
Neuroscience Institute, National Research Council, viale G. Colombo 3, 35131 Padua, Italy
e-mail: diana.pendin@unipd.it

T. Pozzan
Venetian Institute of Molecular Medicine, Via G. Orus 2, 35129 Padua, Italy

Introduction

The majority of extracellular signals, such as hormones, neurotransmitters and growth factors, cannot enter the cell freely and act by binding to plasma membrane receptors that in turn propagate such signals intracellularly. The intracellular signalling depends either on the activation of a cascade of events initiated by the intrinsic enzymatic activity of the receptors (e.g., the tyrosine kinase activity of growth factor receptors) or through the generation of second messengers. In turn, the latter (e.g., cAMP, InsP3) can be synthesized by specific enzymes coupled to the receptors via G-proteins, or, in the case of Ca^{2+}, they diffuse into the cytoplasm through selective ion channels. Second messengers are able to trigger and modulate a wide range of cellular functions such as proliferation, differentiation, contraction, migration, survival, apoptosis and gene transcription. As far as Ca^{2+} is concerned, such a broad spectrum of functions is ensured by a fine spatial and temporal regulation of its concentration. Indeed, cells are able to store Ca^{2+} in specific subcellular compartments, releasing it into the cytosol upon specific stimuli. The complex toolkit that ensures the tight control of Ca^{2+} concentration comprises a variety of channels, antiporters, pumps and Ca^{2+}-binding proteins that are expressed differentially in the different cell compartments and are subjected to specific regulation of activity and expression level. Because of the importance of Ca^{2+} in cell biology and pathology, many efforts have been made to develop tools to study the mechanisms of Ca^{2+} homeostasis and the dynamic changes in its intracellular concentration. Moreover, given the complex spatial nature of Ca^{2+} signals, the interest of many researchers in the field moved from $[Ca^{2+}]$ analysis in the cytoplasm as a whole to the subcellular level, i.e., inside organelles

or specific cytoplasmic subcompartments. In this contribution, we briefly discuss the basic principles that led to the development of the intracellular Ca^{2+} indicators, focusing in particular on the tools that have allowed the measurement and the characterization of the role of Ca^{2+} in the mitochondrial matrix.

Ca^{2+} measurement in living cells

An adequate measurement of Ca^{2+} dynamics in living cells requires tools with sufficient sensitivity and spatio-temporal accuracy. Indeed, $[Ca^{2+}]$ inside the cell can change quickly, depending from the cell's state (at rest or stimulated) and the specific subcellular compartment analysed. For example, cytoplasmic Ca^{2+} concentration, $[Ca^{2+}]_c$, can increase from ~100 nM (in basal conditions) to peaks of 1–3 μM, reached in just a few tens or hundreds of ms upon cell stimulation. Over the past decades, many classes of indicators, both chemical or protein based, have been developed and can be classified according to their physicochemical characteristics and spectral changes occurring upon Ca^{2+} binding: single wavelength indicators change fluorescence intensity without shifting their excitation or emission wavelengths, while ratiometric indicators change their excitation and/or emission spectra. Single wavelength indicators are generally brighter and facilitate Ca^{2+} detection when more than one fluorophore is used. Ratiometric dyes can be calibrated very precisely and minimize the most common problems associated with synthetic indicators, including heterogeneous dye loading, photobleaching, dye leakage and changes in focal plane [1].

Optical measurements of $[Ca^{2+}]_c$ were performed initially using synthetic molecules that change their fluorescence or absorbance properties upon Ca^{2+} binding. Problems with these indicators included their modest Ca^{2+}/Mg^{2+} selectivity and the variable stoichiometry of cation/dye complexes. The field of Ca^{2+} imaging moved a significant step forward in the 1980s when R.Y. Tsien developed a new class of synthetic fluorescent Ca^{2+} indicators (among them quin2, Fura-2 and Indo-1), combining a fluorophore with the Ca^{2+} chelator EGTA [2]. Key to the success of these indicators was also the discovery of a simple method to load these indicators into living cells, thus avoiding the complex procedure of microinjection. The acetoxymethyl ester (AM) form of these chemical dyes is in fact hydrophobic and easily diffuses into living cells; once in the cytoplasm, the AM groups are rapidly hydrolysed by cellular esterases, regenerating the water-soluble indicator within the cell and at the same time preventing its release back into the medium. The structural changes induced by Ca^{2+}-binding lead to modifications in the spectral emission and/or excitation properties of the probes, thus allowing a rapid and reliable estimation of $[Ca^{2+}]$ variations. Over the years, many Ca^{2+} indicators were developed based on the same rationale, with different Ca^{2+} affinities and spectral properties, allowing intracellular Ca^{2+} detection in practically any cell type over a large range of concentrations (from <50 nM to >50 μM). High-affinity indicators are useful to quantify $[Ca^{2+}]_c$ while lower affinity indicators can be in principle optimized for measuring Ca^{2+} within subcellular compartments with higher concentrations [1].

Genetically encoded Ca^{2+} indicators

The esterases that cleave the AM groups in the fluorescent probes mentioned above are primarily located in the cytosol; accordingly, these indicators, with the only exception of Rhod-2, cannot be targeted to organelles with adequate selectivity. One way that has been proposed to overcome this limitation is to overexpress esterases in specific subcompartments of the cell [3]. This approach, although interesting, is still poorly exploited.

The most important breakthrough in the field of Ca^{2+} imaging at the subcellular level stemmed from the use of two proteins, aequorin (Aeq) and green fluorescent protein (GFP), opening the field of genetically encoded calcium indicators (GECIs). Specific targeting signals can be fused to the GECI sequence, achieving selective targeting of the transfected proteins to organelles or cytoplasmic domains. Moreover, GECIs can be placed under the control of tissue-specific or inducible promoters, allowing spatial and temporal control of their expression. They are also suitable for in vivo measurement of Ca^{2+} dynamics in intact tissues as well as in whole organisms, since they can be delivered via transgenesis, viral injection or in utero electroporation [4]. Different classes of GECIs have been created and specific parameters are usually evaluated to characterize each probe, in particular affinity and selectivity for Ca^{2+}, dynamic range and kinetics for Ca^{2+} binding. Usually, the evaluation of these parameters is performed in vitro but, since these probes behave differently in subcellular compartments, it is now clear that an in vivo evaluation is also necessary to obtain reliable measurements of absolute Ca^{2+} concentrations. Moreover, the cited parameters must be carefully assessed to choose the appropriate probe for the designed experiment.

Below, we describe briefly the main characteristics of the two classes of GECIs now available, i.e., bioluminescent probes (based on Aeq) and fluorescent probes (based on GFP), focusing primarily on the GECIs targeted to mitochondria. Needless to say, GECIs targeted to practically all subcellular compartments are now available; sensors of other dynamic parameters (pH, cyclic nucleotides, ATP, O2 radicals, etc.) have also been targeted to organelles using the principles originally invented for the mitochondrial Ca^{2+} sensors.

Bioluminescence-based GECIs: aequorin

Aequorin (Aeq) was the first protein-based Ca^{2+} indicator used in biology. Aeq is a Ca^{2+}-sensitive photoprotein produced by the jellyfish *Aequorea victoria*, with a hydrophobic core that binds the prosthetic group coelenterazine and three EF-hand motifs for Ca^{2+} binding. Upon Ca^{2+} binding, the covalent bond between the prosthetic group and the apoprotein is broken, coelenterazine is oxidized to celenteramide and released with the emission of one photon; this reaction is irreversible. Importantly, the rate of photon emission depends on the $[Ca^{2+}]$, thus allowing the conversion of the speed of emitted light into $[Ca^{2+}]$ through a specific algorithm [5, 6].

The first organelle–targeted GECI was developed in 1992, when the mitochondrial pre-sequence of subunit VIII of cytochrome c oxidase (COX) was fused to an HA1-tagged native Aeq (mtAEQ) [7]. The paper demonstrated that agonist-stimulated elevations of cytosolic free Ca^{2+} evoke rapid and transient increases of intra mitochondrial $[Ca^{2+}]$, $[Ca^{2+}]_m$, which can be prevented by pretreatment with a mitochondrial uncoupler. MtAEQ was particularly important because it was the prototype of the now large family of selectively targeted probes and at the same time because it provided the first direct evidence of fast mitochondrial Ca^{2+} accumulation in living cells in response to a physiological stimulus (see below). Other mitochondrial Aeq-based GECIs were then produced: of particular relevance is the Aeq fused to glycerol phosphate dehydrogenase, a protein of the inner mitochondrial membrane (IMM) with a large C-terminal tail protruding in the mitochondrial intermembrane space (IMS) [8]. This recombinant aequorin (mimsAEQ), provided the first direct evidence of the existence of high $[Ca^{2+}]$ microdomains near the regions of close contact between mitochondria and ER [8].

Other Aeq variants have been targeted to the nucleus, endoplasmic reticulum, sarcoplasmic reticulum, subplasmalemmal space, Golgi apparatus, peroxisomes and secretory vesicles (for a recent review, see [9]).

Among the advantages of using recombinant Aeqs, the following four aspects are noteworthy: first, their wide dynamic range (from 0.1 μM to the low millimolar level), obtained both by modification of the native Aeq and through the use of different coelenterazine derivatives. Second, these probes have only a marginal interference with endogenous Ca^{2+} buffering proteins. Third, they show low sensitivity to pH. Finally, they exhibit a high signal-to-noise ratio. The use of Aeqs also entails some disadvantages, however, and among these we note the need to reconstitute the protein with coelentarazine, the low amount of light emitted by the photoprotein (making it difficult to use in single cells) and the irreversibility of the Ca^{2+} triggered reaction. Moreover, the photoluminescence measurements are difficult to calibrate in compartments (or in cell populations) with heterogeneous Ca^{2+} levels [6].

In the past years, other constructs containing Aeq have been generated, such as a dual reporter system comprising of a GFP mutant fused to Aeq [10] and another (named GAP, GFP-aequorin protein) [11] resulting from the fusion of a GFP mutant with apoaequorin that exploits the Ca^{2+} binding to Aeq to induce changes in the excitation/emission properties of GFP.

GFP-based fluorescent GECIs

Fluorescent GECIs consist of a fluorescent protein (FP) fused to a Ca^{2+}-binding domain in such a way that Ca^{2+} binding modifies the fluorescence properties of the FP. Two strategies have been developed to obtain these indicators: the first employs GECIs containing one fluorescent protein, where Ca^{2+} binding alters the chromophore environment, inducing a change in the intensity or the wavelength of the emitted fluorescence. The other takes advantage of Förster (or fluorescence) resonance energy transfer (FRET) changes that occur between two FPs in molecular constructs where the two proteins are linked through a Ca^{2+}–sensitive peptide.

Single fluorophore GECIs

The development of single fluorophore GECIs started with the discovery that GFP and its variants tolerate the insertion of relatively long peptides at position 145 in their aminoacid sequence. When a Ca^{2+}-binding peptide is inserted, the change in protein structure induced by the binding of the ion affects the protonation of the chromophore, changing its dissociation constant pKa, resulting in an increase or decrease of the chromophore fluorescence when exited at a given wavelength [12]. The most widely used Ca^{2+}-binding protein in these types of constructs is Calmodulin (CaM). In probes of the "Camgaroo" family, Ca^{2+} binding causes a shift of the absorbance peak of the FP. Mitochondrially targeted Camgaroos are available [13]. Other Ca^{2+} sensors targeted to the cytosol or the mitochondrial matrix have been obtained with analogous strategies, for example the "Pericam" family [14]. Of interest, one of these sensors was expressed in adult rabbit ventricular cardiomyocytes, using adenoviral infection, allowing Ca^{2+} measurements in intact perfused hearts [15].

A class of sensors that has become considerably popular in the past years was developed starting from the so-called "GCamP" [16]. Extensive structure-guided optimization has been performed to improve the properties of this

sensor, giving birth to a class of probes with different spectral properties and Ca^{2+} affinity, strong brightness, wide dynamic range and fast kinetics [17].

Finally, attempts have been made to increase the colour palette of single wavelength GECIs. Of particular interest are the red GECIs that employ a red isoform of GFP called RCamPs. One of these sensors, RCaMP1e has been targeted to the mitochondrial matrix and expressed in cultures by infection with an AAV [18].

FRET-based GECIs sensing Ca^{2+}

In 1997, Tsien and co-workers [19] (and independently Persechini et al. [20]) generated the first class of ratiometric GFP-based Ca^{2+} sensors, named by Tsien "Cameleons". The Cameleon structure consists of the two Ca^{2+}-responsive elements CaM and CaM-binding domain of myosin light chain kinase M13 connecting two FPs. The working principle of Cameleons is based on FRET changes: the direct transfer of energy from an excited donor FP (generally, a blue FP) to an acceptor FP (generally, a yellow FP). Ca^{2+} binding to CaM triggers a conformational change in the molecule, forcing the two FPs closer together: this results in an increased FRET efficiency that in turn leads to a decrease in the donor fluorescence intensity and an increase in the acceptor fluorescence intensity. It is thus possible to monitor changes in $[Ca^{2+}]$ as changes in the ratio (R) between acceptor and donor fluorescence intensity.

In the past years, many improvements have been made to the original Cameleons: fluorophores have been replaced or modified to obtain decreased sensitivity to pH and Cl^-, decreased photobleaching, variable Ca^{2+} affinity, improved dynamic range of FRET changes upon Ca^{2+} binding and reduced interference of the probe with endogenous calmodulin targets [21–25]. Moreover, different organelle-targeted Cameleons have been generated, directing the probe to the nucleus, plasma membrane, peroxisomes and mitochondria [26], endoplasmic reticulum [27], as well as the *cis*-medial [28] and *trans* Golgi apparatus [29] compartments.

As far as mitochondria are concerned, the Cameleons mentioned above were selectively targeted to the mitochondrial matrix and thus revealed the Ca^{2+} level within the lumen of the organelles. A unique sensor targeted to the outer membrane of mitochondria (OMM) is also presently available [30], allowing the evaluation of the amplitude and dynamics of Ca^{2+} hotspots at the OMM generated by the release of Ca^{2+} from the ER or entering the cell through plasma membrane channels.

To overcome problems related to the use of CaM as a Ca^{2+} sensor in FRET-based GECIs (in particular to avoid the interaction with CaM-binding proteins) one strategy that has been attempted is the complete substitution of this Ca^{2+}-binding domain. To this end, sensors structurally similar to the Cameleons have been generated where the Ca^{2+}-sensitive peptide is chicken skeletal muscle troponin C, TnC (TN-L15) or human cardiac TnC (TN-humTnC) [26].

Beyond the reported advantages, the use of GECIs for Ca^{2+} imaging presents some drawbacks. The first and most obvious is the need of recombinant expression (by transfection, viral infection or use of transgenic animals). For many cellular models these techniques are difficult to use and chemical indicators remain the first choice. In addition, GECIs, in general, have lower dynamic range and lower fluorescence intensity compared to chemical indicators that are still preferred in experiments when signal-to-noise is a critical aspect of the investigation. Moreover, giving the relatively low kinetics of most GECIs, rapid (ms) Ca^{2+} transients may be difficult to measure. Another flaw intrinsic in the structure of FPs is their pH sensitivity in the physiological range. In conclusion, in the near future we predict that the efforts of the specialists in the field will be focused on the increase of the dynamic range, the amelioration of the sensitivity and of the signal-to-noise ratio, the improvement of the kinetics and the reduction of the pH sensitivity of the FPs.

Role of mitochondria in Ca^{2+} homeostasis

In the previous paragraphs we have briefly described the main families of probes now available to monitor Ca^{2+} dynamics in living cells and the strategies to target them to different cellular compartments. We have focused our attention on probes targeted to mitochondria as these tools have led to some of the most important novel observations in the field of Ca^{2+} signalling. In the last section of this work, we will briefly summarize the state of the art in the field of mitochondria Ca^{2+} handling and future perspectives, in particular applications in in vivo models.

The ability of mitochondria to take up Ca^{2+} from the medium was first documented more than 50 years ago [31–33]. These seminal studies revealed that isolated mitochondria can rapidly accumulate into their matrix massive amounts of Ca^{2+} in an energy-dependent process. A few years later, the chemiosmotic hypothesis [34] provided the thermodynamic basis for explaining this mechanism. Indeed, in respiring mitochondria supplemented with oxygen and a carbon source, the proton pumping by the respiratory chain complexes from the matrix to the IMS generates an electrochemical H^+ gradient across the IMM, comprising a concentration component (around 1 pH unit) and an electrical component, which is negative inside the matrix (about −180 mV). This electrical gradient is the driving force that permits the accumulation of Ca^{2+} into the matrix.

Although the molecular identity of the mitochondrial Ca^{2+} uniporter (MCU) has been elucidated only very recently [35, 36], the idea that mitochondria exert an important role in the maintenance of cellular Ca^{2+} homeostasis was a dominant, although debated, concept since the 70s. The development of genetically encoded Ca^{2+} probes targeted to the mitochondrial matrix [7] helped to prove that, despite the apparent low affinity for Ca^{2+} of the uniporter, the location of mitochondria in very close proximity to the channels eliciting the release of Ca^{2+} from the endoplasmic reticulum (ER) [8, 37, 38] or the entry across the plasma membrane [30] allows the formation of transient microdomains of high [Ca^{2+}] near the mouth of these channels, with a prompt Ca^{2+} accumulation into the matrix. In the past few years, other components of the channel have been discovered that are capable of modulating the channel activity, making MCU-complex one of the most sophisticated ion channels described thus far (for an updated review, see [39].

The functional role of mitochondrial Ca^{2+} uptake is multifaceted. Three main functions have been reported: modulation of ATP synthesis, cell death activation and buffering/shaping of cytosolic Ca^{2+} rises. As far as ATP synthesis is concerned, a general consensus exists on the stimulatory role of mitochondrial Ca^{2+} uptake on the activity of three key dehydrogenases that feed electrons into the respiratory chain (i.e., pyruvate dehydrogenase, isocitrate dehydrogenase and α-ketoglutarate dehydrogenase), resulting ultimately in increased ATP synthesis [40]. Recently, Foskett's group reported that mitochondrial Ca^{2+} uptake, upon a constitutive IP3-mediated Ca^{2+} release from the ER, inhibits pro-survival mitophagy, promotes efficient mitochondrial respiration and maintains cellular bioenergetics [41]. Along the same line, Di Benedetto et al. demonstrated that the rise in matrix Ca^{2+} results in an increase in intramitochondrial cAMP that results in activation of ATP synthesis [42]. On the other hand, mitochondrial Ca^{2+} overload can result in cell death (for a review, see [43]). Excessive Ca^{2+} uptake in fact collapses the proton gradient between the two sides of IMM, by activating a large conductance pore on the IMM named permeability transition pore, PTP, thus causing bioenergetic impairment, release of proapoptotic factors, and eventually cell death. Physiological mitochondrial Ca^{2+} rises do not induce mitochondrial PTP opening per se, as demonstrated in a recent work by Pinton and colleagues using mitochondria targeted Aeq [44], but when concomitant with pro-apoptotic stimuli, like ceramide, PTP opening is facilitated by matrix Ca^{2+} increases (for a review, see [45]).

Finally, considering their buffering function, mitochondria are able to actively buffer and shape both local and bulk cytosolic Ca^{2+} rises. In the first case, the sites are those in close contact with ER or plasma membrane [30], where Ca^{2+} microdomains are created near the mouth of Ca^{2+} channels. The second case is exemplified in pancreatic acinar cells, where active mitochondria surrounding the granule region prevent the spreading of IP3–evoked cytosolic Ca^{2+} signals from the apical region towards the basolateral part of the cell [46].

Mitochondrially targeted GECIs in vivo

As far as chemical dyes are concerned, Rhod-2 (the only example of a mitochondrially targeted dye) appears quite difficult for in vivo use. Indeed, Rhod-2 is a non-ratiometric indicator, thus offering no compensation for movement artefacts inherent to live tissues. Moreover, a general limitation of chemical dyes is the difficulty to supply the dye to the tissue of interest.

On the contrary, GECIs can be delivered by standard approaches such as transgenesis, electroporation in situ and viral infection. Moreover, the use of ratiometric GECIs has the great advantage of avoiding movement artefacts. Despite these advantages, the examples of mitochondrially targeted GECIs in vivo are still scarce. Although mammalian cells have ben the main focus of attention (see below), other eukaryotic cells have also been used. For example, in plants, mitochondria-targeted YFP-fused Aeq [47] and Cameleons have been reported [48, 49]. Mitochondrially targeted ratiometric pericam, camgaroo-2 and Cameleons [50, 51] have also been expressed in vivo in Drosophila motor nerve terminals. A transgenic zebrafish expressing the Cameleon 2mt8YC2.60 was created to Ca^{2+} waves in embryonic myocytes [52].

Transgenic mice were developed targeting GFP-apo-Aeq to the mitochondrial matrix, and coelenterazine was introduced via injection into the tail vein [53]. Mitochondrial Ca^{2+} transients were then recorded in different conditions and whole body Ca^{2+} patterns recorded also in freely moving mice. Ca^{2+} uptake into mitochondria of mouse skeletal muscle during contraction was studied using Cameleon 2mt-YC2, electroporated into hindlimb muscles [54]. Similarly, mitochondrial Ca^{2+}-handling in fast skeletal muscle fibres was evaluated employing 4mtD3cpv [55].

The few examples reported above, though scarce, show an ample variety of in vivo models, ranging from plants to invertebrates to mammals, and have contributed to a deeper understanding of Ca^{2+} dynamics in vivo. In the near future, taking advantage of newly developed in vivo delivery techniques such as viral infection, it will be possible to expand the possibilities of in vivo GECIs applications, overcoming the requirement of crossing multiple strains and other laborious tasks typical of the current protocols for in vivo expression. Most relevant, with these new probes it will be possible to investigate the role of mitochondrial Ca^{2+} handling in vivo in animal models of human diseases.

Conclusions

Many examples are available underlying how the development of GECIs helped new advances in our understanding of cell pathophysiology in different fields, including endocrinology. For example, a milestone in our understanding of the role of mitochondria in cell physiology was obtained for the first time in chromaffin cells upon agonist stimulation of catecholamine release [56]. Similarly, the role of Ca^{2+} microdomains in the spatial control of insulin secretion in β-cells was dissected thanks to the development of a Ratiometric Pericam targeted to the plasma membrane [57]. In another work, Aequorin was targeted to OMM to unravel the role of high $[Ca^{2+}]_c$ in the proximity of mitochondria in the stimulation of steroidogenesis of bovine adrenal glomerulosa cells [58]. Moreover, a transgenic mouse expressing Ratiometric Pericam selectively in gonadotropin-releasing hormone (GnRH) neurons was generated, allowing real-time monitoring of $[Ca^{2+}]$ oscillations in brain slices [59]. These are only a few examples underlying the importance of studying intracellular Ca^{2+} dynamics and validating GECIs as useful probes, in particular for the evaluation of $[Ca^{2+}]$ in subcellular compartments. The ongoing efforts in developing new indicators and in ameliorating existing ones will further help increasing our knowledge in fields, like endocrinology, where Ca^{2+} fulfil a crucial role.

Furthermore, GECI-based approaches seem also to be the most promising for monitoring mitochondrial Ca^{2+} in live animals. Indeed, some examples are now available for in vivo Ca^{2+} measurements mainly with cytosolic probes, since they are better characterized and in general are easier to handle. On the contrary, organelle-targeted GECIs require a profound knowledge of both the sensor and the targeted organelle, since probes can be profoundly affected by the environmental conditions of each specific subcellular compartment.

A vast number of mitochondrial targeted GECI are now available with different Ca^{2+} affinities and require different microscope set-ups. Further improvements in instrumentation and the features of GECIs should be obtained to fully exploit these tools for in vivo. In the past years, a growing colour palette of FPs has been developed, and many efforts have been made to red-shift the excitation wavelength of the indicators, reducing phototoxicity, background fluorescence and facilitating deep imaging into the tissues. Moreover, an interesting challenge is the combination of differently coloured GECIs targeted to distinct organelles, to unravel the interconnection of Ca^{2+} signalling across different organelles, both in physiological and pathological animal models.

Acknowledgments We are grateful to Dr. Paulo Magalhães for critically reading the manuscript. The original work by the group was supported by grant FIRB from the Italian Ministry of University and Research (MIUR), by the Veneto Region "RISIB" and "Vegecell" projects, by the National Research Council (CNR) "Ageing" and "Eurobioimaging" projects to T.P.

Conflict of interest The authors declare that there are no conflicts of interest.

References

1. Paredes RM et al (2008) Chemical calcium indicators. Methods 46(3):143–151
2. Grynkiewicz G, Poenie M, Tsien RY (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 260(6):3440–3450
3. Rehberg M et al (2008) A new non-disruptive strategy to target calcium indicator dyes to the endoplasmic reticulum. Cell Calcium 44(4):386–399
4. Mank M et al (2008) A genetically encoded calcium indicator for chronic in vivo two-photon imaging. Nat Methods 5(9): 805–811
5. Allen DG, Blinks JR, Prendergast FG (1977) Aequorin luminescence: relation of light emission to calcium concentration–a calcium-independent component. Science 195(4282):996–998
6. Brini M et al (1995) Transfected aequorin in the measurement of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$). A critical evaluation. J Biol Chem 270(17):9896–9903
7. Rizzuto R et al (1992) Rapid changes of mitochondrial Ca^{2+} revealed by specifically targeted recombinant aequorin. Nature 358(6384):325–327
8. Rizzuto R et al (1998) Close contacts with the endoplasmic reticulum as determinants of mitochondrial Ca^{2+} responses. Science 280(5370):1763–1766
9. Bonora M et al (2013) Subcellular calcium measurements in mammalian cells using jellyfish photoprotein aequorin-based probes. Nat Protoc 8(11):2105–2118
10. Baubet V et al (2000) Chimeric green fluorescent protein-aequorin as bioluminescent Ca^{2+} reporters at the single-cell level. Proc Natl Acad Sci USA 97(13):7260–7265
11. Rodriguez-Garcia A et al (2014) GAP, an aequorin-based fluorescent indicator for imaging Ca^{2+} in organelles. Proc Natl Acad Sci USA 111(7):2584–2589
12. Baird GS, Zacharias DA, Tsien RY (1999) Circular permutation and receptor insertion within green fluorescent proteins. Proc Natl Acad Sci USA 96(20):11241–11246
13. Griesbeck O et al (2001) Reducing the environmental sensitivity of yellow fluorescent protein. Mechanism and applications. J Biol Chem 276(31):29188–29194
14. Nagai T et al (2001) Circularly permuted green fluorescent proteins engineered to sense Ca^{2+}. Proc Natl Acad Sci USA 98(6):3197–3202
15. Kettlewell S et al (2009) Changes of intra-mitochondrial Ca^{2+} in adult ventricular cardiomyocytes examined using a novel fluorescent Ca^{2+} indicator targeted to mitochondria. J Mol Cell Cardiol 46(6):891–901
16. Nakai J, Ohkura M, Imoto K (2001) A high signal-to-noise Ca(2+) probe composed of a single green fluorescent protein. Nat Biotechnol 19(2):137–141
17. Chen TW et al (2013) Ultrasensitive fluorescent proteins for imaging neuronal activity. Nature 499(7458):295–300
18. Akerboom J et al (2013) Genetically encoded calcium indicators for multi-color neural activity imaging and combination with optogenetics. Front Mol Neurosci 6:2
19. Miyawaki A et al (1997) Fluorescent indicators for Ca^{2+} based on green fluorescent proteins and calmodulin. Nature 388(6645):882–887

20. Persechini A, Lynch JA, Romoser VA (1997) Novel fluorescent indicator proteins for monitoring free intracellular Ca^{2+}. Cell Calcium 22(3):209–216
21. Nagai T et al (2004) Expanded dynamic range of fluorescent indicators for Ca(2+) by circularly permuted yellow fluorescent proteins. Proc Natl Acad Sci USA 101(29):10554–10559
22. Palmer AE et al (2006) Ca^{2+} indicators based on computationally redesigned calmodulin-peptide pairs. Chem Biol 13(5):521–530
23. Horikawa K et al (2010) Spontaneous network activity visualized by ultrasensitive Ca(2+) indicators, yellow Cameleon-Nano. Nat Methods 7(9):729–732
24. Evanko DS, Haydon PG (2005) Elimination of environmental sensitivity in a cameleon FRET-based calcium sensor via replacement of the acceptor with Venus. Cell Calcium 37(4):341–348
25. Truong K et al (2001) FRET-based in vivo Ca^{2+} imaging by a new calmodulin-GFP fusion molecule. Nat Struct Biol 8(12):1069–1073
26. Whitaker M (2010) Genetically encoded probes for measurement of intracellular calcium. Methods Cell Biol 99:153–182
27. Kipanyula MJ et al (2012) Ca^{2+} dysregulation in neurons from transgenic mice expressing mutant presenilin 2. Aging Cell 11(5):885–893
28. Wong AK et al (2013) Heterogeneity of Ca^{2+} handling among and within Golgi compartments. J Mol Cell Biol 5(4):266–276
29. Lissandron V et al (2010) Unique characteristics of Ca^{2+} homeostasis of the *trans*-Golgi compartment. Proc Natl Acad Sci USA 107(20):9198–9203
30. Giacomello M et al (2010) Ca^{2+} hot spots on the mitochondrial surface are generated by Ca^{2+} mobilization from stores, but not by activation of store-operated Ca^{2+} channels. Mol Cell 38(2):280–290
31. Deluca HF, Engstrom GW (1961) Calcium uptake by rat kidney mitochondria. Proc Natl Acad Sci USA 47:1744–1750
32. Vasington FD, Murphy JV (1962) Ca ion uptake by rat kidney mitochondria and its dependence on respiration and phosphorylation. J Biol Chem 237:2670–2677
33. Lehninger AL, Rossi CS, Greenawalt JW (1963) Respiration-dependent accumulation of inorganic phosphate and Ca ions by rat liver mitochondria. Biochem Biophys Res Commun 10:444–448
34. Mitchell P, Moyle J (1967) Chemiosmotic hypothesis of oxidative phosphorylation. Nature 213(5072):137–139
35. De Stefani D et al (2011) A forty-kilodalton protein of the inner membrane is the mitochondrial calcium uniporter. Nature 476(7360):336–340
36. Baughman JM et al (2011) Integrative genomics identifies MCU as an essential component of the mitochondrial calcium uniporter. Nature 476(7360):341–345
37. Rizzuto R et al (1993) Microdomains with high Ca^{2+} close to IP3-sensitive channels that are sensed by neighboring mitochondria. Science 262(5134):744–747
38. Csordas G, Thomas AP, Hajnoczky G (1999) Quasi-synaptic calcium signal transmission between endoplasmic reticulum and mitochondria. EMBO J 18(1):96–108
39. Marchi S, Pinton P (2014) The mitochondrial calcium uniporter complex: molecular components, structure and physiopathological implications. J Physiol 592(Pt 5):829–839
40. Denton RM (2009) Regulation of mitochondrial dehydrogenases by calcium ions. Biochim Biophys Acta 1787(11):1309–1316
41. Cardenas C et al (2010) Essential regulation of cell bioenergetics by constitutive InsP3 receptor Ca^{2+} transfer to mitochondria. Cell 142(2):270–283
42. Di Benedetto G et al (2013) Mitochondrial Ca(2)(+) uptake induces cyclic AMP generation in the matrix and modulates organelle ATP levels. Cell Metab 17(6):965–975
43. Pinton P et al (2008) Calcium and apoptosis: ER-mitochondria Ca^{2+} transfer in the control of apoptosis. Oncogene 27(50):6407–6418
44. De Marchi E et al (2014) The mitochondrial permeability transition pore is a dispensable element for mitochondrial calcium efflux. Cell Calcium 56(1):1–13
45. Rasola A, Bernardi P (2011) Mitochondrial permeability transition in Ca(2+)-dependent apoptosis and necrosis. Cell Calcium 50(3):222–233
46. Tinel H et al (1999) Active mitochondria surrounding the pancreatic acinar granule region prevent spreading of inositol trisphosphate-evoked local cytosolic Ca(2+) signals. EMBO J 18(18):4999–5008
47. Mehlmer N et al (2012) A toolset of aequorin expression vectors for in planta studies of subcellular calcium concentrations in Arabidopsis thaliana. J Exp Bot 63(4):1751–1761
48. Loro G et al (2013) The D3cpv Cameleon reports Ca(2)(+) dynamics in plant mitochondria with similar kinetics of the YC3.6 Cameleon, but with a lower sensitivity. J Microsc 249(1):8–12
49. Loro G et al (2012) Targeting of Cameleons to various subcellular compartments reveals a strict cytoplasmic/mitochondrial Ca(2)(+) handling relationship in plant cells. Plant J 71(1):1–13
50. Chouhan AK et al (2010) Presynaptic mitochondria in functionally different motor neurons exhibit similar affinities for Ca^{2+} but exert little influence as Ca^{2+} buffers at nerve firing rates in situ. J Neurosci 30(5):1869–1881
51. Ivannikov MV, Macleod GT (2013) Mitochondrial free Ca(2)(+) levels and their effects on energy metabolism in Drosophila motor nerve terminals. Biophys J 104(11):2353–2361
52. Mizuno H et al (2013) Transgenic zebrafish for ratiometric imaging of cytosolic and mitochondrial Ca^{2+} response in teleost embryo. Cell Calcium 54(3):236–245
53. Rogers KL et al (2007) Non-invasive in vivo imaging of calcium signaling in mice. PLoS One 2(10):e974
54. Rudolf R et al (2004) In vivo monitoring of Ca(2+) uptake into mitochondria of mouse skeletal muscle during contraction. J Cell Biol 166(4):527–536
55. Scorzeto M et al (2013) Mitochondrial Ca^{2+}-handling in fast skeletal muscle fibers from wild type and calsequestrin-null mice. PLoS One 8(10):e74919
56. Montero M et al (2000) Chromaffin-cell stimulation triggers fast millimolar mitochondrial Ca^{2+} transients that modulate secretion. Nat Cell Biol 2(2):57–61
57. Pinton P et al (2002) Dynamics of glucose-induced membrane recruitment of protein kinase C beta II in living pancreatic islet beta-cells. J Biol Chem 277(40):37702–37710
58. Brandenburger Y et al (1999) Measurement of perimitochondrial Ca^{2+} concentration in bovine adrenal glomerulosa cells with aequorin targeted to the outer mitochondrial membrane. Biochem J 341(Pt 3):745–753
59. Jasoni CL et al (2007) Cell type-specific expression of a genetically encoded calcium indicator reveals intrinsic calcium oscillations in adult gonadotropin-releasing hormone neurons. J Neurosci 27(4):860–867