Biochimica et Biophysica Acta 1787 (2009) 1317–1323

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Review

Measurements of mitochondrial calcium *in vivo*

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ARTICLE INFO

Article history:
Received 12 September 2008
Received in revised form 20 November 2008
Accepted 21 November 2008
Available online 6 December 2008

Keywords:
Aequorin
Cameleon
GFP
Microscopy
Mouse
Rhod-2

ABSTRACT

Mitochondria play a pivotal role in intracellular Ca^{2+} signalling by taking up and releasing the ion upon specific conditions. In order to do so, mitochondria depend on a number of factors, such as the mitochondrial membrane potential and spatio-temporal constraints. Whereas most of the basic principles underlying mitochondrial Ca^{2+} handling have been successfully deciphered over the last 50 years using assays based on *in vitro* preparations of mitochondria or cultured cells, we have only just started to understand the actual physiological relevance of these processes in the whole animal. Recent advancements in imaging and genetically encoded sensor technologies have allowed us to visualise mitochondrial Ca^{2+} transients in live mice. These studies used either two-photon microscopy or bioluminescence imaging of cameleon or aequorin-GFP Ca^{2+} sensors, respectively. Both methods revealed a consistent picture of Ca^{2+} uptake into mitochondria under physiological conditions even during very short-lasting elevations of cytosolic Ca^{2+} levels. The big future challenge is to understand the functional impact of such Ca^{2+} signals on the physiology of the observed tissue as well as of the whole organism. To that end, the development of multiparametric *in vivo* approaches will be mandatory.

1. Functional roles of Ca^{2+} in mitochondria

According to the endosymbiont hypothesis mitochondria are derived from some form of prokaryotes which were eventually taken up by the ancestors of eukaryotic cells [for reviews, see 1,2]. Instead of being digested, the engulfed organisms apparently managed to start a very successful mutual interaction with their hosts. To date, many important cellular functions of mitochondria have been deciphered, one of which being its role in the handling of Ca^{2+} ions [for review, see 3]. As described in detail elsewhere, in many eukaryotic cell types mitochondria play distinct roles in the Ca^{2+} game [for review, see 4,5], such as i) they can transiently accumulate large amounts of Ca^{2+} as a consequence of cytosolic Ca^{2+} increases, thus shaping the kinetics and amplitude of the Ca^{2+} signal, and ii) they can modulate the activity of Ca^{2+} channels by local buffering of Ca^{2+} influx through the plasma membrane or intracellular Ca^{2+} channels. Furthermore, Ca^{2+} itself may also modulate important processes inside mitochondria, such as energy metabolism [for review, see 6,7], hormone metabolism [for review, see 8], or the release of apoptotic factors [for review, see 9]. Thus, on the one hand, Ca^{2+} is managed by mitochondria which affects its cytosolic levels but, on the other hand, the ion also actively influences the metabolism of the organelle. An intriguing feature of mitochondrial Ca^{2+} handling is its interaction with the endomembrane system, mainly with the endoplasmic reticulum [for review, see 7,10]. This strategic cross-connection of two major endomembrane network systems would be ideally suited to control many vital cellular functions. Through the levels of cytosolic Ca^{2+} as well as the stimulation of ER-Ca^{2+} release by a range of external cues, mitochondria are hooked to the outside world and thus act as important homeostatic sensors of the cellular environment [for review, see 8]. Mitochondrial Ca^{2+} handling has been extensively studied by different approaches, ranging from mitochondrial suspensions (*in vitro*), cell culture, tissue slices (*in situ*), and live animals (*in vivo*). Although most of the mechanisms concerning mitochondrial Ca^{2+} handling have been elucidated *in vitro* or in cultured cells, we still know very little about the actual physiological role of these processes in the context of the whole organism. This is mainly due to technical limitations, but recent sensor developments have provided new insights into mitochondrial Ca^{2+} signalling under highly physiological conditions *in vivo*. This contribution aims at summarizing the results obtained in the last few years regarding *in vivo* measurement of mitochondrial Ca^{2+} handling, a field that is still in its infancy. Readers interested in *in vitro* mitochondrial Ca^{2+} homeostasis are referred to other contributions in this special issue for detailed discussions of the vast literature available.

In the following paragraphs, we will first try to outline the major considerations of when and where *in vivo* measurement of mitochondrial Ca^{2+} handling is the most desirable approach. We will then analyze the current challenges in these *in vivo* studies. We will conclude with an overview of possible solutions to overcome at least some of the present technical limitations.

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doi:10.1016/j.bbabio.2008.11.012

2. Limitations of *in vitro* approaches

2.1. Mitochondrial membrane potential

Initial observations of Ca^{2+} transport conducted *in vitro* using isolated mitochondria were already made in the 1960s and 1970s [for review, see 5]. At that time, mitochondrial Ca^{2+} uptake and release were perceived as active and passive processes, respectively. The chemiosmotic hypothesis allowed a better clarification of these vague concepts and, at present, the uptake of Ca^{2+} into mitochondria is understood as an electrogenic process mediated by the ruthenium red-sensitive mitochondrial Ca^{2+} uniporter (MCU), with the mitochondrial membrane potential acting as the driving force for Ca^{2+} accumulation into the matrix [for reviews, see 11,12]. This concept was recently reinforced by the electrophysiological characterisation of mitoplasts showing high Ca^{2+} currents that occur across the inwardly rectifying MCU in the presence of physiologically relevant values of membrane potential [13]. Under physiological conditions, the rate limiting step in mitochondrial Ca^{2+} accumulation is the Ca^{2+} concentration to which the organelle is exposed (i.e., most often the local Ca^{2+} concentration). It is still unclear whether and to what extent physiological changes in membrane potential can affect the rate of mitochondrial Ca^{2+} uptake.

Regarding Ca^{2+} efflux from mitochondria, there is now a general consensus that this process is mediated by $Na^{+}(H^{+})/Ca^{2+}$ antiporters, with the driving force being the ion gradients across the inner mitochondrial membrane. Given that the antiporters are electrogenic (they exchange 3 $Na^{+}(H^{+})$ for 1 Ca^{2+}), their function is also affected by membrane potential [for review, see 12]. A number of other parameters may also be of importance for the efficient transport of Ca^{2+} into and out of mitochondria. As far as these are relevant for our considerations of *in vivo* measurements, they will be addressed below.

2.2. Space, time and amplitude

Apart from the mitochondrial inner membrane potential, the quantity and the speed of Ca^{2+} taken up into mitochondria mostly depends on the amount of this ion present at mitochondrial Ca^{2+} uptake sites and the duration of such events. Since Ca^{2+} levels are typically highest in close proximity to Ca^{2+} channels, the spatial distance of MCUs from these spots is of crucial relevance for the uptake of the ion into the matrix. In fact, whereas most text books traditionally depict mitochondria as dot- or worm-like structures distributed all over the cytoplasm in a seemingly random fashion, these organelles are now increasingly understood as being highly interactive, maintaining intense mutual signalling and metabolite exchange with the rest of the cell. Regarding Ca^{2+} signals, the ER seems to be the prime interaction partner [for review, see 10]. A wealth of literature has demonstrated tight morphological and functional links between ER-Ca^{2+} exit and putative mitochondrial Ca^{2+} uptake sites [e.g. 14,15] [for reviews, see 7,10]. Furthermore, although randomly distributed mitochondrial networks are commonly found in many tissue culture cells, this is only rarely the case in the organism. Indeed, mitochondrial networks *in situ* mostly exhibit striking localisation patterns, which reflect their physiological roles in a given tissue. Examples for such specific patterns are (i) mitochondrial perigranular localisation in pancreatic acinar cells [16,17], (ii) enrichment at vesicle release sites of adrenal chromaffin cells [18], and (iii) striated localisation in skeletal and cardiac muscle fibres [for review, see 19]. Such specific subcellular distribution is apparently further enhanced by the occurrence of distinct mitochondrial microdomains, wherein the matrix Ca^{2+} content may vary by orders of magnitude [18]. For some of these cases, strong evidence has been provided suggesting that the microdomains play pivotal roles in shaping Ca^{2+} signals which might be important for synaptic plasticity, transcriptional regulation, exocytic processes, muscle tissue contractility, or tunnelling of Ca^{2+} transients from one cell pole to the other, to name just a few [for reviews, see 8,17,20]. In other words, apart from mitochondrial integrity and membrane potential, also the spatial arrangement of different organelles towards mitochondria as well as tissue-specific mitochondrial localisation patterns are critical for a proper understanding of this organelle function.

Besides spatial separation, the duration and frequency of Ca^{2+} transients is key to the transformation of external cues into intracellular responses. Mitochondria have been shown to participate in both the shaping of Ca^{2+} transient kinetics as well as the transformation of these signals into matching outputs. Mitochondria may shape Ca^{2+} signals by different means, including propagation of Ca^{2+} waves, induction of oscillations, and sustained release of Ca^{2+} in synapses upon intense stimulation [for review, see 20]. A prime functional output of mitochondria in response to Ca^{2+} transients lies in the adaptation of energy metabolism to the cellular demand [for review, see 6]. Again, these functions depend on a number of systemic parameters, which are hard to predict in a bottom-up approach that is just based on the molecular understanding of mitochondria.

Finally, the amplitude of Ca^{2+} transients might considerably change depending on experimental conditions. In cell culture approaches, often supramaximal stimuli leading to large and long-lasting elevation of cytosolic Ca^{2+} levels are used to show the principal existence of a certain postulated uptake mechanism [e.g. 14,16,21–23]. However, rises in cytosolic Ca^{2+} occurring under physiological conditions may be often highly localised and limited in amplitude, such as mitochondrial Ca^{2+} marks in striated muscle [24], and cytosolic Ca^{2+} sparks in neurons and muscle [for review, see 25] which might eventually trigger a full-blown global Ca^{2+} transient by calcium-induced–calcium release (CICR) mechanisms.

2.3. Metabolic aspects and external cues

Mitochondrial Ca^{2+} uptake is driven by the large mitochondrial inner membrane potential which either arises from respiration or from ATP consumption. A corollary is that the robustness of Ca^{2+} uptake, as well as the interpretation of physiological consequences of such uptake, critically depends on the metabolic activity and route (anaerobic/oxidative) of a given experimental system. Interestingly, in a number of cancer cell lines (including the frequently studied HeLa cells) anaerobic metabolism prevails over oxidative, while the situation might be considerably different in many primary cell lines, intact tissues and organisms [26].

Finally, although mitochondria are located inside cells, ensheathed by two membranes, apparently lacking any direct contact to the cell surface, these organelles are far from being buried in the cell; indeed, they are known to be, for example, prime sensors and actuators of blood glucose and oxygen levels in the pancreas, the hypothalamus and the carotid arteries [for review, see 8]. By different means, including a rise of the cellular ATP/ADP.Pi ratio or of the cytosolic Ca^{2+} level, mitochondria then modulate in their sensing tissues the appropriate release of insulin or catecholamines.

3. *In vivo* measurements

So far, measurements of mitochondrial Ca^{2+} uptake and release, as well as the role(s) of these ion fluxes on cellular and tissue homeostasis, have been almost exclusively investigated in biochemical assays or using tissue culture cells. Collectively, these studies have undoubtedly led to a broad understanding of the basic mechanisms underlying Ca^{2+} handling by mitochondria. Unavoidably, they have also triggered a plethora of questions concerning their actual physiological relevance in the live organism. Critical factors like the amplitude and the spatio-temporal arrangement of Ca^{2+} transients, the orientation and organisation of mitochondrial networks and their interacting organelles, the metabolic activity of a given tissue of

interest and its influence on the general physiology of the organism can hardly be mimicked *in vitro*. Thus, wherever these features might play any significant role, it is highly desirable to carry out all measurements *in situ* and *in vivo*. At present, a series of probes allows for the direct monitoring of Ca^{2+} fluxes into and out of mitochondria, but their application to *in vivo* studies is still quite limited. In the following, we will briefly introduce these currently available probes and discuss their advantages and draw-backs.

3.1. Chemical probes

As discussed above, energised mitochondria are endowed with a highly negative membrane potential across the inner membrane, enabling the accumulation of cationic dyes, such as tetramethylrhodamine, which is frequently used as marker for mitochondrial localisation and potential. A Ca^{2+}-dependent derivative, rhod-2 [27], was introduced in the mid-nineties as a selective mitochondrial Ca^{2+} sensor [28,29]. Although targeting of rhod-2 into mitochondria has been shown to be problematic in a some cases (see below), it has been used in a number of studies investigating mitochondrial Ca^{2+} handling of cultured cells and slice preparations. The K_D for Ca^{2+} of this probe, which is now commercially available from a couple of sources, was found to be 570 nM *in vitro*, while calibrations in live cells usually revealed slightly higher values, a phenomenon that is often observed for calcium sensors in a biological environment. As in the case of other chemical probes, rhod-2 is mainly used in its cell-permeant acetoxymethyl (AM) ester. Unlike other probes, the delocalised positive charge present in rhod-2's AM moiety allows the accumulation of this dye within the mitochondrial matrix, where it is hydrolysed and trapped [30]. This property, allied to its spectral characteristics (excitation and emission in the green-red part of the visible spectrum make it less susceptible to cellular autofluorescence), have made rhod-2 the fluorescent Ca^{2+} indicator of choice amongst the currently available chemical dyes to study mitochondrial Ca^{2+} handling. However, compared to other Ca^{2+} sensors such as the ratiometric dyes fura-2 or indo-1, rhod-2 responds to increasing Ca^{2+} levels not by a change in the emission and/or excitation spectrum, but by a rise in fluorescence emission; i.e., rhod-2 is an intensity-based Ca^{2+} sensor. This is one of its major drawbacks for measurements in live tissue, since there is no compensation for movement artefacts. Another serious issue concerns uneven distribution of the dye within mitochondria [31], which could lead to over- or underestimation of the Ca^{2+} content in some regions.

Furthermore, targeting efficiency is often problematic with rhod dyes. While rhod-2 was reported to show reasonably good mitochondrial localisation in a number of preparations, such as striated muscle cells [24,32–53], glial [54–61], neuronal [59,62–77], or acinar cell cultures [78–84], in other cases important quantities of the probe were detected in the cytosol, in non-specific accumulations or extramitochondrial localisations [85]. In perfused rabbit heart, rhod-2 was actually found to be completely cytosolic [86–88]. The overt discrepancies in probe distribution in different experimental systems may be at least partially explained by the uptake mode of the dye into cells and mitochondria: since rhod-2 as such is membrane impermeant, rhod-2 AM is used for loading. Once inside the cell, the ester is cleaved, leaving the charged rhod-2 compound trapped intracellularly. However, rhod-2 AM has to penetrate also the mitochondrial membranes before being hydrolysed and it is likely that removal of only one of the four AM groups is sufficient to prevent the accumulation of the dye into the matrix; thus, tissue-dependent differential activity of cytosolic esterases may at least in part explain the variable efficacy of mitochondrial accumulation in different tissues. This hypothesis is corroborated by the observation that loading of rhod-2 AM at low temperatures – and thus at low esterase activity – is beneficial for the targeting efficiency of the dye [32,89]. Evidently, such protocols are hardly applicable in many living organisms, in particular in homeothermic animals.

Additional problems that, to our knowledge, have not been addressed until now concern the Ca^{2+} buffering capacity and the potential toxicity of the AM hydrolysis. As to the first, usually investigators tend to choose brighter cells for imaging; this also means that the selected cells bear elevated concentrations of a high affinity Ca^{2+} chelator, and are therefore kept under conditions where mitochondrial Ca^{2+} buffering may be severely increased. As to the second, it has to be stressed that, as for all other AM esters, the hydrolysis products are acetate and formaldehyde, and, to our knowledge, no systematic investigation of the potentially harmful effects of these compounds on mitochondrial functions has been carried out.

A more general limitation of any chemical dye with respect to its use *in vivo* is its supply to the tissue of interest. Whereas genetically encoded probes can be delivered by standard transgenetic approaches (see below), chemical probes have to be applied in any case either directly to the tissue or systemically, e.g., via the blood stream. Although this might pose severe problems in a number tissues, recent technological advancements, such as the multi-cell bolus loading technique [90,91], now allow a series of cellular parameters to be studied *in situ* and *in vivo* using chemical sensors.

3.2. Genetically encoded probes

A major advantage of genetically encoded over chemical Ca^{2+} sensors is that they can be efficiently targeted into subcellular compartments, such as the endoplasmic reticulum, the nucleus or mitochondria. For this purpose, an arsenal of targeting sequences is now available to guide proteins according to the signal hypothesis [92,93] into various specific localisations within cells. In the case of mitochondrial probes, mostly short N-terminal peptides from the yeast cytochrome *c* oxidase (COX) subunit IV (12 amino acids) or the human COX subunit VIII (36 amino acids, used in all *in vivo* studies so far) were cloned in front of the actual sensor coding sequence. Notably, both, sensor activity as well as targeting efficiency were found to vary according to the used peptide sequence. Furthermore, targeting efficiency appears to inversely correlate with sensor complexity. Finally, a systematic analysis of these factors using distinct GFP-based Ca^{2+} sensors targeted with different sequences into mitochondria [94] revealed that the use of duplicated N-terminal targeting sequences can strongly increase targeting efficiency. As to aequorin, its targeting to the mitochondrial matrix is highly efficient with a single COX VIII targeting sequence and there is no need to change this import peptide. In any case, efficiency of targeting needs to be properly verified, in particular for GFP-based probes, for any new experimental setup.

3.2.1. Aequorin

Together with GFP, the photoprotein aequorin is responsible for the green bioluminescent touch response of the marine hydrozoan jellyfish, *Aequorea victoria* [95]. While GFP plays the role of a bioluminescence resonance energy transfer (BRET) acceptor in this response, aequorin plays the role of donor and delivers the energy necessary to trigger the BRET reaction [96]. It does so by peroxidizing (and thus consuming) an aromatic substrate, coelenterazine, upon activation by calcium ions [for review, see 97]. In the absence of the GFP acceptor molecule, the aequorin-coelenterazine reaction releases blue light in a stoichiometrically calcium-dependent manner. This phenomenon was exploited soon after its discovery in 1962 with scientists using biochemically isolated aequorin to measure intracellular Ca^{2+} transients [98,99]. With the publication of the aequorin sequence in 1985 [100], Ca^{2+} measurements in live cells using genetically encoded aequorin became an important tool for the understanding of cytosolic calcium handling. Regarding the concept of

subcellular compartmentation of calcium signalling and particularly with respect to the importance of mitochondria in this phenomenon, targeting of aequorin into these organelles was a major step forward [14,101]. In fact, this and a number of following investigations using mitochondrial aequorin, led to a revival of mitochondria as key players in the Ca^{2+} game, showing their tight interaction with the ER network and many of the physiological functions now well-known to the community [for review, see 10].

For a number of reasons, aequorin could be an almost ideal sensor for mitochondrial calcium measurements in live organisms. First, it can be genetically expressed and can therefore be introduced into model organisms such as *Drosophila* or mouse by standard transgenic methods [102,103]. Second, aequorin efficiently folds in most cell types into a fully functional protein. Third, using appropriate targeting sequences, aequorin can be efficiently directed into virtually any specific location inside the cell [for review, see 104]. Fourth, aequorin can be genetically modified, yielding mutants that allow us to measure Ca^{2+} from the submicromolar to the millimolar range of concentrations [18]. Fifth, aequorin consumes its substrate in a stoichiometric and almost irreversible manner; aequorin-based light production is therefore highly quantitative [105]. Sixth, aequorin actively produces light upon binding of calcium; therefore, no excitation light producing severe background signals in whole animal imaging approaches is needed [102,103]. Finally, aequorins can be coupled by genetic means to appropriate BRET acceptor molecules, such as GFP, thereby permitting the construction and simultaneous usage of differently targeted sensors or tuning the emission wavelength for higher tissue penetration efficiency [106,107]. A limitation of the aequorin approach is the low amount of Ca^{2+}-dependent light production, which can be particularly challenging upon targeting of the sensor into subcellular compartments [108]. Therefore, aequorin measurements usually employ cell population analyses with relatively low spatio-temporal resolution.

Recently, Rogers et al. have described the first aequorin-based *in vivo* monitoring of mitochondrial Ca^{2+} handling [103]. These authors show the generation of a conditional knock-in mouse line ubiquitously expressing a mitochondrially targeted GFP-aequorin (mtGA) fusion protein. In this mtGA sensor, the aequorin moiety acts as Ca^{2+} sensor, which delivers emission energy to the GFP acceptor molecule in a BRET process similar to the one observed in the *Aequorea* medusae. Although BRET leads to a partial loss of emission energy, addition of the GFP moiety is beneficial, because it emits longer wavelength light with higher tissue penetration depth than the aequorin alone would do. The mtGA could be shown to be nicely localised in the mitochondrial compartment [103]. Upon systemic application of coelenterazine, mitochondrial Ca^{2+} signalling could be reliably studied in different tissues using a bioluminescence imaging device over several hours. This finding came as a positive surprise, since coelenterazine (in this case applied systemically to the animal) is known to be consumed immediately upon interaction with aequorin and Ca^{2+}. Using nerve-stimulation of hindlimb muscles in anaesthetised animals the authors demonstrated the uptake of Ca^{2+} into mitochondria during single twitch and tetanic contractions, thereby confirming a precedent *in vivo* study employing a mitochondrial cameleon (2mtYC2, see below). While the spatio-temporal resolution of the 2mtYC2 measurements were superior to the ones obtained with mtGA, the latter approach profited from being completely non-invasive, allowing also the observation of Ca^{2+} signals in the live, unrestrained and non-anaesthetised animal. This delivered insights into the development-dependent formation of whole body mitochondrial calcium wave patterns. Future studies employing tissue-selective expression patterns of mtGA in combination with appropriate physiological questions promise to provide important new data regarding the role of mitochondrial calcium signalling in physiology.

3.2.2. GFP-based Ca^{2+} sensors

GFP, as well as its spectral mutants and functionally similar fluorescent proteins (XFPs) from other cnidarian species, have revolutionised cell biological research. Although described already in 1962 as a side product of the aequorin purification protocol [95], GFP became a major focus of biological research only more than 30 years later upon the publication of its DNA sequence [109] and the proof of its heterologous expression in *C. elegans* [110] and mammalian cells [111]. Apart from its early employment as gene reporter or protein localisation tool, XFPs were soon developed further to be usable as *bona fide* measuring instruments. In particular, Roger Tsien, Atsushi Miyawaki and colleagues have been quite successful in this field, setting up custom-made, modular tools that allow to monitor dynamic changes of intracellular parameters by changes in the fluorescent properties of modified XFPs [for review, see 112]. Typically, such probes consist of a sensing fluorescence modulator domain (e.g., calmodulin and its binding peptide in the Ca^{2+}-sensing cameleon probes) and one or more XFPs. Upon binding of the cellular factor which is under investigation (e.g., Ca^{2+}) the sensor-modulator domain changes its conformation and influences the fluorescence of the XFP(s). Currently, two major groups of probes employ inducible changes either in fluorescence intensity (and/or excitation spectrum) or in FRET (Förster resonance energy transfer). Well known examples for the first group are camgaroo, pericams [113], pHfluorins [114] and mtAlpHi [115]. Widely used exponents of the second group are cameleons [116,117], cAMP and cGMP probes [118–121], and enzyme activity probes [122–125]. Like aequorin, also XFP-based sensors can be easily targeted into virtually any kind of subcellular compartment by means of appropriate targeting sequences, although targeting efficiency needs to be controlled properly and sometimes necessitates tuning [94]. Currently, different XFP-based sensors are employed for *in vitro* and – more importantly for the topic of this review – *in vivo* visualisation of Ca^{2+} dynamics. Henceforth these will be termed FCIPs (fluorescent Ca^{2+} indicator proteins) and include, to date, FIP-CB_{SM} [126], camgaroo [127], pericam [127], GCaMP [128] and, in particular, cameleons [116]. Originally, most of these probes were designed using the calmodulin-M13 peptide (the 25-residue long calmodulin binding peptide of myosin light chain kinase) pair as Ca^{2+}-dependent modulator of XFP fluorescence. Since calmodulin is an abundant signalling molecule in many cell types, interference between the FCIPs and endogenous calmodulin could not be excluded. Nonetheless, these sensors worked rather well in many different tissue culture approaches, in *C. elegans* [129–137], and the fruit fly [138–150] upon stable expression.

In vertebrates *in vivo*, stably expressed FCIPs, particularly first generation yellow cameleons, showed weak performance and the FRET responses upon Ca^{2+} transients were severely dampened [151,152]. This led to the exploration of inducible or transient FCIPs expression in mammals [153–155] as well as of different molecular designs of FCIPs. The most prominent examples of such modified FCIPs include high dynamic range cameleons (YC6.1, with CaM-dependent kinase kinase sandwiched between the N- and C-termini of calmodulin) [156], cameleons with re-engineered calmodulin and M13 peptide [117,157,158], and cameleon-like Ca^{2+} sensors based on troponin C as 'Ca^{2+} nose' [128,159]. Altogether, these different approaches led to a number of insights mainly into cytosolic Ca^{2+} handling in brain [for recent reviews, see 160,161].

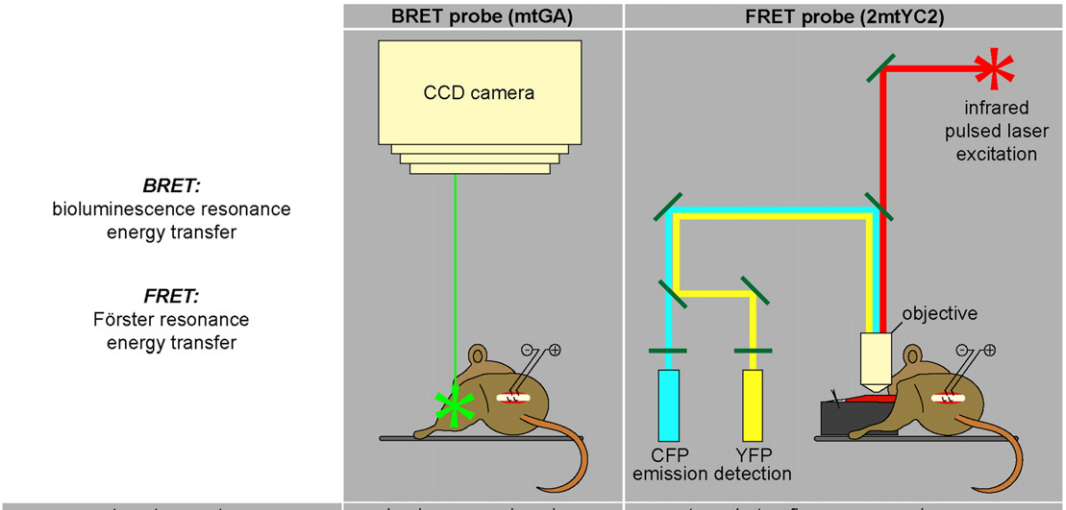
The literature on organellar Ca^{2+} handling in live vertebrate tissue using FCIPs is up to now much more limited [154,155]. Both studies employed *in vivo* electro-transfer of cDNAs encoding different cameleon FCIPs into hindlimb muscles, leading to consistent sensor expression for at least three weeks upon transfection. Ca^{2+} transients in the cytosol [154], in mitochondria [154] or in the sarcoplasmic reticulum [155] were measured upon application of different skeletal muscle contraction regimes using two-photon *in vivo* microscopy. These investigations first showed that also FCIPs containing the

calmodulin-M13 interaction pair can, in principle, be used in vertebrate tissue, at least in transient transfections. Second, they demonstrated the high capability of the ratiometric cameleon FCIPs to correct even for the comparably strong movement artefacts, which naturally occur during muscle contraction. With respect to mitochondria in vertebrate muscle, regular uptake of Ca^{2+} into these organelles during single twitches as well as upon tetanic stimulation was demonstrated for the first time under highly physiological conditions and *in vivo* [154] in a fast-twitch tibialis anterior muscle. Given that very similar results were recently obtained also *in vivo* with the aequorin-based sensor mtGA [103], Ca^{2+} uptake into vertebrate muscle mitochondria appears to be a basic and robust feature. It is therefore tempting to speculate that vertebrate muscle actually uses this activity-dependent uptake for the adaptation of the mitochondrial energy metabolism to the muscle contraction-driven ATP-demand, as suggested also for other tissue types [for review, see 8].

Direct comparison of the data employing mitochondrial GFP-aequorin (mtGA) [103] or cameleon (2mtYC2) [154] (Fig. 1, schematic of experimental setups) reveals further similarities. First, in both cases intense tetanic stimulation led to mitochondrial Ca^{2+} elevations that persisted for a couple of seconds longer than the actual duration of the stimulus, suggesting a transient storage function of the organelle at high Ca^{2+} loads. Second, upon single twitches, the Ca^{2+} transients were apparently much shorter, with a completion time in the millisecond range. Pharmacologic interference with the mitochondrial Na^{+}/Ca^{2+} exchanger slowed down the Ca^{2+} decay, suggesting that this was actually due to active expulsion of Ca^{2+} from mitochondria rather than due to Ca^{2+} precipitation or other mechanisms [154]. Finally, a clear positive correlation between muscle activity and the dimension of mitochondrial Ca^{2+} was observed [103]. Both, the mtGA and the 2mtYC2 approaches display advantages and disadvantages, which are largely complementary. While the mtGA measurements can be carried out in a completely non-invasive manner and thus also on behaving animals, fluorescence microscopy usually asks for sterical fixation of the object of interest, unless particular, sophisticated methods such as implanted miniature microscopes [162] are used. A further advantage of the mtGA approach is its wide field of observation, i.e., a whole animal can be surveyed, in contrast to fluorescence microscopy, which will be limited to an optical field of at most few square millimetres. Again, new imaging technologies such as selective plane illumination microscopy (SPIM) might, in future, overcome this limitation of fluorescence microscopy [163]. As a bioluminescence approach, mtGA can also be advantageous, because it deals with virtually zero background noise, whereas any type of fluorescence microscopy has to cope with signals originating from sources other than the sensor itself, which deteriorates its signal-to-noise ratio. However, aequorin bioluminescence also comes at the expense of comparably low photon yield, leading to an important trade-off between image acquisition speed and spatial resolution. As such, fluorescence microscopy is usually much faster than bioluminescence measurements and it exhibits significantly better spatial resolution. In conclusion, the mtGA- and 2mtYC2-based approaches appear to be pretty complementary raising big hopes for future research addressing mitochondrial Ca^{2+} handling in live animals.

4. Future challenges

At the time of writing, the aequorin-based bioluminescence and the cameleon-based fluorescence approaches seem to hold most promises for a reliable monitoring of mitochondrial Ca^{2+} handling in live animals. We are still far from a satisfying tool set, though. First of all, both assay systems exhibit severe inherent limitations (see above), which might be partially solved by future technological achievements,



	BRET probe (mtGA)	FRET probe (2mtYC2)
imaging mode	luminescence imaging	two-photon fluorescence microscopy
observation field	wide (cm range)	small (µm - mm range)
spatial resolution	low (supracellular, ≥ 100 µm)	high (subcellular, ≤ 0.1 µm)
temporal resolution	medium - low (0.03-25 Hz)	medium (1-4 Hz)
useful with slightly moving samples, anaesthetised animals, behaving animals?	yes yes yes	yes yes no

Fig. 1. Schematic of the experimental setups currently used to measure mitochondrial Ca^{2+} signalling *in vivo*. Left picture: BRET imaging approach; the anaesthetised, stably mtGA expressing animal is placed under a luminescence imager equipped with a high-sensitivity CCD camera. During nerve-electrode evoked muscle contraction, which leads to a transient rise of mitochondrial Ca^{2+} levels, bioluminescence light is emitted from the coelenterazine-injected animal. BRET imaging can also be performed with the unrestrained, behaving animal. Right picture: FRET imaging approach; the anaesthetised, 2mtYC2 cameleon expressing animal is placed under a two-photon laser scanning microscope, equipped with a high resolution water immersion objective. Upon excitation of Cyan Fluorescent Protein (CFP) using pulsed infrared laser light, CFP and Yellow Fluorescent Protein (YFP) emission signals of the cameleon sensor are detected simultaneously by photomultiplier tubes. Upon nerve-electrode evoked muscle contraction, the increase in mitochondrial Ca^{2+} levels leads to a change in FRET, measurable by a variation in the YFP-to-CFP emission ratio. The table below the drawings compares major features of the BRET and FRET imaging methods.

including better BRET acceptors and higher sensitivity cameras for the bioluminescence assay and fast, large field microscopy with high spatial resolution and low photodamage [163] for the fluorescence approach. Second, being able to just see mitochondrial Ca^{2+} dynamics in a live, complex organism does not lead to the understanding of their physiological meaning unless they can be correlated to further cellular parameters under well-defined experimental conditions. Apparently, such parameters can be manifold, depending on the question of interest. In the context of muscle physiology, these could be factors like contractile kinetics and muscle force, mitochondrial membrane potential and metabolic activity, pH, Ca^{2+} levels in cytosol and sarcoplasmic reticulum, to name just a few. Actually, bioindicators are available for some of these, but their implementation in combination with mitochondrial Ca^{2+} measurement *in vivo* remains a major challenge. However, our increasingly comprehensive understanding of XFPs [164–166], is leading to a growing colour palette of fluorescent proteins [167–171]. Combined with recent advancements in the fields of spectral unmixing of fluorescent dye labels [172], multiple ratiometric FRET imaging [173] and novel imaging techniques this may soon allow for deeper insights into the signal–function relationship of mitochondrial Ca^{2+} handling in live organisms.

Acknowledgements

RR was supported by the European Community (HPMF CT200201543.01), the University of Padova and the Deutsche Forschungsgemeinschaft (RU923/1-1). The original work of the authors has been supported by grants from the Italian Ministry of University, the Veneto Region grant “Biotech 2” and grants from Italian Telethon. We are grateful to Dr. Magalhães for critical reading of the manuscript.

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