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Organelles in focus

Mitochondrial biosensors

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ABSTRACT

Biosensors offer an innovative tool for measuring the dynamics of a wide range of metabolites in living organisms. Biosensors are genetically encoded, and thus can be specifically targeted to specific compartments of organelles by fusion to proteins or targeting sequences. Mitochondria are central to eukaryotic cell metabolism and present a complex structure with multiple compartments. Over the past decade, genetically encoded sensors for molecules involved in energy production, reactive oxygen species and secondary messengers have helped to unravel key aspects of mitochondrial physiology. To date, sensors for ATP, NADH, pH, hydrogen peroxide, superoxide anion, redox state, cAMP, calcium and zinc have been used in the matrix, intermembrane space and in the outer membrane region of mitochondria of animal and plant cells. This review summarizes the different types of sensors employed in mitochondria and their main limits and advantages, and it provides an outlook for the future application of biosensor technology in studying mitochondrial biology.

1. Introduction

Genetically encoded sensors have revolutionized the fields of biochemistry, since they offer a convenient tool to measure, in vivo, several parameters, e.g. metabolite dynamics, redox state and molecular dynamics. Since their first appearance about two decades ago, over 200 different biosensors are currently available and this list is constantly expanding (http://biosensor.dpb.carnegiescience.edu/). Genetically encoded sensors were engineered on the basis of proteins able to report availability of a substrate, or a physical–chemical state, and they provide reports in the form of luminescence or, more commonly, fluorescence changes. In their simplest form, the reporter itself is sensitive to the condition tested. This is the case for aequorin, a jellyfish protein that emits light in response to Ca^{2+} binding, or of different members of the green fluorescent protein (GFP) family, used as direct sensors for pH, superoxide or the redox status (Rizzuto et al., 1992; Hanson et al., 2004; Wei-LaPierre et al., 2013). For many other substrates, however, it was necessary to couple the reporter (usually, one or more FP) to a protein that specifically changes its conformation upon binding to its substrate. Ideally, sensors are ratiometric, meaning that their response is independent from protein concentration. In order to engineer ratiometric sensors, often two FPs with different spectral properties are employed. The FP pair may either interact via Förster resonance energy transfer (FRET), or the additional FP is used as a normalizing value for sensor concentration. Another type of genetically encoded sensors employs a single FP. However, some single-FP sensors can be considered ratiometric when their excitation spectra present two peaks which are inversely affected by the substrate binding to the sensor. Hence their ratio, rather than the absolute fluorescence intensity, can be used as response readout (Jones et al., 2013).

Biosensors offer several advantages over chemical dyes and traditional quantification analyses: they are generally more substrate-specific and, being genetically encoded, are minimally invasive and do not pose problems of heterogeneity of probe uptake by different cells (Jones et al., 2013). Moreover, by selecting the adequate promoter it is possible to restrict expression of the sensor either temporally (by conditional promoters, as for the redox sensor roGFP under control of *GAL4* (Liu et al., 2012)) or spatially (by tissue-specific promoters). Likewise, spatial expression can be modulated within a cell, by attaching leading peptides that direct the sensors to specific organelles or compartments, or by creating chimaeras of sensor fused to localized proteins. It is therefore possible to measure metabolites in individual organelles of single cells, rather than populations.

Mitochondria are complex organelles present in every eukaryotic cell. In addition to producing energy, mitochondria are a source of reactive oxygen species (ROS) and play a central role in the regulation of many processes, such as apoptosis or as storage of secondary messengers (Brookes et al., 2004). Such a complexity in the physiology is reflected in the elaborate structure of mitochondria. The presence of a double membrane system defines at least

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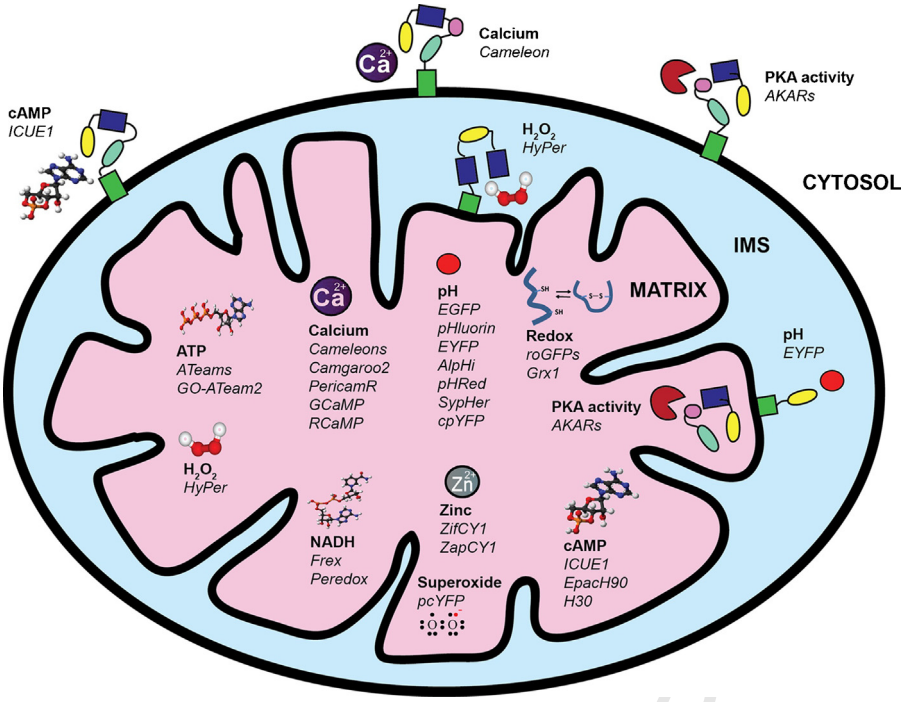

Fig. 1. Localization of genetically encoded sensors in mitochondrial matrix, IMS and peri-organellar region.

five different regions: a peri-organellar cytoplasmic space, an outer membrane (OM), an inner membrane (IM), an intermembrane space (IMS), and the matrix, each with its own characteristics and functions.

2. Strategies for targeting sensors to mitochondrial compartments

Due to their importance in the cell metabolism, mitochondria have been among the first organelles to be targeted by genetically encoded sensors, with aequorin used to study Ca^{2+} signaling in this organelle (Rizzuto et al., 1992, 1994; Rutter et al., 1996; Montero and Alonso, 1999). Directed targeting of sensors to mitochondria has been eased by the characterization of specific leader peptides that, when fused in frame to the N-terminus of a protein, drive import of the proteins into this organelle and the compartment of interest. It is possible to direct the sensor to the matrix, or to anchor it to either side of OM or IM (Fig. 1). For matrix localization, several leader peptides have been used in sensors. Most of sensors employ the targeting sequence of subunit VIIIa of the human cytochrome c oxidase (COX8) (MSVLTPLLLRGLTGSARRLPVPRAKIHSLGDP). Whereas small sensors such as the single-FP Ca^{2+} sensors Camgaroo and Pericam are correctly delivered into the mitochondrial matrix, many studies show mislocalization for larger sensors such as the Ca^{2+} FRET Cameleons, possibly because the structural complexity of these sensors hinder efficient translocation across mitochondrial membranes (Arnaudeau and Kelley, 2001; Filippin et al., 2003, 2005; Jean-Quartier et al., 2012). To address this problem, multiple (2×, 4×, 6×, 8×) copies of COX8 were tested for correct localization of Cameleon in the matrix. Increasing copy number improves localization but affects the organelle stability and morphology, therefore four tandem copies are indicated as a good compromise for Cameleons (Palmer et al., 2006). A single copy of the leader peptide may also affect the affinity and dynamic range of a sensor, as shown for Pericam, possibly because of incomplete cleavage following import into the matrix (Filippin et al., 2005). Other leading peptides commonly used are the 12 amino acid of subunit IV of yeast cytochrome c oxidase (COX4) (MLSLRQSIRFFK) (Llopis and McCaffery, 1998; Nagai et al., 2001; Dipilato et al., 2004; Wang et al., 2008; Park et al., 2010), the 36 residues of pyruvate dehydrogenase E1α (PDHE1) (MRKMLAAVSRVLSGASQKPASRVLVASRNFANDATF) (Hanson et al., 2004; Zhao et al., 2011; Liu et al., 2012), or the leader peptide of *Neurospora crassa* ATP-synthase (Gutscher et al., 2008). In most cases, mitochondrial sensors were deployed in animal cells (human, mouse, rat, Drosophila) and only few studies exist in the plant field. In Arabidopsis, cpYFP (pH/superoxide sensor, see below) and the redox state sensor roGFP1 were targeted to the mitochondrial matrix by the β-ATP synthase (ATPS) leader peptide of tobacco (Jiang et al., 2006; Schwarzländer et al., 2011). The glutathione redox potential sensors Grx1s were fused to the Arabidopsis serine hydroxymethyltransferase (SHMT) for import into plant mitochondria (Albrecht et al., 2013). In the case of Cameleon, even the human COX8 proved effective in plants (Loro et al., 2012).

For targeting to the IMS, sensors can be fused to full proteins or domains anchoring to the IM or OM. The hydrogen peroxide sensor HyPer and the pH-sensitive EYFP were fused to the C-terminus of the mouse glycerol phosphate dehydrogenase 2 (GPDH), localizing in the outer face of the IM (Porcelli et al., 2005; Malinouski et al., 2011). Cameleons and the PKA activity sensors AKARs were targeted to the peri-organellar space by fusion with the OM yeast anchoring peptides TOM20, yTOM70 and the 15 residues of DAKAP1a, respectively (Allen and Zhang, 2006; Giacomello et al., 2010; Lefkimmiatis et al., 2013).

3. Biosensors

To date, only a limited number of sensors have been used in mitochondria. Attention has been paid to molecules involved in energy production (ATP, NADH, pH), ROS (superoxide, hydrogen peroxide), the redox state, second messengers (cAMP, Ca^{2+}) and the metal cofactor Zn^{2+}. Table 1 shows the current sensors employed in different mitochondrial compartments.

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Table 1
List of biosensors targeted to mitochondrial matrix, IMS or peri-organellar space.

Name of sensor	Metabolite	FPs	Targeting sequence	Cell type	Reference
ATeams	ATP	FRET: mseCFP/mVenus mseCFP/cpVenus	2× COX8	HeLa	Imamura et al. (2009)
GO-ATeam2	ATP	FRET: cpEGFP173/mKO	2× COX8	HeLa, HEK	Nakano et al. (2011) and Lefkimmiatis et al. (2013)
AEQ	Ca^{2+}	Aequorin (bioluminescence)	cyt c COX8	BAEC, HeLa, rat skeletal myotubes	Rizzuto et al. (1992), Rizzuto et al. (1994), Rapizzi et al. (2002) and Filippin et al. (2003)
Cameleons	Ca^{2+}	FRET: ECFP/EYFP (YC2) ECFP/Citrine (YC3, YC4) ECFP/cpVenus (YC3.6, D1cpv, D2cpv, D3cpv, D4cpv) ECFP + EYFP (split Cameleon)	1×, 2×, 4×, 6×, 8× COX8 TOM20 (OM)	HeLa, INS-1 832/13, EA.hy926, OP-9, HL-1, Arabidopsis root and guard cells	Arnaudeau and Kelley (2001), Filippin et al. (2003), Filippin et al. (2005), Palmer et al. (2006), Giacomello et al. (2010), Jean-Quartier et al. (2012), Loro et al. (2012) and Alam et al. (2012)
Camgaroo2	Ca^{2+}	Single FP: Citrine	1×, 2× COX8	HeLa	Griesbeck et al. (2001), Rapizzi et al. (2002), Filippin et al. (2003) and Filippin et al. (2005)
PericamR or RPericam	Ca^{2+}	Single FP: cpYFP	COX4 1×, 2× COX8	HeLa, INS-1 832/13, EA.hy926, OP-9, HL-1	Nagai et al. (2001), Filippin et al. (2003), Filippin et al. (2005) and Jean-Quartier et al. (2012)
GCaMP1.6	Ca^{2+}	Single FP: cpGFP	COX4	CAD	Park et al. (2010)
RCaMP	Ca^{2+}	Single FP: cpRuby	COX8	Rat neurons	Akerboom et al. (2013)
ICUE1	cAMP	FRET: ECFP/Citrine	COX4 DAKAP1a (OM)	HEK293	Dipilato et al. (2004)
EpacH90	cAMP	FRET: CFP/YFP	4× COX8	HeLa, HEK293	Lefkimmiatis et al. (2013)
H30	cAMP	FRET: CFP/YFP	4× COX8	Rat cardiomyocites, HeLa, CHO	Di Benedetto et al. (2013)
HyPer	H_2O_2	Single FP: cpYFP	2× COX8 (matrix), GPDH (IMS)	HEK293, NIH 3T3, HeLa	Belousov et al. (2006) and Malinouski et al. (2011)
Frex, FrexH	NADH	Single FP: cpYFP	PDHE1	COS-7, HEK293	Zhao et al. (2011)
Peredox	NADH/NAD^+	Double FPs: cpT-Sapphire/mCherry	?	Neuro-2a	Hung et al. (2011)
EGFP	pH	Single FP: EGFP (F64L/S65T)	COX8	CHO	Kneen et al. (1998)
Ecliptic pHluorin	pH	Single FP: EGFP with mutations	4× COX	HeLa	Dittmer et al. (2013)
EYFP	pH	Single FP: EYFP	COX4	HeLa, rat cardiomyocytes	Llopis and McCaffery (1998)
MIMS-EYFP	pH	Single FP: EYFP	GPDH	ECV304, Mouse cortical astrocytes	Porcelli et al. (2005) and Azarias and Chatton (2001)
AlpHi	pH	Single FP: EYFP	COX8	HeLa, rat pancreatic β-cells	Abad et al. (2004)
pHRed	pH	Single FP: mKeima with mutations	COX8	neurons	Tantama et al. (2011)
SypHer	pH	Single FP: cpYFP with mutations	(2×?) COX8	Mouse muscle cells, astrocytes, HeLa	Azarias and Chatton (2001), Poburko et al. (2011), Quatresous et al. (2012) and Santo-Domingo and Demaurex (2012)
cpYFP	O_2^- pH	Single FP: cpEYFP (V68L/Q69K)	COX4, β-ATPase (plants)	Mouse muscle cells, neurons, cardiomyocites, HeLa, Arabidopsis	Schwarzländer et al. (2011, 2012), Quatresous et al. (2012), Wei-LaPierre et al. (2013) and Zhang et al. (2013)
AKAR3 AKAR4	PKA activity	FRET: ECFP-Cerulean/cpVenus	4× COX8 (matrix) yTom70 (OM), DAKAP1 (OM)	HeLa, HEK293	Allen and Zhang (2006) and Lefkimmiatis et al. (2013)
roGFP1 roGFP2	Redox potential	Single FP: GFP with disulfide bonds 147–204	PDHE1	HeLa, mouse neurons, Drosophila neurons	Hanson et al. (2004), Jiang et al. (2006), Guzman et al. (2010) and Liu et al. (2012)
Grx1-roGFP2 roGFP2-Grx1	Redox potential (glutathione)	Single FP: GFP with disulfide bonds 147–204	ATPS COX8 SHMT (plant)	HeLa, Schneider's cells (fly) Arabidopsis guard cells	Gutscher et al. (2008) and Albrecht et al. (2013)
ZifCY1/Cys_2His_2 ZapCY1	Zn^{2+}	FRET: CFP/Citrine	4× COX8	HeLa, neurons, MIN6, HC11	Dittmer et al. (2013), Miranda et al. (2012) and Park et al. (2012)

Abbreviations of targeting sequences: human cytochrome c oxidase subunit VIIIa (COX8), yeast cytochrome c oxidase subunit IV (COX4), pyruvate dehydrogenase E1α (PDHE1), mouse glycerol phosphate dehydrogenase 2 (GPDH), *Neurospora crassa* ATP synthase (ATPS), Arabidopsis serine hydroxymethyltransferase (SHMT).

3.1. ATP

Despite its importance as an energy currency in cells, accurate techniques for measuring ATP dynamics at the subcellular levels of intact cells are lacking. The development of genetically encoded sensors for ATP represented a turning point in the study of this important molecule. The ATeam sensor family consists of the FRET pair msECFP and circularly permuted Venus (cpVenus) fused to the ε subunit of the bacterial F_0F_1-ATP synthase, a protein which specifically binds ATP (Imamura et al., 2009). Importantly, since ATP is not hydrolyzed upon binding, cell homeostasis is not affected. Differential targeting allowed the researchers to compare cytosolic and matrix ATP levels in HeLa cells. Surprisingly, sensor responses revealed that ATP was lower in the mitochondrial matrix than in the cytosol, possibly as result of ATP pumps. It was argued that a low ATP concentration might be necessary to avoid feedback inhibition of the ATP synthase machinery (Imamura et al., 2009). Improved versions of ATeams (GO-ATeams) contain the red-shifted FP pair cpGFP and mKO$_k$, allowing the parallel visualization of a blue-excited sensor or dye. Using GO-ATeam together with the Ca^{2+} dye fura-2, concurrent increases of ATP and Ca^{2+} following histamine treatment were observed (Nakano et al., 2011).

3.2. NADH

NADH and its oxidated form NAD^+ are important coenzymes involved in several redox processes, and they regulate mitochondrial metabolism. Traditional assays for NADH quantification are either destructive or do not discriminate between NADH and NADPH (Hung et al., 2011). Two different sensors for NADH were independently developed at the same time, both relying on the binding of the bacterial dimeric repressor protein Rex to NADH. The sensors Frex and FrexH (a high affinity version) are composed by cpYFP inserted between a full Rex monomer and a second, truncated Rex protein (Zhao et al., 2011). Despite one single FP is present, the sensor is ratiometric in its excitation peaks. As many sensors employing cpFPs, a major limitation of Frex is the sensitivity of cpFP to pH, especially since the pK_a of the FP is similar to the pH of the mitochondrial matrix (pH ~8), thus requiring a careful calibration and controls. The second sensor, Peredox, was engineered by inserting cpSapphire between two Rex monomers (Hung et al., 2011). Addition of a second red FP, mCherry, allows normalization for protein expression level, and targeted mutagenesis eliminated pH sensitivity. Peredox reports NADH/NAD^+ ratio rather than NADH concentration. However, mitochondrial concentration of NADH appeared to saturate the sensor, thus making Peredox, in the present form, insensitive in this compartment (Hung et al., 2011).

3.3. pH

Intrinsic pH sensitivity, a major limitation for many sensors, can be exploited to construct pH sensors. pH sensitivity is due to the effect of protonation of chromophore or chromophore environment on the spectral properties of many FPs. Early pH sensors simply consisted of modified EGFPs (among which, pHluorins (Miesenbock et al., 1998)). The acidic pK_a (~6), however, make EGFP sensors unsuitable in the alkaline mitochondrial matrix (Kneen et al., 1998). A slightly higher pK_a (~7) is offered by EYFP (Llopis and McCaffery, 1998). EYFP was also targeted to the IMS and reported lower pH in this compartment (pH = 6.9) than in the cytosol (pH = 7.6) of endothelial cells, indicating that diffusion of protons across the OM is restricted (Porcelli et al., 2005). Spectral properties may change when FPs are attached to other proteins. When EYFP is fused to calmodulin in the Ca^{2+} sensor Camgaroo2, it then reports pH changes in the alkaline range. mtAlpHi, where the calmodulin moiety is substituted by an inactive aequorin counterpart, is a single-FP pH sensor with a pK_a of 8.5 (Abad et al., 2004). A convenient red-shifted pH indicator, pHRed, allows concurrent monitoring of other metabolites with green-emitting sensors (Tantama et al., 2011). pH sensitivity of FPs can be increased by exposing the chromophore region by means of circular permutation (cpFP). cpYFP is particularly sensitive to pH, either in its free form or within chimeric sensors. Accordingly, a number of pH sensors are based on cpYFP. SypHer is a cpYFP-based ratiometric pH sensor with a pK_a = 8.71, particularly suited for the mitochondrial matrix. Use of SypHer allowed to unravel fluctuation in the mitochondrial matrix pH that paralleled those of Na^+ in the matrix (Azarias and Chatton, 2001) and of Ca^{2+} in the cytosol (Poburko et al., 2011). Furthermore, spontaneous pH fluctuations of the matrix, called "flashes", coincided with a decrease of $\Delta\psi_m$, likely reflecting changes in proton pump activity (Santo-Domingo and Demaurex, 2012). Similar flashes of pH have been observed in plant mitochondria using free cpYFP (Schwarzländer et al., 2011, 2012).

3.4. ROS

ROS, and in particular the anion superoxide and hydrogen peroxide, are produced in mitochondria during respiration. ROS have a double-edged role, acting both as agents of oxidative damage and as signaling molecules (Brookes et al., 2004). By using free cpYFP as superoxide sensor, several groups have observed flashes of superoxide production in mitochondria (reviewed by Quatresous et al., 2012; Wei-LaPierre et al., 2013; Zhang et al., 2013). However, it is unclear how superoxide can chemically affect the fluorescence output of cpYFP, and it has been questioned whether this sensor is actually recording pH change (Schwarzländer et al., 2011, 2012; Quatresous et al., 2012). Similar flashes have been observed in mitochondria expressing HyPer, a H_2O_2 sensor (Belousov et al., 2006). Interestingly, HyPer also contains cpYFP, inserted into the bacterial peroxide-regulated protein OxyR. Targeting of HyPer to IMS of HEK293 cells showed surprisingly low levels of H_2O_2, despite disulphide bonds, an indicator of an oxidant status, can form in this compartment. H_2O_2 levels in matrix and IMS, however, rapidly increase following inhibition of electron chain transfer by rotenone treatment (Malinouski et al., 2011).

3.5. Redox

The versatility of GFPs in creating a wide range of sensors is exemplified by roGFPs, a family of sensors of the redox status. roGFPs were generated by replacing two amino acids in the β-strands of the GFP barrel with cysteines. Depending on the redox status, these cysteine pairs can form a disulfide bond which affects the spectral properties of the chromophore. The resulting roGFP is a small redox sensor with ratiometric response. When targeted to the mitochondrial matrix of HeLa or Arabidopsis root cells, roGFP1 reported a redox potential of −360 mV (Hanson et al., 2004; Jiang et al., 2006). roGFPs have been also used to study the role of mitochondrial oxidative stress in neurological pathogenesis, by imaging transgenic mice and flies (Guzman et al., 2010; Liu et al., 2012). When fused to glutaredoxin-1, roGFP2 was converted into a sensor for glutathione redox potential, Grx1-roGFP2. Comparison of responses of Grx1 targeted to the cytosol and mitochondria of HeLa cells showed that glutathione oxidation is directly coupled to $\Delta\psi_m$ drop in mitochondria, whereas in the cytosol oxidation is delayed (Gutscher et al., 2008). Grx1-roGFP2 is correctly imported into mitochondria of mammal cells and oocytes (Gutscher et al., 2008; Eichenlaub-Ritter et al., 2011), but mislocalizes when used in plant and Drosophila cells (Albrecht

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et al., 2013). In order to correctly target the sensor into plant and fly mitochondria, roGFP2 was fused to the N-terminus of Grx1, instead than to the C-terminus. The inverted sensor, roGFP2-Grx1, is also characterized by a faster response (Albrecht et al., 2013).

3.6. cAMP

cAMP is the paradigm of second messenger and it regulates the activity of the downstream proteins protein kinase A (PKA), exchange protein directly activated by cAMP (EPAC) and cyclic nucleotide-gated channels (CNGC). Recent evidences suggest the existence of an intramitochondrial cAMP signaling circuit (Lefkimmiatis et al., 2013). Sensors for cAMP are based on PKA, EPAC and CNGC. To date, however, only EPAC-based sensors have been used in mitochondria. Whereas an early study employing the FRET sensor ICUE1 reported similar changes in cAMP levels in mitochondria and cytosol following simulation (Dipilato et al., 2004), it was later shown that the coupled response was probably an artefact due to mislocalization of the sensor in the cytosol (Di Benedetto et al., 2013). When correctly localized in the matrix, both sensors H30 and EpacH90 were insensitive to the cytosolic cAMP pool, indicating that the IM is impermeable to cAMP and that this second messenger is autonomously generated in the matrix (Di Benedetto et al., 2013; Lefkimmiatis et al., 2013). However, at least one target of cAMP signaling, PKA, seems to be absent in the matrix, since the PKA activity FRET sensors AKARs fails to respond in this compartment. Conversely, when expressed in the peri-organellar space, AKAR sensors showed stronger response than in the rest of the cytosol, suggesting an intense activity of PKA in this region (Allen and Zhang, 2006; Lefkimmiatis et al., 2013).

3.7. Calcium

Ca^{2+} regulates several key aspects of mitochondrial functionality, from enzyme activity to gene expression, and impairment of these signaling circuits in mitochondria has been linked to pathologies (Brookes et al., 2004). Ca^{2+} was the first molecule to be studied by genetically encoded sensors, first in cytosol and then in mitochondria. Early investigations in the '90s employed a matrix-targeted aequorin (mtAEQ), a protein that emits light in presence of Ca^{2+} (Rizzuto et al., 1992, 1994; Rutter et al., 1996; Montero and Alonso, 1999). However, due to the dim emission of aequorin and the irreversibility of its reaction, fluorescence-based sensors offer now a better alternative. Several Ca^{2+} sensors have been developed and targeted to mitochondria. Among the most popular sensors, Cameleons consist of the Ca^{2+}-binding protein calmodulin, the M13 peptide and a FRET FP pair. Several versions of Cameleon exist, with different FPs and affinities. The most recent versions present mutations in the calmodulin moiety, preventing perturbation of calmodulin signaling (Palmer et al., 2006). Due probably to their size and complex structure, Cameleons often do not localize correctly when targeted to the mitochondria, especially when expressed at high level (Arnaudeau and Kelley, 2001; Filippin et al., 2003, 2005; Jean-Quartier et al., 2012). Nevertheless, several members of Cameleon have successfully been used in mitochondrial matrix of animal and plant cells (Alam et al., 2012; Loro et al., 2012) and also in the peri-organellar space (Giacomello et al., 2010). A more accurate mitochondrial localization is generally achieved with smaller single-FP Ca^{2+} sensors, such as Camgaroo2, Pericam, GCaMP and the related red version RCaMP (Griesbeck and Baird 2001; Nagai et al., 2001; Filippin et al., 2003, 2005; Park et al., 2010; Jean-Quartier et al., 2012; Akerboom et al., 2013). However, it was observed that Camgaroo2 may photoconvert into an unresponsive form during visualization, whereas Pericam appeared more stable. On the other hand, like many other cpYFP-based sensor, Pericam is highly pH-sensitive, thus requiring careful control during measurements (Filippin et al., 2005).

3.8. Zinc

Zn^{2+} is an essential cofactor for many proteins but can be toxic when present in excess amount. Cellular and mitochondrial Zn^{2+} homeostasis is therefore tightly controlled, yet its mechanism is poorly understood. The Zn^{2+} sensors ZapCY1 and ZifCY1/Cys_2His_2 consist of a zinc fingers from the transcription factors Zap1 or Zif268, respectively, fused between CFP and Citrine (Park et al., 2012). Binding of Zn^{2+} to the zinc fingers of the linker results in a FRET change. Unlike chemical indicators such as RhodZin-3, the ZifCY1 sensor was retained in mitochondria even after depolarization (Dittmer et al., 2013). Imaging of ZifCY1 and ZapCY1 targeted in the matrix showed that the level of Zn^{2+} in mitochondria varies greatly among different cell types and that in HeLa cells, Zn^{2+} in mitochondria is three orders of magnitude lower than in the cytosol (Park et al., 2012). In hippocampal neurons, mitochondria may act as Zn^{2+} reservoir and release it after stimulation with glutamate (Dittmer et al., 2013). Co-expression with red-shifted versions of the sensor in the cytosol allowed to track Zn^{2+} dynamics in multiple organelles, and showed that sequestration in the mitochondria is a slower event than in the nucleus (Miranda et al., 2012).

4. Future outlook

There is a need to obtain subcellular information on metabolic activity. While it is possible to isolate mitochondria, the analysis of purified organelles likely does not reflect the biology of the organelle within a cell, and novel tools are required to monitor activity in vivo. The engineering and use of genetically encoded sensors is a thriving area of research and many molecules can now be monitored and quantified with this technology in vivo with subcellular and exceptional temporal resolution. In addition to measuring molecule concentrations and dynamics, a new class of activity sensors now allows us to directly quantify the activity state of proteins, such as transporters, in vivo (De Michele et al., 2013). Thanks to the improvement in microscopes and the development of microfluidic platforms enabling high quality time-lapse imaging, we foresee that in the in the coming years many of these sensors will be used to address important biological questions at the subcellular resolution. Mitochondria, due to their structural and physiological complexity, certainly represent a prime target for investigation. An exciting alternative to targeting sensor to mitochondria by fusion to leader peptides will be direct transformation of mtDNA (Lightowlers, 2011). The recent expansion of the FP color palette in sensors will furthermore allow concurrent analysis of multiple parameters.

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