Tracking intra- and inter-organelle signaling of mitochondria

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Introduction
Mitochondria have undergone a drastic transition from free-living bacteria to fundamental compartments within eukaryotic cells [1]. Therefore, the mitochondria’s broad field of contributions, ranging from production of ATP by oxidative phosphorylation (OXPHOS), buffering of Ca²⁺, and contribution to various cellular signaling

Abbreviations
CJ, cristae junction; CM, cristae membrane; DRP1, dynamin-related protein 1; dSTORM, direct stochastic optical reconstruction microscopy; EMRE, essential MCU regulator; ETC, electron transport chain; FADH₂, flavin adenine dinucleotide; FRET, Förster resonance energy transfer; GE, genetically encoded; GPS2, G protein pathway suppressor 2; IMM, inner mitochondrial membrane; IP3R, inositol triphosphate receptor; LETM1, leucine zipper-EF-hand-containing transmembrane protein 1; LIT, Light-inducible tethering; MAMs, mitochondria-associated ER membranes; MCU, mitochondrial calcium uniporter; MFN1, mitofusin 1; MFN2, mitofusin 2; MICOS complex, mitochondrial contact site and cristae organizing complex; MICU1, mitochondrial calcium uptake 1; MICU2, mitochondrial calcium uptake 2; mPTP, mitochondrial permeability transition pore; mtUPR, mitochondrial unfolded protein response; NADH, nicotinamide adenine dinucleotide; OMA1, metalloprotease-related protein 1; OMM, outer mitochondrial membrane; OPA1, optic atrophy 1; OXPHOS, oxidative phosphorylation; PALM, photo-activated localization microscopy; PE, phosphatidylethanolamine; PS, phosphatidylserine; ROS, reactive oxygen species; SIM, structured illumination microscopy; TMMR, tetramethylrhodamine methyl ester; TOM, translocase of the outer membrane; UCP2, uncoupling protein 2; UCP3, uncoupling protein 3; VDAC, voltage-dependent anion channel; YME1L, ATP-dependent metalloprotease; ΔΨ𝑚 hümt, Mitochondrial membrane potential.
pathways, was supplemented with highly dynamic structural and functional adaptations [2,3]. Stretching throughout the cell as a highly dynamic network along the cytoskeleton, mitochondria are constantly undergoing fusion and fission to meet the requirements of cellular subdomains under various conditions [2,3], as discussed in our `Mitochondria as highly specialized working unit’ section. Besides by structure and shape, mitochondria’s activity is largely controlled by the homeostasis of ions (Fig. 1), including H⁺ [4–6], K⁺ [7] and Ca²⁺ [8]. These ions are not just powerful tools to fine-tune mitochondrial activity, but also operate as messengers for intra-organelle and intercellular communication, as explained in the chapter ‘Fine-tuning of mitochondrial activity’. While fulfilling their multiple tasks, mitochondria strongly rely on the support of their cellular environment. This results in a busy interplay between mitochondria and various organelles [9], as summarized in ‘Mitochondria as signaling hubs: Give&Get’. Having the majority of proteins encoded by nuclear DNA makes a constant communication between mitochondria and the nucleus indispensable [10], for instance. Moreover, mitochondria form highly specialized signaling hubs with the endoplasmic reticulum (ER) to ensure and control lipid and Ca²⁺ transfer in restricted subdomains [11]. Therefore, it is obvious that interplay between mitochondria and different cellular compartments takes place at specific contact sites or through the exchange of second messengers. To track these subcellular processes, cutting-edge techniques are required to make investigation possible, including high-resolution microscopy as well as highly sensitive organelle-targeted biosensors. We provide an overview about techniques that enable us to study all of these processes at each chapter as well as in the table (Table 1), highlighting the importance of technological progress to reveal further mysteries about our cellular powerplants.

**Mitochondria as highly specialized working units**

**Dynamic changes in structure and shape of mitochondria**

The mitochondrial network is a highly specialized working unit capable of undergoing dynamic adaptation in order to meet metabolic needs and to allow internal and external signaling [2,3]. Thereby, constant fission and fusion of the inner (IMM) and outer mitochondrial membrane (OMM) play a crucial role in maintaining mitochondrial integrity. The fusion and fission homeostasis affects the opening probability of the mPTP, facilitating uncontrolled efflux of ions [12], as well as oxidative capacity [13,14], production of reactive oxygen species (ROS) [15], mitophagy [16].
### Table 1. Technical approaches to track mitochondrial structure, activity, and interorganelle interplay.

| Technical approach | Advantages/disadvantages | References |
|--------------------|--------------------------|------------|
| **Mitochondrial structure and shape** | | |
| Fluorescence microscopy | | |
| Confocal microscopy | ○ Conventional resolution of ~280 nm | [230] |
| | + Imaging of living cells and fixed cells possible | |
| | – Resolution of maximal 150 nm (4Pi) | |
| SIM | + Live cell and time lapse imaging | [66] |
| STED | + Superior spatial resolution over confocal microscopy | |
| | + Analysis of submitochondrial structures | |
| PALM/dSTORM | + Highest spatial resolution possible with fluorescence microscopy | [65] |
| | + Easy specific targeting with high labeling density using fluorescent marker | |
| | – Fixation of the sample is often necessary | |
| | – Temporal resolution of PALM displays a problem for moving structures in living cells | |
| Electron microscopy | + Precise analysis of submitochondrial structures possible | [20] |
| | + Very high spatial resolution | |
| | + Immunogold preparation allows protein localization but lacks in labeling density | |
| | – Fixation and embedding of the sample necessary | |
| **Mitochondrial energy production** | | |
| Oroboros O2k | ○ Oxygen consumption is measured by a polarographic oxygen electrode | [74] |
| | + Analysis of cells, tissues, and isolated mitochondria possible | |
| | + Sequential injection/titration of compounds possible | |
| | – Not suitable for high-throughput screening | |
| Seahorse technology | + Oxygen consumption and extracellular acidification are measured in parallel by fluorescent sensors | [75] |
| | + Analysis of adherent cells, suspensions cells, permeabilized cells, isolated mitochondria, and—using specific tissue plates—tissues possible | |
| | – Only four injections possible | |
| | – 24- and 96-well-based assay platform | |
| GE ATP probes | + Organelle-targeting makes analysis of ATP levels in various cellular compartments possible in real time | [76,77] |
| ATeams | – Proper transfection/infection efficiency is required | |
| **pHmito** | | |
| GE pH probes | ○ Organelle-targeted pH sensor that allows pH measurements in the lumen of mitochondria | [99] |
| mtAlpHi | + Excitation of the probe at 498 nm reduces phototoxicity | |
| | – Sensor provides intensiometric read-out, hampering pH quantification | |
| | – Proper transfection/infection efficiency is required | |
| SypHer | ○ Organelle-targeted pH sensor that allows pH measurements in the lumen of mitochondria | [92] |
| | + Excitation at 430 nm and 480 nm with the detection of a constant emission wavelength at 530 nm allows a ratiometric read-out and an easy pH quantification | |
| | – Excitation of the probe at 430 nm might cause phototoxicity | |
| | – Proper transfection/infection efficiency is required | |
| **Fluorescent dyes** | | |
| BCECF | ○ Chemical pH sensor allowing global intracellular pH measurements | [101] |
| | + Cell loading with BCECF-AM yields high fluorescent cell number | |
| | + pKa of ~ 6.98 is close to the cytosolic pH | |
| | + Excitation at 440 and 490 nm with the detection of a constant emission wavelength at 530 nm allows a ratiometric read-out and an easy pH quantification | |
| | – Excitation of the probe at 430 nm might cause phototoxicity | |
| | – Presence of cellular esterases required for BCECF-AM to BCECF cleavage | |
Table 1. (Continued).

| Technical approach | Advantages/disadvantages | References |
|---------------------|--------------------------|------------|
| SNARF               | ○ Chemical pH sensor allowing global intracellular pH measurements  
+ Cell loading with SNARF-AM yields high fluorescent cell number  
+ pKa of ~ 7.5 is close to the cytosolic intracellular pH  
+ Excitation at ~ 500 nm with the detection of two emission wavelengths at 580 and 640 nm allows a ratiometric read-out and an easy pH quantification  
+ Long excitation wavelength of the probe reduces phototoxicity  
− Presence of cellular esterases required for SNARF-1-AM to SNARF-1 cleavage  
− Although global intracellular staining, probe was used to measure pHmito using high-resolution microscopy  
− Measurements require a sophisticated microscope setup due to separation of two emission wavelengths | [102] |
| Mito-pH             | ○ Chemical pH sensor allowing specific pHmito measurements  
+ Cell loading with Mito-pH yields high fluorescent cell number  
+ pKa of ~ 7.33 is close to the cytosolic pH  
+ Excitation at 490 and 560 nm with the detection of emission wavelengths at 520 and 600 nm allows a ratiometric read-out and an easy pH quantification  
− Long excitation wavelengths of the probe reduce phototoxicity | [103] |
| Δψm Fluorescent dyes | TMRM                     | [107] |
|                     | ○ Monochromatic dye (λex = 555 nm, λem = 570 nm) for semiquantitative analysis of Δψm  
+ Cell loading with TMRM yields high fluorescent cell number  
− Mitochondria with low Δψm are possibly not stained and cannot be measured  
− Alteration of cellular respiration and binding to mitochondrial membrane might affect results | |
|                     | TMRE                     | [105] |
|                     | ○ Monochromatic dye (λex = 549 nm, λem = 574 nm) for semiquantitative analysis of Δψm  
+ Cell loading with TMRE yields high fluorescent cell number  
− Mitochondria with low Δψm are possibly not stained and cannot be measured  
− Alteration of cellular respiration and binding to mitochondrial membrane might affect results | |
|                     | JC-1                     | [106] |
|                     | ○ Chemical, ratiometric dye (λex = 488 nm, λem = 530 and 595 nm) for semiquantitative analysis of Δψm  
+ All mitochondria are stained, independent of their Δψm  
+ Cell loading with JC-1 yields high fluorescent cell number  
− Photosensitive  
− Fluorescence may be changed independently of Δψm by, for instance, H2O2 or disturbed equilibrium between monomers and aggregates | |
| Mitochondrial K+ homeostasis | Patch-clamp  | [118,126,128,137] |
|                     | + Gold standard method for K+ fluctuation measurements  
+ Very sensitive method  
− Isolation of mitochondria and preparation of mitoblasts required to measure K+ fluctuations across the IMM  
− Usage of isolated mitochondria might be far from the physiologic intracellular situation | |
| GE K+ probes        | GEPiII                   | [111] |
|                     | ○ Organelle-targeted K+ sensor that allows K+ measurements in the lumen of mitochondria  
+ EC50 of 60.95 mM is suitable for measurements of [K+] within the mitochondrial matrix | |
Mitochondrial fission is mainly mediated by the cytosolic dynamin-related protein 1 (DRP1). It binds to the OMM and constricts the mitochondrion, an action facilitated by its GTPase activity [23]. Furthermore, dynamin is necessary to achieve complete fission, as DRP1 activity leads to constriction, but not cleavage, during the fission process [24,25]. Nevertheless, it is still unknown whether a specific process or piece of protein machinery is necessary for the cleavage of the IMM.

Mitochondrial fusion is mainly driven by the proteins optic atrophy 1 (OPA1) and dynamin-like protein mitofusin 1 and 2 (MFN1 and MFN2) [26,27]. MFN1 and MFN2 are located at the OMM and serve to facilitate fusion of two organelles. The necessary energy to

Table 1. (Continued).

| Technical approach | Advantages/disadvantages | References |
|--------------------|--------------------------|------------|
| **Mitochondrial Ca\(^{2+}\) homeostasis** | | |
| **Fluorescent dyes** | | |
| Fura-2 | ○ Indirect measurement of mitochondrial Ca\(^{2+}\) movement | [174] |
| | + Simple experimental preparation | |
| | – Experimental preparation limited to simple cellular incubation | |
| | – Not suitable for direct measurement of intra-organelle Ca\(^{2+}\) | |
| Fluo-3/Fluo-4 | ○ Indirect measurement of mitochondrial Ca\(^{2+}\) movement | [179] |
| | + Simple experimental preparation | |
| Rhod-2 | + AM ester dye that allows for mitochondria-specific Ca\(^{2+}\) measurement | [180] |
| | – Only suited to short experimental protocols | |
| **GE Ca\(^{2+}\) probes** | | |
| 4mtD3cpv | ○ Organelle-targeted, FRET-based (\(\lambda_{\text{ex}} = 430\text{ nm}, \lambda_{\text{em}} = 480\text{ and }535\text{ nm}\)) mitochondrial Ca\(^{2+}\) sensor | [185] |
| | ○ \(K_0\) for Ca\(^{2+}\) around 600 nM | |
| | + Highly sensitive cameleon with a wide monitoring range | |
| | – Proper transfection/infection efficiency is required | |
| 4mtD1GO-CAM | ○ Organelle-targeted, FRET-based (\(\lambda_{\text{ex}} = 477\text{ nm}, \lambda_{\text{em}} = 510\text{ and }560\text{ nm}\)) mitochondrial Ca\(^{2+}\) sensor | [186] |
| | ○ \(K_0\) for Ca\(^{2+}\) around 1.53 \(\mu\)M | |
| | + Red-shifted cameleon with a wide monitoring range | |
| | + Very well suitable for combination with other organelle-targeted Ca\(^{2+}\) indicators and to be used simultaneously with Fura-2 to correlate cytosolic and mitochondrial Ca\(^{2+}\) signals | |
| | – Proper transfection/infection efficiency is required | |
| mtGEM-GECO1 | ○ Organelle-targeted, FRET-based (\(\lambda_{\text{ex}} = 394\text{ nm}, \lambda_{\text{em}} = 455\text{ and }511\text{ nm}\)) mitochondrial Ca\(^{2+}\) sensor | [187] |
| | ○ \(K_0\) for calcium around 340 nM | |
| | + Very well suitable for combination with other organelle-targeted Ca\(^{2+}\) indicators | |
| | – Proper transfection/infection efficiency is required | |
| mtCAR-GECO1 | ○ Organelle-targeted, intensiometric-based (\(\lambda_{\text{ex}} = 565\text{ nm}, \lambda_{\text{em}} = 620\text{ nm}\)) mitochondrial Ca\(^{2+}\) sensor | [188] |
| | + Very well suitable for combination with other organelle-targeted Ca\(^{2+}\) indicators | |
| | – Proper transfection/infection efficiency is required | |

apoptosis [17,18], Ca\(^{2+}\) signaling [18,19], and mitochondrial interactions with other cell compartments [20–22].
fuse membranes is provided predominantly by the GTPase activity of MFN1 [28]. Extracellular-signal-regulated kinase is able to reduce MFN1 activity by phosphorylation, resulting in the fragmentation of mitochondria [29]. Analogous to the OMM-located MFN1 and MFN2, OPA1 is one of the main regulators responsible for the fusion of the IMM [30]. Two forms of OPA1, L-OPA1 and S-OPA, are kept in balance by the metalloprotease-related protein 1 (OMA1) and ATP-dependent metalloprotease YME1L via proteolytic cleavage of the IMM bound L-OPA1 to soluble S-OPA1. A predominance of the L-form yields mitochondrial fusion, while metabolic stimuli or cell stress signals activate L-OPA1 proteolysis by OMA1 and YME1L, respectively, resulting in fission [31,32].

Recently, intermitochondrial signaling by either nano-tubes or direct interaction via intermitochondrial junctions has been intensively studied. These intermitochondrial contact sites have a high electron density and are coordinated pairs of cristae oriented orthogonally to the OMM in two adjoined mitochondria [33]. Nano tunnels are either a result of stalled and incomplete fission events [34] or are de novo generated mitochondrial protrusions formed by members of the kinesin family, like the protein kinesin-1 heavy chain along the cellular tubulin cytoskeleton [35]. These intermitochondrial contact sites are thought to transmit Ca\(^{2+}\) or apoptotic signals across mitochondria [36] or even a coupling of the mitochondrial membrane potential (ΔΨ\textsubscript{m}) of neighboring mitochondria [37]. Thereby, kissing and nanotunneling of mitochondria represent an alternative form of intermitochondrial communication [38,39]. Notably, exchange of, for instance, matrix proteins through nanotunnels follows a slower kinetic compared to conventional fusion most likely due to their small diameter of < 100 nm [38].

The IMM is morphologically separated into two compartments, divided by the cristae junction (CJ): (a) the inner boundary membrane directly facing the inner leaflet of the OMM; and (b) the cristae membrane (CM), forming the protrusions and invaginations of the IMM into the mitochondrial matrix [40,41]. Both compartments differ in protein composition and functional activity [42,43]. OPA1 is involved in the stabilization of the CJ and is interconnected with the mitochondrial contact site and cristae organizing complex (MICOS complex) [44]. Loss of OPA1 by knockdown or knockout leads to widened cristae lumen and CJ [17,45], reduction of IMM potential [46], increase in basal mitochondrial Ca\(^{2+}\) levels [47], and apoptosis induction [48]. Similar to OPA1, the inner boundary membrane localized mitochondrial Ca\(^{2+}\) uptake 1 (MICU1) is involved in CJ stabilization, maintenance of IMM potential, and cytochrome c restriction to the cristae lumen. The Ca\(^{2+}\) sensing ability of MICU1 and its thereby affected quaternary structure might influence the CJ stability and permeability [49]. The MICOS complex is composed of several subunits with mitofilin representing the biggest [50]. MICOS proteins are especially enriched in the CJ and form IMM-IMM and IMM-OMM contact sites with proteins like OPA1 [44,51], translocase of the outer membrane (TOM) [52,53] and S-adenosylmethionine synthetase [54] to ensure CJ biogenesis and stabilization. The structure of the CJ also restricts the F_{0}F_{1}-ATP synthase and the respiratory chain complexes to the CM, leading to a closed compartment of the cristae lumen [55,56]. Since the OMM is generally permeable due to voltage-dependent anion channel (VDAC) [57,58], the CJ seems to form a diffusion barrier for protons, creating an isolated space for the activity of F_{0}F_{1}-ATP synthase and respiratory chain complexes with lateral pH gradients [59]. Computational models and in vitro experiments have shown that low pH causes cristae invaginations of the IMM based on the electrostatic induced negative curvature of the outer CM leaflet [60,61]. Besides the direct involvement of the F_{0}F_{1}-ATP synthase in cristae formation and morphology [42,56,62], an indirect effect of CM localized respiratory supercomplexes mediated by the decreased pH in the CJ might be an important factor in cristae biogenesis and shape definition [63].

Techniques to analyze structure and shape

While the current knowledge about the ultrastructure of mitochondria relies mainly on electron microscopy data, recent advancements in fluorescence microscopy, in particular super-resolution microscopy techniques like structured illumination microscopy (SIM), stimulated emission depletion (STED), or photo-activated localization microscopy (PALM), enable researchers to visualize and investigate mitochondrial ultrastructure in living cells.

direct stochastic optical reconstruction microscopy (dSTORM) is an alternative to immunogold labeling and electron microscopy for analysis of submitochondrial localization of proteins like the F_{0}F_{1}-ATP synthase [64] or uncoupling protein 4 (UCP4) with high spatial resolution [65].

However, dSTORM is not suitable for live cell imaging and PALM approaches in live cells do not reach the necessary temporal resolution necessary to analyze dynamics of the IMM. Therefore, STED [66] or SIM [67] microscopy have been used to image, for instance, binding of fluorescent dyes dependent on
NADH and flavin adenine dinucleotide (FADH\textsubscript{2}). Beta-oxidation, fuels the citric acid cycle to produce derived from oxidative decarboxylation of pyruvate or ER, pointing to a Ca\textsuperscript{2+} sively in mitochondrial areas in close proximity to the mitochondria. The dynamics of the IMM in regions of close mitochondrial areas in close proximity to the ER, pointing to a Ca\textsuperscript{2+}-regulated mechanism of IMM rearrangement \cite{67}. The submitochondrial dynamic and MICU1-dependent relocalization of mitochondrial Ca\textsuperscript{2+} uniporter (MCU) and UCP2 upon ER-Ca\textsuperscript{2+} release form the entire IMM into the inner boundary membrane, which was visualized recently using SIM \cite{49}. Similar observations of MCU inner boundary membrane localization under oxidizing conditions were made with STED and SIM showing the superior performance of super-resolution over conventional microscopy in live cells \cite{69}.

The STED technique has been used to visualize spiral MICOS-cluster arrays along the inner boundary membrane of yeast and human mitochondria, making an estimation of mitochondrial microarchitecture in combination with the use of immunogold labeling possible \cite{43,70}. However, while STED microscopy achieves better resolution than SIM, it comes with the drawback of generally higher excitation intensity, leading to photobleaching and toxicity and, thus, limited acquisition time. SIM can be used with low excitation intensities, high frame rate, and over extended periods of time \cite{71}. Also, nonsaturation fluorescence microscopy along with intensive deconvolution was used to analyze the cristae kinetics in live cells and to distinguish between the IMM and matrix structures \cite{68}.

**Energy production as principal task**

The mitochondria’s core working unit is the mitochondrial respiratory chain located in the IMM within the cristae \cite{55,56}. Oxidation of glycolysis derived pyruvate and nicotinamide adenine dinucleotide (NADH) by mitochondria yields about 15 times more ATP than glycolysis itself \cite{72}. Thereby, acetyl coenzyme A, derived from oxidative decarboxylation of pyruvate or beta-oxidation, fuels the citric acid cycle to produce NADH and flavin adenine dinucleotide (FADH\textsubscript{2}).

These reducing equivalents serve as electron donors for the electron transport chain (ETC), consisting of NADH dehydrogenase (complex I), succinate dehydrogenase (complex II), ubiquinone, cytochrome bc\textsubscript{1} complex (complex III), and cytochrome c and cytochrome c oxidase (complex IV). After transfer of electrons, derived from the NADH and FADH\textsubscript{2} as hydrogen molecules, to the ETC via complex I and II, the electron transport through complex I to complex IV is coupled to proton pumping from the mitochondrial matrix into the locally restricted cristae lumen. This process causes a negative charge in the matrix and a positive charge in the IMS resulting in an electrochemical gradient, used for the proton transport back from the IMS into the mitochondrial matrix through ATP synthase (complex V). The released energy is, finally, utilized by FoF\textsubscript{1}-ATP synthase to gain the cellular energy carrier, ATP, by phosphorylation of adenosine diphosphate \cite{73}.

**Techniques to measure activity of mitochondrial activity**

Besides a broad range of biochemical approaches such as western blot, ROS, and ATP assays, there are specific measurements for mitochondrial bioenergetics available. Based on a polarographic oxygen electrode measuring the concentration and consumption of oxygen before and after injection of various substrates, the Oroboros O2k has been in use since the 1990s. This machine offers the possibility to analyze cells, tissues as well as isolated mitochondria with high resolution. Furthermore, sequential injection and titration of compounds inhibiting different complexes of the mitochondrial respiratory chain or boosting maximal mitochondrial activity can be done during the ongoing measurement. However, the Oroboros O2k is not suitable for high-throughput screenings as only two samples can be analyzed at the same time \cite{74}. Therefore, the Seahorse XF Extracellular Flux Analyzer has been introduced about 10 years ago, offering 24- and 96-well-based assay platforms. Thereby, the oxygen consumption rate and the extracellular acidification are measured in parallel by fluorescent sensors. However, this brings the limitation that injectable compounds may interfere with the fluorescent sensor. Moreover, in contrast to the Oroboros O2k, only four compounds can be injected during the measurements. Since the sensor-containing biocartridge has to be loaded with the injectable compounds before starting the actual measurement, it is not possible to change or adapt the compound concentrations during the measurement. Notably, various cell and tissues plates and protocol are meanwhile available, making the analysis of not just adherent cells but also tissues, suspension cells, permeabilized cells, and isolated mitochondria possible \cite{75}. Another appealing approach is the usage of genetically encoded (GE) ATP indicators based on a Förster resonance energy transfer (FRET) and...
equipped with an ATP-sensing subunit of the bacterial F$_0$F$_1$-ATP synthase. Usage of these so-called ATeams sensors enabled tracking of ATP levels ranging from 7.4 $\mu$M to 3.3 mM in different cellular compartments in real time [76,77].

**Fine-tuning of mitochondrial activity**

**The H$^+$ ion**

Mitochondrial activity and function are not solely controlled by mitochondrial structure, but also by various fine-tuning mechanisms, including homeostasis of ions (Fig. 1). In mitochondria, the H$^+$ has a unique role. It is well known that the concentration of protons (H$^+$) needs to be tightly regulated to preserve essential functions on a single cell level as well as in organisms [78,79]. While the cytosol, the ER, and the nucleus have a neutral pH of 7.0 to 7.4, lysosomes or secretory vesicles maintain an extremely acidic pH for degradation or secretion purposes [80,81]. Strikingly, we can find acidic as well as alkaline areas in mitochondria [82]. While the mitochondrial intermembrane space represents a slightly acidic environment with a pH of ~6.8, the mitochondrial matrix is the most alkaline compartment within a cell with pH values around 7.6–8.0 [82]. This difference in the proton concentration is mainly caused by the activity of the respiratory chain, transporting electrons along respiratory complexes. The serial reduction of electrons provides enough energy to shuttle protons via complex I, III, and IV from the reduction of electrons. The catalytic activity of the respiratory chain, transporting electrons via complex I, III, and IV results in increased permeability of the IMM to protons, decreasing $\Delta\psi_m$ as well as the mitochondrial pH gradient [92] and initiating cell death [93]. Since the mitochondrial metabolism is tightly regulated, it is not surprising that dysregulations and sustained changes of the $\Delta\psi_m$ lead to severe mitochondrial dysfunctions and have been associated with various disease conditions, including cancer [94,95] and neurodegeneration [96,97]. For instance, some types of tumors have been associated with elevated $\Delta\psi_m$ linked to increased glycolytic rates and resistance to regulated cell death [98]. Consistent with these reports, some lung cancer cell lines (A549, H446, SPC), breast cancer MCF7 cells, and glioblastoma MO59K cells exhibited higher $\Delta\psi_m$ than the correspondent healthy, noncancerous cell types [94]. Moreover, higher $\Delta\psi_m$ was linked to increased tumorigenicity and malignancy of cancer stem cells, linked to development and (re)occurrence of malignant tumors, while cells with lower $\Delta\psi_m$ were more prone to differentiation. Since reduction of $\Delta\psi_m$ by rapamycin decreased tumorigenicity significantly in these cells, targeting $\Delta\psi_m$ might be a potential strategy to prevent development of malignant tumors [94].

**Techniques to measure pH$_{mito}$ and $\Delta\psi_m$**

Nowadays, a huge variety of indicators and GE sensors is available to determine pH$_{mito}$ and $\Delta\psi_m$ and the most prominent candidates will be presented below.

mtAlpHi was described as one of the first encoded pH$_{mito}$ indicators, visualizing and characterizing metabolic changes within mitochondria [99]. The calmodulin of the Ca$_{2^+}$ indicator camgaroo was substituted by a portion of aequorin comprising only two EF hands, resulting in a Ca$_{2^+}$-insensitive probe with an estimated pKa of 8.5, excitation at 498 nm, and emission at 522 nm.

One of the most commonly used GE pH$_{mito}$ sensors is SypHer [92], based on a circular permuted yellow fluorescent proteins (YFP) derived from mutating the cysteine residues of the H$_2$O$_2$ sensor Hyper [100]. SypHer exhibits ratiometric responses at 430 nm and 480 nm upon changing the pH, but is insensitive to H$_2$O$_2$, Ca$_{2^+}$, and PO$_{4^{2-}}$. SypHer proved perfectly suitable for the detection of cytosolic as well as mitochondrial pH values due to a pK$_a$ of 8.71, a 20-fold increase upon switching from pH 7 to 10, and a four-fold increase in the range between pH 7 and 8. Furthermore, SypHer has been also used in simultaneous measurements in combination with other sensors performing two- or multi-color imaging [92]. The development of small chemical fluorescent dyes to specifically monitor pH$_{mito}$ has been challenging, but was finally accomplished a few years ago. While dyes like BCECF [101] or SNARF [102] have been used to measure pH in the cytosol, probes like Mitochondrium as signaling organelles
of a pH-sensitive FITC fluorophore fused to a pH-insensitive hemicyanine group. This pH-insensitive part of the probe not only acts as the reference fluorophore for a ratiometric read-out, but allows for localization in mitochondria, due to their lipophilic cationic nature. The sensor reacts reversibly to changes in pH, thereby exhibiting a double ratiometric read-out of dual excitation/dual emission and dual excitation between pH 6.1 and 8.4 [103]. Another approach to monitor pH changes was made by using a chemical system composed of a piperazine-linked naphthalimide being responsible for the fluorescent off and on signal, a cationic triphenylphosphonium group for specific mitochondrial targeting, as well as a reactive benzy1 chloride subunit for fixation in mitochondria. This probe accumulates within mitochondria due to the effect of the Δψm on the cationic triphenylphosphonium group. Additionally, the benzy1 chloride was thought to undergo nucleophilic substitution with reactive thiols of mitochondrial proteins, ensuring mitochondrial localization even upon acidification or membrane depolarization [104].

While several GE pH sensors have been developed in the last decade, to the best of our knowledge, there is no GE sensor available for measuring Δψm.

Frequently used indicators for semiquantitative analysis of Δψm are small fluorescent dyes based on a lipophilic cation structure like TMRM, tetramethylrhodamine ethyl ester (TMRE) [105], and 5,5',6,6'-tetrachloro-1,1',3,3'-tetracyanobenzilizem-dazoylcarbocyanine iodide (JC-1) [106]. TMRM and TMRE are single wavelength indicators emitting red light upon Δψm-dependent accumulation within mitochondria. The excitation and emission wavelength of these monochromatic dyes are quite similar, with excitation at 555 nm and emission at 570 nm for TMRM and excitation at 549 nm and emission at 574 nm for TMRE [105]. If used in higher concentrations, TMRM and TMRE might alter the cellular respiration and bind to mitochondrial membranes [105,107]. In contrast, JC-1 (λexc: 488 nm) is a ratiometric dye, existing as green fluorescent monomer at depolarized membrane potential (λem: 530 nm) and forming red aggregates (λem: 595 nm) at hyperpolarized membrane potential [106,108]. Notably, JC-1 is very photosensitive and the fluorescence may be changed independently of Δψm by, for instance, H2O2 or disturbed equilibrium between monomers and aggregates. All these dyes are differently permeant and require specifically adjusted loading protocols dependent on the respective cell type [108]. Moreover, complete depolarization of mitochondria, by for instance FCCP, might be necessary to achieve a baseline, which makes comparison of different Δψm measurements possible [109].

**The K+ ion**

Potassium ions (K+) are essentially involved in various processes and represent the most abundant intracellular cation. The cytosolic K+ concentration is important for the maintenance of the cell’s membrane potential, works as cofactor for various enzymes, and regulates cell volume as well as endo- and exocytosis. Also, mitochondrial functions rely on intact mitochondrial K+ (Kmito) homeostasis. While cytosolic K+ concentration is close to 140 mM in vital cells, the concentration of Kmito ranges between 20 and 60 mM [110,111].

Since mitochondrial volume [112], Δψm [113], mitochondrial metabolism [114], and ROS production [115] are tightly coupled to Kmito, the transport of this ion has to be strictly controlled by K+ channels and transporters located in the IMM [116]. Δψm drives K+ influx by diffusion across the membrane, referred to as K+ leak, mostly via two-pore domain K+ (K2P) channels, and via ATP-sensitive K+ channels located in the IMM (mitoKATP). Besides, voltage gated K+ (Kv) channels and Ca2+ activated K+ (KCa) channels are located within the IMM [117,118]. The role of mitoKATP, in particular, has been extensively studied. The generation of high levels of ATP by F0F1-ATP synthase causes a decrease in Δψm and leads to a decreased flux of K+ across the mitoKATP channels into the matrix, probably preventing devastating mitochondrial depolarization. However, under conditions of oxidative stress, increased levels of ROS activate mitoKATP channels, dissipating Δψm and counteracting further ROS production. The interplay between K+ and H+ becomes evident when considering the presence of K+/H+ transporters (KHX) in the IMM, transporting K+ from and H+ into the mitochondrial matrix. One of these transporters is the leucine zipper-EF-hand-containing transmembrane protein 1 (LETM1), facilitating K+ extrusion from the mitochondrial matrix and, thereby, also modulating Na+ and Ca2+ homeostasis [119].

High levels of matrix K+ assist in modulating the transmembrane H+ gradient, altering ATP production, but may also promote the controlled re-generation of the H+ gradient toward the IMM via the ETC [120]. Interestingly, the administration of nonselective KATP channel blockers such as glibenclamide, widely used for the treatment of type 2 diabetes, was found to ameliorate ischemia reperfusion injury in the brain, kidney, intestine, and lungs. The effect was associated
with a modulation of the oxidative stress caused by releasing ischemia, after starting reperfusion [121–123], thus highlighting the importance of proper K+mito homeostasis.

Besides the interplay of K+mito with Δψm and H+, K+mito is fundamentally affected by mitochondrial Ca2+ homeostasis. The most prominent example of Ca2+ activated K+ channels represents the large conductance KCa (BKCa) channel, found in the plasma membrane of excitable cells such as neurons and skeletal muscle cells. Several types of KCa channels could be also found in the IMM in various cell types, including the mitochondrial localized large conductance KCa (mitoBKCa) in LN299 human glioma cells [124]. Activation of these channel types is caused by elevated cytosolic Ca2+ levels as well as by changes in the Δψm, leading to K+ fluctuations across the membrane. While the pore vestibule of mitoBKCa faces the intermembrane space, the Ca2+ sensitive domain is located in the mitochondrial matrix, indicating that activation of mitoBKCa requires elevation of mitochondrial matrix Ca2+ [125–127]. Notably, charybdotoxin, which strongly inhibits BKCa channels at the plasma membrane, failed to affect mitoBKCa, raising the question whether mitoBKCa and BKCa are structurally possibly unrelated to each other [127–129].

Notably, application of compounds acting as plasma membrane BKCa channel openers like NS1619 [126], accelerating mitochondrial K+ uptake twofold, halved the size of a myocardial infarct in guinea pig hearts after reperfusion of ischemic regions. These results suggest the participation of mitoBKCa channels against necrotic and apoptotic cell injury after ischemic tissue damage, possibly by modulation of the mitochondrial respiratory chain [7], prevention of [Ca2+]mito overload, maintenance of Δψm, and/or normalization of ROS levels [126,130,131]. Moreover, NS1619 as well as CGS7184, another BKCa channel opener, also exhibited protective effects on neuronal cells [132,133]. Application of these compounds resulted in K+ flux from the extracellular space into the mitochondrial matrix in isolated rat brain mitochondria, causing Δψm depolarization and reduced ROS production [134]. In addition, application of the BKCa channel activator NS11021 was reported to inhibit glutamate-induced oxidative stress by attenuating ER stress and mitochondrial dysfunction [135]. Notably, CGS7184 was shown to directly activate the mitoBKCa by single-channel recordings. While this compound boosted oxygen consumption rate in isolated rat brain mitochondria, it exhibited cytotoxic effects by increase of cytoplasmic Ca2+ concentration and consequent activation of calpains in intact neuronal HT22 cells [136].

These results highlight the therapeutic potential but also the risk for side effects of BKCa channel modulating compounds.

Techniques to measure mitochondrial K+

Considering the importance of intact cellular K+ homeostasis, scientists have been eager to find ways to investigate cellular and mitochondrial K+ levels. K+mito dynamics are frequently measured via patch-clamp approaches using isolated mitochondria or mitoblasts [118,126,128,137]. A valuable alternative to investigate subcellular and especially K+mito dynamics within single living cells is provided by fluorescent indicators [138–140]. These indicators represent small chemical dyes, either unspecifically labeling the whole cell or specifically localizing within mitochondria. K+ binding to the sensors results in an alteration of their fluorescence depending on the K+ concentration [138–140]. Recently, the first GE indicators suitable for intracellular K+ measurements have been developed [111,141]. These probes, referred to as GE potassium ion indicators (GEPIIs), in combination with available chemical fluorescent indicators sensitive for K+ will in future deepen our understanding of subcellular and particularly mitochondrial K+ homeostasis [142].

The Ca2+ ion

While mitochondrial function and activity is heavily dependent on Ca2+ homeostasis, mitochondria, in turn, also affect the ion’s intricate role as a widespread signaling molecule within the cell. Previously thought to function primarily as a regulator of cytosolic Ca2+, it was later determined that Ca2+mito influx and efflux machinery are geared more toward control of the organelle’s own Ca2+ levels [143]. Changes to Ca2+mito concentration are known to have an effect on cellular ATP production [144,145], respiration [146], ER–mitochondria crosstalk ability (as reviewed by [147]), the onset of cellular apoptosis [148,149], autophagy [150], and many other processes related to cellular health. The mitochondria’s function also depends heavily on its ability to send messages to other organelles and receive them from the rest of the cell through Ca2+ movement. For example, the mitochondrion is known to preferentially take up Ca2+ released by the ER [151], a process that usually involves close contact sites between the two organelles [150]. Importantly, such association of the mitochondria with other cellular components is not limited to the ER; rather, other interaction has been documented between mitochondria and the nucleus [152], cytoskeleton [142], and
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plasma membrane [153], among others. Of the signals that pass to and from these parts of the cell, Ca\textsuperscript{2+}\textsubscript{mito} uptake, specifically, has multiple significant consequences pertaining to proper overall function; Ca\textsuperscript{2+} being sequestered into the matrix ultimately has an effect on local and more widespread cellular Ca\textsuperscript{2+} signals. Homeostasis between this organelle’s Ca\textsuperscript{2+} stores and the rest of the cell is crucial, as an overload of intraluminal Ca\textsuperscript{2+} can lead to the initiation of the apoptotic pathway [8].

Mitochondria are able to activate the intrinsic apoptotic pathway following Ca\textsuperscript{2+} overload through the release of multiple proteins from their lumen into the cytosol [154]. For example, cytochrome c, an important player in the ETC, is under normal circumstances found attached to the IMM. Following Ca\textsuperscript{2+} overload, mitochondria release this protein into the cytosol, where it stimulates the formation of a caspase-activating complex otherwise known as the apoptosome. The apoptosome’s overall function of activating killer caspses ultimately results in the death of the cell [155]. Concerning another important aspect of metabolism and health, cellular respiration, Ca\textsuperscript{2+} is again vital. It is known that the close positioning of mitochondria to large sources of Ca\textsuperscript{2+} (namely, the ER or the plasma membrane) allows for significant accumulation of the ion inside the mitochondrial matrix upon physiological Ca\textsuperscript{2+} release. This increase in matrix Ca\textsuperscript{2+} levels, in turn, affects mitochondrial metabolism through stimulation of mitochondrial effector molecules such as particular dehydrogenases of the Krebs cycle which are functionally dependent on Ca\textsuperscript{2+}, ultimately leading to ATP production [156]. The ability of mitochondria to accumulate Ca\textsuperscript{2+}, and the existence of a Ca\textsuperscript{2+}\textsubscript{mito} uniporter, has been reported on since the 1960s [157,158], but the molecular identity of the MCU was only determined in 2011 [159,160]. MCU is a 40 kilodalton (kDa; 35 kDa in its cleaved form) IMM protein with two transmembrane domains, which forms the core of the uniporter complex [159–161] Ca\textsuperscript{2+}\textsubscript{mito} uptake occurs primarily through MCU activity, and this uptake is highly selective but has a low apparent \( K_D \) for Ca\textsuperscript{2+}.

These effects are achieved through MCU regulators. Essential MCU regulator (EMRE) was shown to be a core component of the uniporter complex and is essential for MCU mediated- MCU [162]. In addition, EMRE was shown to regulate MCU activity based on matrix Ca\textsuperscript{2+} levels [163]. MICU1 and MICU2 are considered to be MCU gatekeepers, setting a Ca\textsuperscript{2+}\textsubscript{mito} uptake threshold, which is higher than that of the MCU [164,165]. Cells with MICU1 knockdown were shown to have elevated basal Ca\textsuperscript{2+}\textsubscript{mito} levels [166]. MICU2 is considered to be a negative regulator of MCU [165]. Thereby, the stoichiometry of MICU1 to MICU2 was shown be an important factor in Ca\textsuperscript{2+} uptake and was also reported to vary across different tissues and organs [167]. Besides, also UCP2 and UCP3 were shown to influence MCU-dependent Ca\textsuperscript{2+} uptake at higher Ca\textsuperscript{2+} concentrations, whereas LETM1 was shown to influence a potentially different uptake mechanism, likely more pronounced when Ca\textsuperscript{2+} levels are lower [168]. It was shown that UCP2 normalizes MICU in case of protein methyl transferase I-driven methylation of MICU1, resulting in sensitivity loss of MICU1 to Ca\textsuperscript{2+} [169]. An important, but as of yet unanswered, aspect of Ca\textsuperscript{2+}\textsubscript{mito} uptake is whether there are different uptake pathways operating in relation to low versus high cellular Ca\textsuperscript{2+} concentration sources, as well as what the physiological implications of these potential different uptake pathways may be. Another interesting phenomenon that awaits clarification is the spatial resolution of Ca\textsuperscript{2+}\textsubscript{mito} uptake, namely whether it occurs along the full length of the IMM, or whether this activity is restricted to certain membrane regions. Important to consider when discussing uptake of Ca\textsuperscript{2+} into the mitochondrial matrix is the organelle’s ability to balance overall charge through extrusion mechanisms. In the case of the mitochondrion, Ca\textsuperscript{2+} influx is mainly kept in check through an exchange with other ions. The major protein playing a role in this process was identified to be Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCLX) [170]. Its presence thus necessitates another mechanism to remove the excess Na\textsuperscript{+} that accumulates in the matrix following the exchanger’s activity. This is proposed to be achieved by members of the Na\textsuperscript{+}/H\textsuperscript{+} exchanger family, consisting of multiple plasma membrane and organellar transporters [171]. LETM1 has also been considered as having a role in Ca\textsuperscript{2+} movement, though its exact function in this capacity is as yet unclear. It has been proposed to act as a Ca\textsuperscript{2+}/H\textsuperscript{+} antiporter, which implies a function in Ca\textsuperscript{2+} extrusion [172]. Other publications indicate that it is instead a K\textsuperscript{+}/H\textsuperscript{+} antiporter, thereby influencing Ca\textsuperscript{2+} uptake and/or extrusion via secondary means [119,173].

Techniques to measure mitochondrial Ca\textsuperscript{2+}

Mitochondrial Ca\textsuperscript{2+} homeostasis is clearly a complex and wide-ranging process; therefore, the techniques required are also spread across multiple disciplines. In particular, the use of various sensors to study intracellular Ca\textsuperscript{2+}, as well as more specific indicators able to determine the ion’s movement to, within and from the mitochondrion itself, provide the quickest insight into the intricate web of Ca\textsuperscript{2+} signals constantly present throughout the cell.
Probes for overall intracellular Ca\(^{2+}\) measurement can generally be clustered into chemically engineered fluorophores and GE FPs.

In the recent past, the number of Ca\(^{2+}\) sensors specific to mitochondria and other organelles has reached new heights. Sensors used to measure general intracellular Ca\(^{2+}\) movement, including the ubiquitously employed cytosolic Ca\(^{2+}\) sensor Fura-2 [174], as well as the Calcium green family of indicators, are frequently being supplemented, improved upon or replaced by novel developments in sensor technology that allow for a more direct view of the processes occurring within mitochondria and other organelles. With the huge variety currently available, many criteria must be considered to optimally measure intracellular or mitochondrial Ca\(^{2+}\), dependent on the desired experimental read-out. Choosing the best-suited Ca\(^{2+}\) sensor necessitates consideration of factors such as the probe’s original form and modification required for expression in cells, its affinity for Ca\(^{2+}\), and its spectral properties, among others (as thoroughly reviewed by [175]).

Briefly, chemical indicators, compounds which change their fluorescence properties following binding to Ca\(^{2+}\), are perhaps best suited for observation of the ion’s cytoplasmic movement. While they are much simpler to employ than the average GE sensor (no cellular transfection required; simple cell-loading steps are sufficient), a main disadvantage of chemical sensors for organelle-specific Ca\(^{2+}\) measurement is the lack of controlled localization once loaded into target cells. Use of such probes will therefore not guarantee mitochondria-exclusive expression and Ca\(^{2+}\) monitoring. Chemical fluorophores are thus mainly suitable for drawing indirect conclusions on Ca\(^{2+}\)mito signaling as it pertains to the rest of the cell.

Fura-2 is a classic example of a ratiometric sensor designed for such purposes. Compared to what was available prior to its characterization, Fura-2 offered ~30× increased fluorescence signals and improved ability to bind Ca\(^{2+}\) specifically over other divalent cations, among other advantages [174]. This sensor and its variants continue to appear prominently in cellular Ca\(^{2+}\) research related to crosstalk between mitochondria and the rest of the cell; for example, investigation into the mechanism whereby the ER and mitochondria interact through mitochondria-associated ER membranes (MAMs) commonly employs the cytosolic Ca\(^{2+}\) sensor Fura-2 to draw conclusions on Ca\(^{2+}\)signaling between the two organelles [176,177]. Fluo-3 is another fluorescent dye that has been widely used to measure cytoplasmic Ca\(^{2+}\) movement, but has its own purported disadvantages. For example, Lee et al. [178] showed that in certain cell types this indicator leaks significantly, leading to lowered fluorescence measurements for intracellular Ca\(^{2+}\), combined with increased background fluorescence as leaked dye binds to Ca\(^{2+}\) present in surrounding medium. Fluo-3’s close relative, Fluo-4, proves similar in structure and other properties, but exhibits increased fluorescence and range for Ca\(^{2+}\) [179]. There are also cell-permeant dyes that target the mitochondria specifically, such as the Rhod-2 dye of the rhodamine-based family of indicators. These dyes were first introduced in the late 1980s and include Rhod-2, X-rhod-1, and many variants, all of which exhibit increased fluorescence upon binding Ca\(^{2+}\) [180]. The AM ester varieties of these indicators are cationic, which causes ion-potential-centric uptake of Ca\(^{2+}\) into the mitochondria. Due to these properties, the rhodamine-based AM esters have been employed in the literature as mitochondrial-Ca\(^{2+}\) selective indicators [181,182]. The appeal of Rhod-2, for example, is easy to see, as just like Fura-2 and other common cytosolic Ca\(^{2+}\) dyes, only cellular incubation prior to experiments is required. However, Rhod-2 is known to diffuse out of the mitochondria and into the cytosol after a relatively short period of time, making longer experiments unreliable insofar as accurate mitochondrial Ca\(^{2+}\) measurement goes. These are but a few examples, and, importantly, each of the outlined tools come with their own advantages and drawbacks. Nevertheless, together, they exemplify the broad range of sensors currently available for cytoplasmic Ca\(^{2+}\) measurement.

Among those sensors that are continuously evolving are the new generation of GE fluorescent sensors; compounds comprised of a FP fused to some form of sensing polypeptide. In most cases, the FP is bound to a protein that undergoes a conformational change in response to substrate binding. These sensors are generally considered advantageous over their chemical counterparts due to their substrate specificity, and the fact that their GE nature prevents variance in probe uptake across different cells [183]. Touted as being perhaps the most useful advantage of such sensors is the ability to target them to highly specific cellular regions [184], as shown at the turn of the century by Arnaudeau et al. (2001) with the use of a cameleon indicator targeted to each of the cytosol, mitochondrial matrix, and ER lumen.

Development of tools for accurate measurement of Ca\(^{2+}\)mito levels specifically has provided new insights into Ca\(^{2+}\)mito fluxes and their regulation. For example, 4mtD3cpV is a FRET-based ratiometric Ca\(^{2+}\) sensor that is currently widely used. It is excited with blue light (430 nanometers—nm) and
exhibits dual emission at 480 and 535 nm. The ratio of the 480 nm emission to the FRET signal (535 nm) provides investigators with insight into and enhanced understanding of basal Ca\(^{2+}\) levels as well as its fluctuations [185].

Another FRET-based ratiometric Ca\(^{2+}\) sensor, which can be combined with cytosolic Fura-2 dye, is 4mtD1GO-CAM [186]. It is a red-shifted sensor, which allows the researcher to measure Ca\(^{2+}\) in the mitochondria while simultaneously employing Fura-2 to measure cytosolic Ca\(^{2+}\). As Fura-2 has a wide excitation–emission spectrum, combined measurement with 4mtD1GO-CAM proves extremely useful for investigation into the spatiotemporal fluxes of Ca\(^{2+}\). In addition, as both the Fura-2 dye and mtD1GO-CAM sensor are ratiometric, accurate observation of basal Ca\(^{2+}\) levels is also possible [186].

Ca\(^{2+}\) sensors of the GECO series provide a good opportunity to measure Ca\(^{2+}\) levels simultaneously in different compartments [187,188]. mtGEM-GECO1 is a ratiometric sensor with a very low \(K_D\) for Ca\(^{2+}\) (340 nm) and has an excitation wavelength of 394 nm and dual emission of 455 and 511 nm [187]. Its relative mtCAR-GECO1 is an intensiometric sensor with excitation and emission spectra of 565 and 620 nm, respectively [188]. Excitation and emission spectra of CAR-GECO1 and GEM-GECO1 sensors allow measurement of Ca\(^{2+}\) levels in two different organelles or mitochondrial compartments simultaneously with almost no spectral overlap.

From this simple examination of a handful of the more prominent cytosolic and mitochondria-specific sensors in use today, it is clear that investigators have no lack of options when it comes to studying the intricate movements of Ca\(^{2+}\) in this organelle and throughout the cell.

**Mitochondria as signaling hubs: Give & Get**

**Interplay between mitochondria and nucleus**

Although mitochondria are equipped with their own circular deoxyribonucleic acid containing 37 genes (i.e., 13 genes encoding for proteins, such as subunits of the respiration complexes and the ATP synthase, 24 genes encoding for tRNAs), almost all mitochondrial proteins are encoded by the nuclear genome, making constant communication between mitochondria and the nucleus indispensable [10]. Consequently, mitochondrial biogenesis strongly depends on the contribution of the nucleus, and properly controlled signaling cascades are required to fine-tune mitochondrial protein synthesis, counteract mitochondrial dysfunction, and initiate compensatory mechanisms [189]. Constant mitochondrial status control by the nucleus helps to prevent mitochondrial malfunction, to counteract damage, and to restore mitochondrial homeostasis via situation-induced activation of transcriptional response [190]. The most prominent example of mitochondrial–nucleus crosstalk is retrograde signaling pathways. In these pathways, the mitochondrial unfolded protein response (mtUPR) initiates a protective transcriptional program upon proteotoxic perturbations, aiming to re-achieve homeostasis in mitochondrial protein biosynthesis and recover the defective organelle [191,192].

**Techniques to measure mitochondrial–nucleus interplay**

Dually targeted proteins, localizing to the nucleus as well as to mitochondria, are used as communication indicators for mtUPR (retrograde) signaling. As mitochondrial protein import strongly affects cell viability, the transport of these proteins, visualized by tagging them with a FP, can be used as an indicator of mitochondrial fitness [193–195]. For instance, the mammalian activating transcription factor 5 is imported into mitochondria under physiological conditions, but trapped and consequently translocated to the nucleus to activate transcriptional adaption in case of mitochondrial dysfunction [196]. In addition, transcriptional cofactor G protein pathway suppressor 2, another modulator of mtUPR, has also been presented as such an indicator protein, translocating between mitochondria and the nucleus depending on the functionality of mitochondria [197]. Interestingly, cytosolic proteins prone to aggregation are imported into mitochondria for degradation. This translocation of misfolded or aggregated proteins from the cytosol to mitochondria, associated with increased mitochondrial stress levels, can be visualized using split FP techniques [198].

**Interplay between mitochondria and ER**

Contact between mitochondria and the ER occurs at very specialized junctions stabilized by MAMs, forming locally restricted signaling hubs to restrict and protect the transfer of lipids and Ca\(^{2+}\) between mitochondria and the ER [199]. First discovered by electron microscopy in the 1950s [200] but their basic function only described in the 1990s [201,202], investigation of these structures has been further pushed by the development of cutting-edge technologies like high-resolution microscopy over the last 20 years [199].
Various proteins stabilizing and modulating mitochondrial–ER interplay, including Ras-related protein RAB32 [203], MFN2 [204], or phosphofurin acidic cluster sorting protein 2 [205], as well as protein tethering complexes like inositol triphosphate receptor (IP3R), inositol-requiring enzyme 1 α [206], glucose-related protein 75 and VDAC, have been identified and characterized [207]. Disrupted communication between the ER and mitochondria has been associated with pathological conditions and human diseases [208], such as Alzheimer’s disease [209,210], Charcot-Marie Tooth [211], Parkinson’s disease [212,213], viral infections [214], cancer [215], diabetes mellitus, and age-related dysfunction [216].

Techniques to measure ER–mitochondrial interplay

Different approaches have been developed to explore the physical and functional sides of ER-mitochondria tethering. The current state-of-the-art technique to visualize MAMs is electron microscopy, making quantification of the distance between the membranes of the ER and mitochondria, as well as the number of contact sites, possible [217]. Coupling electron microscopy with tomography has provided 3D models and information about the structure and plasticity of the contact points between the two organelles [20]. While these techniques come with unbeatable resolution, they also bring the disadvantage of fixation, possibly affecting mitochondrial structure. Therefore, large efforts have recently been placed to develop methods that allow the visualization of MAMs in living cells and still provide high spatial resolution. As discussed in our “Techniques to analyze structure and shape” section, advancements in high-resolution fluorescence microscopy and in the design of organelle-targeted FP-tagged proteins or sensors allow to visualize and investigate structural and functional mitochondrial and ER interplay in living cells [216]. Moreover, split FP (split-FP) approaches or so-called bimolecular fluorescence complementation technology have been used to study mitochondrial–ER interaction [218]. Cieri et al. (2017) have designed split-GFP-based contact site sensors (SPLICS), fusing the GFP1–10 moiety to an OMM targeting signal and the GFP11 β-strand to an ER leading peptide and varying the linker length between the targeting signals and the split-FP to visualize narrow (approximately 8–10 nm) and wide (approximately 40–50 nm) distances between the ER and mitochondria. As soon as the split-FPs are in close vicinity due to the interactions between proteins fused to each fragment, they hit a full fluorescent FP [219]. Using split-FP technology based on GFP, dynamic changes in the structure of MAMs could be visualized and enhanced formation of ER–mitochondrial contact by mitochondrial uncouplers shown [220]. Moreover, Harmon et al. have developed a split-FP approach to study mitochondrial–ER interaction based on a YFP Venus by fusing the n-terminal fragment of Venus128 to a mouse ER–protein and targeting the c-terminal fragment of Venus to the OMM via the n-terminal leading peptide of TOMM20. As a result, alterations in the MAM structure in response to ER stress, starvation, and protein level changes could be detected [221].

Recently, Ding et al. [222] have developed a novel FP approach using a pair of quenched and nonfluorescent FP-derived monomers that become a fluorescent heterodimer upon FP association. Alford et al. [223] exploited this FP technology and generated probes by fusing one monomer to the C-terminus of TOMM20 and targeting the other monomer of the dimerization-dependent FP pair to the ER–membrane via calnexin. Hajnoczykzer et al. exploited the FKBPFRB heterodimerization system by fusing FKBP to an OMM targeting signal and combining FRB with an ER retention signal to enable rapamycin inducible tethering between the two organelles. By using this approach, tethering of the ER and mitochondria results in an increase of already in close apposition located contact sites, rather than creating new organelle contacts [224].

Furthermore, recently developed light-inducible tethering systems allow the induction of ER–mitochondrial interaction, which facilitates the functional study of ER–mitochondrial contacts [225].

Besides various techniques based on cutting-edge microscopy, an IP3R–VDAC proximity ligation assay has been developed for the quantification of ER–mitochondria interplay. Proximity ligations assays, as an in situ tool, enable the detection of endogenous proteins, protein modifications, and protein interactions with high specificity and sensitivity by using antibodies to detect two unique protein targets [226]. Furthermore, Percoll density gradient was used to purify MAMs in order to analyze their composition [227]. Functional characterization of MAM regions taking lipid exchange into account has been performed using phosphatidylserine (PS) to phosphatidylethanolamine (PE) conversions or as mitochondrial PS content with regard to lipid transfer [228]. Since mitochondria are not governed by classical vesicular trafficking mechanisms, required membrane phospholipids for the mitochondrial membrane biogenesis have to be imported into the organelle. Therefore, mitochondria rely on the efficient supply of lipids from the ER, meaning that biosynthesis of some phospholipids depends on the mitochondria–ER crosstalk. PS, coming from the ER and being directly transferred to
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Conclusion

As presented in the current review, mitochondria are versatile organelles within eukaryotic cells, which deliver utilisable energy and function as signaling hubs, communicating with various cellular compartments to maintain their own function but also to provide their service.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

CTM, JR, GZ, SB, HB, ZK, and BG contributed chapters to the manuscript. CTM together with WFG and RM planned the manuscript’s structure and content.

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