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Measuring mitochondrial function in intact cardiac myocytes

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Abstract

Mitochondria are involved in cellular functions that go beyond the traditional role of these organelles as the power plants of the cell. Mitochondria have been implicated in several human diseases, including cardiac dysfunction, and play a role in the aging process. Many aspects of our knowledge of mitochondria stem from studies performed on the isolated organelle. Their relative inaccessibility imposes experimental difficulties to study mitochondria in their natural environment – the cytosol of intact cells – and has hampered a comprehensive understanding of the plethora of mitochondrial functions. Here we review currently available methods to study mitochondrial function in *intact* cardiomyocytes. These methods primarily use different flavors of fluorescent dyes and genetically encoded fluorescent proteins in conjunction with high-resolution imaging techniques. We review methods to study *mitochondrial morphology*, mitochondrial membrane potential, Ca^{2+} and Na^{+} signaling, mitochondrial pH regulation, redox state and ROS production, NO signaling, *oxygen consumption*, ATP generation and the activity of the mitochondrial permeability transition pore. Where appropriate we complement this review on intact myocytes with seminal studies that were performed on isolated mitochondria, permeabilized cells, and in *whole hearts*.

Keywords

Cardiomyocytes; Mitochondrial function; Methods

Introduction: Mitochondrial structure and function

Mitochondria are cytosolic double-membrane organelles, *which for their ability to generate ATP for most of the cellular energy demands, are often referred to as the ‘power plants’ of the cell* [1, 2]. However, mitochondria are also involved in a range of other processes, such as signaling, cellular ion homeostasis, oxidative stress, apoptotic and necrotic cell death, as well as the control of cell cycle and cell growth [3]. The cellular number of mitochondria varies widely by species, cell and tissue type. An adult ventricular myocyte contains ~7000 mitochondria, which occupy ~35% of the cell volume [4, 5] to match the high energy demands of these cells. Mitochondria dynamically change their morphology through the

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Disclosures

None.

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processes of mitochondrial fusion and fission to form an extensive interconnected mitochondrial network or a fragmented discrete phenotype [6–9]. Indeed, the name “mitochondrion” originating from the Greek words “mitos” (thread), and “chondrion” (grain or granule) reflects the heterogeneity of mitochondrial morphology. In adult cardiomyocytes, the size, shape and metabolic activity of mitochondria also depend on intracellular location. Three subpopulations of mitochondria in the adult heart have been identified as interfibrillar, subsarcolemmal and perinuclear mitochondria [7, 8, 10]. Interfibrillar mitochondria are aligned in longitudinal rows between myofibrils [4, 8, 10] in close proximity to sarcoplasmic reticulum (SR) Ca^{2+} release sites [10]. They often span a single sarcomere from Z-band to Z-band and are relatively uniform in size and shape (rod-shaped organelles 0.5–1 μm in width and 1–2 μm in length) [8, 10]. Subsarcolemmal and perinuclear mitochondria appear less organized and more variable in shape and size [8, 10], possibly as a result of less restraint fission and fusion compared to interfibrillar mitochondria [7, 8]. In contrast to adult myocytes, mitochondria of neonatal cardiomyocytes are organized in extensive cytoplasmic membrane networks undergoing continuous fission, fusion, and movement rather than individual rod-shaped organelles [8]. Mitochondria composed of compartments that carry out specialized functions: the outer mitochondrial membrane (OMM), the intermembrane space (IMS), the inner mitochondrial membrane (IMM) with the cristae and the matrix (Figure 1). Mitochondria contain their own genome that is distinct from the genome of the cell. The OMM encloses the entire organelle but is freely permeable to molecules of up to 5000 daltons due to the presence of pores (about 2–3 nm) formed by the Voltage-Dependent Anion Channel (VDAC). VDAC is the most abundant protein of the OMM and is present in 3 distinct isoforms in eukaryotic cells (VDAC1, VDAC2 and VDAC3) [11, 12]. VDAC is involved in transporting metabolites, including ADP and ATP, between mitochondria and cytosol, and in its ‘closed confirmation’ it maintains a pore of ~1.8 angstroms diameter, that permits passage of protons and other ions [13], making the concentration of small molecules such as ions and sugars in the IMS similar to the cytosol. Although all three VDAC isoforms are equivalent in allowing mitochondrial Ca^{2+} loading upon IP_3 -releasing agonist stimulation in HeLa cells, silencing of VDAC1 selectively impairs the transfer of a low-amplitude apoptotic (e.g., oxidative stress in form of 1 mM H_2O_2) Ca^{2+} signal to mitochondria [14]. Larger molecules like proteins, however, can only cross the OMM by active transport through mitochondrial membrane transport proteins making the IMS a compartment that contains a distinct set of proteins including cytochrome c. The vast majority of proteins destined for the mitochondrial matrix are encoded in the nucleus and synthesized outside mitochondria. Mitochondrial protein import involves the TIM/TOM complex (TIM: Transporter Inner Membrane; TOM: Transporter Outer Membrane) [15, 16]. Besides their protein transport role, members of this translocation machinery also participate in processes leading to apoptosis. For example, the Peripheral Benzodiazepine Receptor (PBR, also known as translocator protein of the outer membrane or TSPO) of the OMM serves the cholesterol transport and steroid synthesis [17], but is also involved in OMM permeabilization in apoptosis in conjunction with the pro-apoptotic Bcl family of proteins [18]. Members of the Bcl-2 protein family regulate apoptosis by controlling the formation of the Mitochondrial Apoptosis-Induced Channel (MAC, see Figure 1) in the OMM in response to certain apoptotic stimuli [19] where the pro-apoptotic members Bax and/or Bak form MAC [19, 20], and the anti-apoptotic members Bcl-2 or Bcl-xL prevent MAC formation. MAC formation is an early marker of the onset of apoptosis [21, 22] and MAC activation mediates the release of cytochrome c to the cytosol, triggering the commitment step of the mitochondrial apoptotic cascade. Pharmacological inhibition (e.g. with Bax Channel Inhibitors [23] and MAC inhibitors [24]) or genetically knocking down main components of MAC result in prevention of cytochrome c release and apoptosis.

In striking contrast to the OMM, the IMM is highly impermeant. Ions, metabolites and proteins require specialized membrane transporters and exchangers to access the matrix compartment. The TIM translocase complex is responsible for protein import and metabolite passage. The IMM forms internal compartments called cristae. The IMM contains the phospholipid Cardiolipin which significantly reduces the IMM permeability to protons and therefore allows to establish a proton-motive force across the IMM. The proton-motive force is generated by the complexes of the IMM respiratory chain (or electron transport chain, ETC) which uses free energy released during electron transport to translocate protons (H^+) from the mitochondrial matrix into the IMS. The main components of the ETC are complexes I to IV, coenzyme Q and cytochrome c. The proton-motive force is composed of the mitochondrial membrane potential ($\Delta\Psi_m$) which arises from the net movement of positive charges across the IMM, and the pH gradient. Influx of protons back into the matrix is mediated by the F_1F_0 -ATP synthase. This multi-subunit protein complex includes a H^+ channel coupled to an ATP synthase which generates ATP. $\Delta\Psi_m$ across the IMM is highly negative (~ -180 mV), due to the chemiosmotic gradient for protons across the IMM. The proton gradient provides the energy for ATP synthesis by the respiratory chain. $\Delta\Psi_m$ also provides the driving force for Ca^{2+} uptake into mitochondria by the Ca^{2+} -uniporter (MCU) [25–29] which has been identified recently as a 40-kDa protein of the IMM [28, 29]. Increases in matrix Ca^{2+} concentration ($[Ca^{2+}]_m$) enhance the activities of ATP synthase and enzymes of the tricarboxylic acid (TCA) cycle, thus stimulating ATP production rates [30]. However cytosolic Ca^{2+} overload and excessive Ca^{2+} uptake by the MCU can depolarize mitochondria and limit Ca^{2+} entry, but may also abolish the driving force for H^+ entry and ATP synthase function [31]. Matrix acidification by the H^+ flow through the ATP synthase required for ATP synthesis also inhibits the MCU and thereby allows to maintain $[Ca^{2+}]_m$ at optimal levels [32, 33]. In order to maintain mitochondrial Ca^{2+} homeostasis, Ca^{2+} uptake is balanced by Ca^{2+} extrusion primarily via mitochondrial Na^+/Ca^{2+} exchanger (NCX_m), with a small contribution of Ca^{2+}/H^+ exchanger and Na^+/H^+ exchanger which serves as the pathway for Na^+ extrusion from the matrix [25, 26]. Elevated levels of matrix Ca^{2+} and reactive oxygen species (ROS) activate the mitochondrial permeability transition pore (mPTP) leading to necrotic or apoptotic cell death [25, 34]. The irreversible mPTP opening is associated with a collapse of $\Delta\Psi_m$, release of cytochrome *c* and perhaps more ROS generation, resulting in a vicious cycle of further amplification of ROS production, mitochondrial Ca^{2+} overload, and increasingly irreversible cell damage [35]. While it has been postulated that the mPTP mediates the lethal permeability changes of the mitochondrial membranes leading to mitochondria-mediated cell death, recent studies indicate that brief transient openings of the mPTP (cf. Figure 2 and [36, 37]) can provide protection against cellular injury by serving as a physiological Ca^{2+} release mechanism during mitochondrial Ca^{2+} overload [38–41]. A rise of intramitochondrial Ca^{2+} can also activate a putative mitochondrial nitric oxide synthase (mtNOS) which can generate NO locally but also can become as source of ROS under certain pathological conditions [26, 42]. Even though the existence of the mitochondrial nitric oxide synthase (mtNOS) in cardiac mitochondria is still controversial and the enzyme has not been identified at the protein level (see [26, 43–46] for reviews), several functional studies demonstrated that an increase in mitochondrial Ca^{2+} uptake could generate local NO [42, 46–53] which can serve as a protective mechanism by inhibiting mitochondrial Ca^{2+} accumulation and respiration.

Methods to measure mitochondrial function

In the following paragraphs we will review methods to study mitochondrial morphology, mitochondrial membrane potential, Ca^{2+} and Na^+ signaling, mitochondrial pH regulation, redox state and ROS production, NO signaling, oxygen consumption, ATP generation and the activity of the mitochondrial permeability transition pore. This review focuses on studies performed on intact cardiac myocytes. Considering the fact that single cell studies have their

own inherent limitations (e.g. elimination of putative interactions with cells of the surrounding tissue, differences in oxygen and metabolite supply) we complement, where appropriate, this review with seminal studies that were performed on isolated mitochondria, permeabilized cells, and in whole hearts.

Measurement of mitochondrial morphology

Given the relatively rigid and well-defined arrangement of mitochondria in the adult heart (see above), it may be difficult to appreciate the relevance of changes in mitochondrial morphology to this organ. This characteristic arrangement of mitochondria develops during cardiac maturation, since in neonatal cardiomyocytes [8] mitochondria are organized in filamentous networks similar to many other non-cardiac cell types [54]. Emerging evidence suggests that changes in mitochondrial morphology may be relevant to various aspects of cardiovascular biology, including cardiac development [55], the response to ischemia-reperfusion injury [56], heart failure [57], diabetes mellitus [55, 58] and apoptosis [59]. Traditionally the vast majority of our knowledge about mitochondrial morphology stems from electron-microscopic studies (see [8, 10] for detailed reviews). However, this method only provides a snapshot in space and time, and cannot visualize mitochondrial dynamics directly. However, several novel techniques have been developed recently to monitor dynamic changes in mitochondrial morphology, fission and fusion. For example, mitochondrial fusion has been studied dynamically with an assay based on mitochondrial expression of red and green fluorescent proteins and monitoring of matrix content mixing after fusion [60]. More recently, a quantitative assay for mitochondrial fusion based on the Renilla luciferase complementation assay has been described [61]. The process of mitochondrial fusion can be tracked also using a photoactivatable mitochondrial green fluorescent protein (PA-GFP_{mt}) [62]. PA-GFP_{mt} is nonfluorescent but can be activated in a highly localized manner by ultraviolet illumination. The photoactivated GFP diffuses rapidly within the entire mitochondrial matrix and can be therefore be used to tag individual mitochondria. In adult murine myocytes infected with adenovirus to deliver photoactivatable mitochondrial-targeted PA-GFP [56] elongated interfibrillar mitochondria could be identified. The length of these elongated mitochondria ranged from 2 to 6 μm (corresponding to 1 to 3 sarcomeres). Cell treatment with mitochondrial division inhibitor-1, a pharmacological inhibitor of the mitochondrial fission protein dynamin-related protein 1 (Drp1), increased the percentage of the elongated mitochondria approximately fourfold [56]. Moreover, inhibition of mitochondrial division reduced cell death and inhibited mPTP opening after simulated ischemia-reperfusion, and in vivo treatment with this inhibitor reduced myocardial infarct size in mice after coronary artery occlusion and reperfusion [56] suggesting that inhibition of the mitochondrial fission could provide a novel pharmacological approach for cardioprotection.

Measurement of mitochondrial membrane potential

In intact cells the mitochondrial matrix compartment is not accessible with standard electrophysiological techniques using sharp or patch-clamp microelectrodes. Therefore, $\Delta\Psi_m$ is typically estimated with fluorescent voltage-sensitive dyes which are membrane-permeant fluorescent lipophilic cationic compounds that distribute across the IMM [63–67] following the Nernst equation. The most commonly used compounds are rhodamine 123 (Rho123), its derivatives tetramethylrhodamine methyl and ethyl esters (TMRM and TMRE) (Figure 2), and the carbocyanine compound JC-1 [68, 69]. While in principle the use of these voltage-sensitive indicators is straight forward, there are several caveats to consider when interpreting results obtained in intact cells. First, the dye must be kept in all superfusion solution throughout the experiment in order to maintain its equilibrium distribution. Otherwise, with each solution change, dye will leak out of the cell, resulting in changes in fluorescence that are independent of in $\Delta\Psi_m$ [70]. For cardiomyocytes, it is

necessary to load cells with dyes for 30–60 min at 37°C in order to assure appropriate mitochondrial localization [70, 71]. Dye concentration should be kept in the low nanomolar range (5–50 nM for TMRE and TMRM, for example). Even when cells are loaded with low (nanomolar) concentrations of the dye, its concentration in mitochondria can reach several hundred micromolar [72]. TMRM, for example, can concentrate about 10 fold across the plasma membrane and 400–600 fold across the mitochondria membrane (with $\Delta\Psi_m \sim -180$ mV) [70, 71]. Moreover, the concentration of these dyes in mitochondria not only depends on $\Delta\Psi_m$ but may also be affected by changes in the volume of these organelles, e.g. when mitochondrial swelling occurs. Mitochondrial movement in and out of the focal plane (e.g. due to fusion and fission, see above) can pose another problem for correct $\Delta\Psi_m$ monitoring. It is possible to correct for movement artifacts by applying a ratiometric approach. For example, the simultaneous loading of mitochondria with targeted PA-GFP_m (a matrix-targeted protein used in mitochondrial morphology studies, see above) and TMRE [62] *has been used to measure $\Delta\Psi_m$ ratiometrically and to circumvent movement-dependent changes in TMRE fluorescence [62]. The simultaneous presence of two fluorophores, however can potentially be an additional source of error resulting from interactions between the two dyes (see below).* Additionally, many of these dyes exhibit significant binding to mitochondrial structures and enhanced mitochondrial accumulation which causes deviations from Nernstian behavior [67, 73]. Dyes can leak out of mitochondria when mPTP opening occurs, due to simple diffusion rather than partition in accordance with membrane potential. All these factors should be considered when $\Delta\Psi_m$ calibration is made (see [62, 67, 72] for details). *In intact cells, $\Delta\Psi_m$ measurements are complicated by the role that the plasma membrane plays for the accumulation and distribution of the probe. First, it has been shown that cells expressing plasma membrane glycoproteins responsible for multidrug resistance (MDR) have a limited ability to accumulate TMRM and JC-1 [74] (an issue to keep in mind when comparing different cell types, cardiac cell lines and species). All potentiometric dyes are good substrates for MDR carriers, which will export the dye from the cells leading to a decreased dye accumulation [70]. Typically, in cells expressing MDR, dye loading will be extremely slow and can be greatly enhanced by treatment with MDR inhibitors (progesterone, verapamin, and cyclosporin A) [70, 74]. Second, the cytosolic dye concentration is also affected by the plasma membrane potential which drives, together with $\Delta\Psi_m$, the distribution of the dye between mitochondrial matrix, cytosol and extracellular space [71]. Typically, upon mitochondrial depolarization, TMRM is released from mitochondria to the cytosol, causing a decrease of mitochondrial fluorescence and an increase of cytosolic fluorescence without any significant changes of the signal averaged over the whole cell. Increased cytosolic TMRM will redistribute into the extracellular solution if the depolarization persists for a sufficiently long period of time [70] leading to a decrease of whole cell fluorescence, or back into mitochondria if depolarization is transient [40] without any significant changes in whole cell fluorescence. Along the same line changes in plasma membrane potential will also cause a redistribution of TMRM from cytosol to the extracellular buffer and may cause a change in the mitochondrial fluorescence signal, even though $\Delta\Psi_m$ remains unchanged.*

Voltage-sensitive dyes have also been used at higher concentration to monitor $\Delta\Psi_m$ (e.g. 1–20 μ M TMRM loaded for 10–15 min with subsequent washout) non-confocally from whole cells [70]. In this case mitochondrial fluorophore accumulation leads to formation of non-fluorescent dye aggregates [75], called J-aggregates [63, 67], that causes fluorescence quenching. J-aggregate formation is not unique to TMRM and has also been observed with Rho123 and other fluorophores. Depolarization of $\Delta\Psi_m$ and subsequent redistribution of TMRM out of the matrix compartment results in TMRM ‘de-quench’ and a fluorescence increase reflecting the average change in $\Delta\Psi_m$ [70, 71, 76]. In this quench/de-quench mode small depolarization in $\Delta\Psi_m$ induced much larger (2-order of magnitude) changes in TMRM fluorescence compared to that obtained in redistribution mode (low concentration approach)

[77]. However, caution should be used when applying the de-quench approach in combination with confocal imaging. Laser illumination of TMRM loaded in mitochondria at high concentrations generates ROS from the fluorescent probe that provokes mPTP opening [37, 76]. (This 'side effect' has actually been used as experimental model for simulation of oxidative stress and to induce mPTP opening observed during ischemia-reperfusion [76, 78] rather than for $\Delta\Psi_m$ monitoring).

Monitoring $\Delta\Psi_m$ has also been achieved successfully in intact heart. This may be of particular importance for studying mitochondrial function in the diseased heart. Despite a number of technical difficulties related to the visualization of mitochondrial function in intact heart, optical mapping in ex vivo perfused guinea pig [79, 80] and rat [81–83] hearts has been performed. Using real time 2-photon [79–81] or high-resolution CCD camera [82, 83] imaging, complex spatiotemporal heterogeneities in $\Delta\Psi_m$, together with complex spatial patterns (e.g. $\Delta\Psi_m$ waves) and dynamic fluctuations in $\Delta\Psi_m$ could be observed during global ischemia.

Overall, the different available voltage-sensitive dyes have been used in cardiac tissue with variable success. Rho123 has turned out to be problematic since mitochondrial uncouplers failed to induce any changes in $\Delta\Psi_m$ in cardiomyocytes [70, 84]. Moreover, Rho123 was reported to inhibit ADP-stimulated respiration and mitochondrial ATP synthase at high matrix concentrations [85]. JC-1 was first reported in adult cardiac myocytes to measure $\Delta\Psi_m$ during anoxia and reoxygenation [86]. JC-1 emits light at red and green wavelengths depending on concentration. At high concentrations J-aggregates form and emit red light, whereas at low concentration the monomer form emits green light [86]. While, in principle JC-1 offers the advantage to measure $\Delta\Psi_m$ ratiometrically by monitoring the ratio of green/red (530/590 nm) fluorescence [86], data obtained with JC-1 should also be interpreted with caution [70, 86]. J-aggregates are formed across the whole cell only when higher concentrations (0.5–1 μM) and longer loading times (30 min to 2 hours) were used. Otherwise J-aggregates tend to accumulate in the periphery only due to slow diffusion and redistribution rates of the dye [70, 74] which may preclude the detection of rapid changes in $\Delta\Psi_m$. Furthermore, the equilibrium between monomer and J-aggregates is also influenced by pH, osmolarity, and oxidative environment of the cells [87, 88]. Moreover, high concentrations of JC-1 can be toxic to mitochondria detected as formation of crystalline-like structures [70, 74]. As a rule, the use of high (more than 1 μM) concentrations of the potentiometric dyes should be avoided since they can cause metabolic inhibition [63]. Of commonly used fluorophores, TMRM exhibits the least mitochondrial toxicity [67]. To minimize phototoxicity and ensure a linear response, low concentrations of TMRM should be used in the dye re-distribution mode described above.

TMRM is often used in combination with other fluorescent dyes to monitor simultaneously additional mitochondrial parameters such as $[\text{Ca}^{2+}]_m$, mitochondrial ROS generation (with dichlorofluorescein diacetate, DCF) or the opening of the mPTP (with calcein). The simultaneous presence of two fluorophores can result in FRET phenomena (Förster resonance energy transfer or fluorescence resonance energy transfer) that can lead to erroneous interpretations of the results. Both DCF [80, 89] and calcein [37, 90] have been described as FRET partners of TMRM.

The plethora of limitations and drawbacks associated with the use of voltage-sensitive dyes also renders these indicators difficult to calibrate quantitatively (for discussion of signal calibration see e.g. (see [62, 67, 72])). Often experimental data with potentiometric probes are expressed as a percentage changes from basal levels. As a minimum recommendation it is suggested to expose cells to the protonophore FCCP (1–10 μM) at the end of an experiment to equilibrate H^+ concentration in all compartments and therefore abolish the

$\Delta\Psi_m$. This provides a minimum $\Delta\Psi_m$ level that is required for signal normalization between different cells. An interesting suggestion for signal calibration takes into consideration the standard deviation of the measured fluorescence signal. Polarized mitochondria concentrate the dye into the organelle causing a bright punctate signal with a high standard deviation (SD) across the cell (i.e., much brighter signal over mitochondria compared to cytosolic signal) while depolarization causes a decrease in SD as the dye is evenly distributed throughout the cell (see [70] for more details). The ratio of mean fluorescence to SD (mean/SD) gives a quantitative measure of dye localization and can be used to calibrate $\Delta\Psi_m$.

Measurement of $[Ca^{2+}]_m$ and mitochondrial Ca^{2+} transport

Different methodologies have been used to measure $[Ca^{2+}]_m$ in intact living cardiomyocytes with fluorescent Ca^{2+} indicators. Typical strategies employed entrapping membrane-permeant forms of fluorescent Ca^{2+} indicators (typically acetoxymethyl (AM) esters) in the matrix compartment and subsequently eliminating the cytosolic contribution to the fluorescence signal. De-esterification by intracellular (cytosolic and intramitochondrial) esterases entraps the indicator in the respective compartments. However, the potential presence of cytosolic dye may confound or even preclude $[Ca^{2+}]_m$ measurements. Cytosolic dye can be eliminated by accelerating extrusion at higher temperature [84, 91], perfusion of the cytosol or by quenching with Mn^{2+} [92–94] or Co^{2+} [71]. De-esterification at 37°C for several hours may preferentially eliminate cytosolic loading [95, 96], however it can also reduce the Ca^{2+} responsiveness of the dye [70, 71]. Cytosolic perfusion with dye-free pipette solution can be achieved using the whole cell patch clamp method [93, 97]. The cytosolic dye quench technique has been used successfully [92–94, 98, 99], however is potentially complicated by the fact that the MCU can transport Mn^{2+} (and thus quench matrix dye) and Mn^{2+} may interfere with mitochondrial Ca^{2+} transport [92, 100]. Among the available fluorescent Ca indicators, indo-1 [92–94, 98], fura-2 [99], fluo-3 [101], dihydorhod-2 [102], rhod-2 [103] and X-rhod-1 [71] have been used for $[Ca^{2+}]_m$ measurements. *Rhodamine compounds (Rhod-2, Rhod-2FF, dihydorhod-2 and X-rhod-1) were originally developed with the goal to measure $[Ca^{2+}]_m$ selectively in mitochondria. This was based on the fact that esterification with AM results in a cationic molecule that will accumulate preferentially (but not exclusively!) in mitochondria due to the large negative $\Delta\Psi_m$ and remain in the mitochondrial matrix after cleavage by intramitochondrial esterases. The K_d of rhod-2 for Ca^{2+} is relatively low (~ 570 nM), which can lead to dye saturation during mitochondrial Ca^{2+} overload, and is affected by changes in pH as they might occur during ischemia [104]. The related probe X-rhod-1 may be more suitable for measuring higher $[Ca^{2+}]_m$ levels because of its higher K_d (~ 700 nM).*

Moreover, it has been reported that rhod-2 and rhod-FF induced Ca^{2+} -dependent inhibition of mitochondrial Ca^{2+} uptake since increase in $[Ca^{2+}]_m$ was blunted during repetitive stimulation [105]. Also, changes in mitochondrial morphology and membrane potential, which were amplified by cell exposure to the excitation light, were observed after cell loading with these dyes at low concentrations (1–2 μ M) [105]. At higher dye concentrations (>5 μ M) cell damage occurred, that partially could be explained by the generation of toxic byproduct induced by dye illumination. A common potential disadvantage of all rhodamin-derived indicators is their non-ratiometric nature, which make $[Ca^{2+}]_m$ measurement susceptible to errors stemming from changes in dye concentration and motion artifacts. Classical ratiometric indicators like fura-2 [99] and indo-1 [92–94, 98] have been used to measure $[Ca^{2+}]_m$, however their relatively low K_d and the potential presence in the cytosol imposes serious limitations to the use of these indicators for $[Ca^{2+}]_m$ measurements in intact cells. Despite their limitations and shortcomings conventional fluorescent Ca^{2+} indicators have been used to study the role of mitochondria for Ca^{2+} homeostasis in cardiac myocytes. For example, Sedova et al. [101] addressed the controversial question (for reviews see [26,

106]) whether $[Ca^{2+}]_m$ reflects the cytosolic beat-to-beat Ca^{2+} transients, using a novel approach which imitates conditions of an intact cytosolic environment in membrane-permeabilized cells. Permeabilized cells have the unique advantage that the cytosolic environment can be controlled precisely while the arrangements and interactions between intracellular membranes and organelles (SR, mitochondria) remain structurally and functionally intact [107, 108]. $[Ca^{2+}]_m$ was measured with fluo-3 trapped inside mitochondria and cytosolic indicator was removed by plasma membrane permeabilization with digitonin (Figure 3). Cells were exposed to rapid ejections of Ca^{2+} from a micropipette mimicking cytosolic Ca^{2+} transients. With this technique the lack of beat-to-beat mitochondrial Ca^{2+} spikes could be demonstrated.

Alternatively, $[Ca^{2+}]_m$ can be measured using a fusion protein consisting of aequorin and a mitochondrial targeting sequence. Advantages of this chemiluminescence approach are its selectivity for measuring $[Ca^{2+}]_m$ and the steadily improved spatial resolution of the technique [109, 110], however its applicability has been restricted to use with cultured cells (e.g. cultured neonatal rat ventricular myocytes [111]). In addition, the signal generated by the photoprotein is typically too weak to be monitored with single cell imaging [109–112]. Ca^{2+} -dependent consumption of the photoprotein and the requirement of the exogenous cofactor co-elenterazine could cause problem during continuous monitoring of $[Ca^{2+}]_m$ [109]. A similar technique (adenoviral infection with aequorin targeted to cytosol and mitochondria) was employed in adult rat ventricular myocytes [112]. Using this technique it has been demonstrated that electrical stimulation at 2 Hz, $[Ca^{2+}]_m$ increased simultaneously with $[Ca^{2+}]_i$, but the decay of $[Ca^{2+}]_m$ was much slower. Adenoviral infection requires myocytes to be put into short-term culture, which might negatively affect the excitation-contraction coupling process due to structural and electrical remodeling.

Recent years have seen the development of novel mitochondrial Ca^{2+} probes that are specifically targeted to the mitochondrial matrix compartment by taking advantage of molecular biology techniques. Mt-pericam, a mitochondrial matrix targeted, circularly permuted green fluorescent protein fused to calmodulin and its target peptide M13 [113] was developed with the goal to monitor $[Ca^{2+}]_m$ ratiometrically. At 405 nm excitation wavelength pericam emission is sensitive to changes in $[Ca^{2+}]_m$, however emission in response to excitation at 488 nm is sensitive to changes in pH [114]. Taking advantage of these properties it has been proposed that this ratiometric pericam can be used for simultaneous Ca^{2+} and H^+ detection, since its K_d and pK_a values are suitable for measuring mitochondrial $[Ca^{2+}]$ and pH [113]. Packaging the pericam into adenoviral vectors allowed for in vivo cardiac infection [115] and subsequent $[Ca^{2+}]_m$ monitoring in mouse myocytes. Using this technique Belke et al. [115] clearly demonstrated that basal $[Ca^{2+}]_m$ increased slowly from resting levels during electrical pacing at 1 Hz, however no distinct $[Ca^{2+}]_m$ transients were observed.

The Ca^{2+} -sensitive inverse pericam Mitycam [113] was targeted to mitochondria with a standard mitochondrial-targeting sequence (subunit VIII of human cytochrome c oxidase) and adenovirally expressed in cardiomyocytes [116]. Mitochondrial location of Mitycam was confirmed by the characteristic fluorescence pattern that overlapped with the signal from the mitochondrial marker MitoTracker Red, and by the sensitivity of the fluorescence signal to mitochondrial inhibitors. Mitycam was capable of reporting beat-to-beat mitochondrial Ca^{2+} transients.

Most recently, a novel fluorescent indicator for $[Ca^{2+}]_m$, GCaMP2-mt, was developed by adding a mitochondrial targeting sequence to a high signal-to-noise Ca^{2+} sensor protein GCaMP2 [117] to measure oxidant-induced responses of $[Ca^{2+}]_m$ in cultured neonatal myocytes.

Several tools are routinely used to study mitochondrial Ca^{2+} transport. Mitochondrial Ca^{2+} uptake via MCU can be inhibited pharmacologically with ruthenium red [118–121] or Ru360 (a more MCU-specific ruthenium red derivative) [122–125], or by collapsing $\Delta\Psi_m$ with the protonophore FCCP [126–128] to eliminate the driving force for uptake. Oligomycin [129–131] should be used in combination with FCCP to prevent ATP depletion by mitochondrial ATP synthase operating in the reverse mode. In addition, mitochondrial Ca^{2+} extrusion via NCX_m can be blocked efficiently by clonazepam [132, 133] or by the more NCX_m -specific benzothiazepine compound CGP-37157 [133–135].

Measurements of mitochondrial Na^+ concentration

Through the action of mitochondrial and plasma membrane NCX mitochondrial and cytosolic Ca^{2+} and Na^+ regulation is tightly interconnected. For example, $[\text{Na}^+]_i$ raises significantly in heart failure [136] which stimulates NCX_m and leads to reduced steady-state $[\text{Ca}^{2+}]_m$ [97, 137] and presumably increased $[\text{Na}^+]_m$, decreased activity of Ca^{2+} -activated dehydrogenases, hampered regeneration of NADH and NAD(P)H levels [137], to ROS generation [138] and energy starvation [97, 139] during excitation-contraction coupling. The majority of $[\text{Na}^+]_m$ measurements was performed in isolated mitochondria [140] or permeabilized cells loaded with fluorescent Na^+ indicators such as SBFI [141], Sodium Green [142] and CoroNa Green [143]. In rat ventricular myocytes loaded with SBFI $[\text{Na}^+]_m$ was significantly lower than $[\text{Na}^+]_i$ but increased after metabolic inhibition [141]. $[\text{Na}^+]_m$ is ~5 mM which was about 2 times lower than $[\text{Na}^+]_i$ reported in these cells [141], and similar to $[\text{Na}^+]_m$ and $[\text{Na}^+]_i$ estimates obtained with electron microprobe analysis [144, 145].

The CoroNa Red sodium indicator is a cationic dye that can be loaded into the cell without an acetoxymethyl ester groups [146, 147]. Because of its positive charge CoroNa Red is taken up preferentially by polarized mitochondria, and has been successfully utilized in intact renal epithelial cells [146] and astrocytes [147, 148].

Measurement of intramitochondrial pH

Mitochondrial matrix pH (pH_m) has been difficult to study in intact cells and attempts have been made using the fluorescent pH indicators BCECF [149] or carboxy-SNARF [150], however measurements are complicated by cytosolic contamination of the fluorescence signal. Improvements come from Green Fluorescent Protein (GFP) mutants that emit light in a pH-dependent fashion [151] and can be directed to various organellar compartments by expression as fusion proteins linked to targeted peptides. One such mutant (pH-GFP) with a near-linear increase fluorescence response in the pH range 7.5–8.5, was directed to the mitochondrial matrix using an N-terminal COX-IV leader sequence and successfully used to measure matrix pH in apoptosis conditions [151, 152].

The recently reported ratiometric pericam used to monitor mitochondrial Ca^{2+} (as described above), also measures changes in mitochondrial pH independently of Ca^{2+} [114] thus allowing recording of mitochondrial Ca^{2+} and pH.

Measurement of mitochondrial redox state

In mitochondria, the major net energy conversion is based on transport of electrons released in the TCA cycle from reduced nicotinamide adenine dinucleotide (NADH) to molecular O_2 via a series of catabolic reactions in the respiratory chain, whereby NADH is oxidized to NAD^+ . The respiratory chain converts the potential energy of the electrons into a proton gradient across the IMM, which drives ATP production. Mitochondrial NADH content is determined by NADH generation and NADH turnover. NADH is generated in the TCA cycle and by β -oxidation of fatty acid carnitine esters, and its turnover is determined by the rate of oxidative phosphorylation, which in turn is governed by ATP demand, NADH redox

state, O₂ supply [153], and the functional state of the respiratory chain complexes. Unlike its conjugate electron acceptor NAD⁺ (which is non-fluorescent) NADH is fluorescent (with emission maximum at 460 nm) upon excitation with near-ultraviolet light of 365 nm. Thus, NADH-fluorometry can be used to estimate mitochondrial NADH/NAD⁺ ratio. NADH is also present in cytosol, however in cardiac tissue the NADH fluorescence signal is primarily mitochondrial [154, 155], and contributions from NADPH (nicotinamide adenine dinucleotide phosphate, a cellular source for reducing equivalents used in protective redox reactions against ROS toxicity and in biosynthesis) are negligible, due to the fact that it is present in much smaller amounts [156], and because its fluorescence quantum yield is 3–4 times lower than that of NADH [157, 158]. When excited at 488 nm, the autofluorescence (520 nm) of cardiac cells arises predominantly from mitochondrial dehydrogenases containing flavin adenine dinucleotide (FAD), whose redox state is linked to that of mitochondrial NADH [159]. Thus, changes in flavoprotein fluorescence serve as endogenous local reporter of mitochondrial matrix redox potential (Figure 4). In contrast to NADH, it is the oxidized FAD that is fluorescent, such that the total fluorescence is inversely proportional to the ratio of reduced to oxidized flavoprotein. The flavoprotein signal decreases to a minimum in the presence of the cytochrome oxidase inhibitor cyanide (CN⁻), and increases to a maximum in the presence of the mitochondrial uncoupler FCCP, allowing an in-situ calibration of the redox signal. NADH/FAD autofluorescence measurements have been conducted successfully in intact hearts to monitor redox state at the organ level [160–163]. In the intact heart ischemia initially caused an increase in NADH autofluorescence and a decrease in FAD autofluorescence indicating a more reduced redox state during the onset of ischemia [160]. During late ischemia mitochondria became less reduced as revealed by the decline in NADH fluorescence without a significant change in FAD fluorescence. Both signals become highly oxidized, however, during reperfusion [160].

Recently developed redox-sensitive green fluorescent proteins (roGFPs) [164, 165] opened the way to monitor the ambient redox potential changes in intact cells ratiometrically (excitation maxima at ~ 400 and 490 nm). This ratiometric approach provides a great advantage of avoiding the differences in protein expression between individual cells and the absolute optical sensitivity of the protein. This indicator is genetically encoded and therefore can be targeted. For example, roGFP1 (GFP with mutations C48S, S147C, and Q204C) is specifically expressed in mitochondrial matrix by fusing the plasmid with the pyruvate dehydrogenase E1 α subunit leader sequence [164]. This probe was used successfully in HeLa cells [164], however its use in larger cells such as cardiomyocytes will require an adenoviral construct.

Measurement of mitochondrial oxygen consumption

Measurements of mitochondrial oxygen (O₂) consumption together with estimation of the rates of oxidative phosphorylation (OXPHOS) are good indicators of the preservation or deterioration of mitochondrial integrity. Traditionally, studies of mitochondrial O₂ consumption and oxidative phosphorylation are performed polarographically on isolated mitochondria with Clark-type oxygen electrodes [166–169] or with an optical approach that is based on fluorescence quenching of a ruthenium compound by O₂ [170, 171]. Growing evidence that structural and functional interactions between mitochondria and cytoskeleton [172], other organelles [173] and cytosol play a significant role in OXPHOS reactions, has led to the development of new methods that can probe these reactions and monitor O₂ consumption in intact [174, 175] and permeabilized [107, 176] cells, and together with the use of specific substrates and inhibitors help determine the activities of various elements of the respiratory chain [107, 176–178].

Measurement of mitochondrial ROS generation

Myocytes have multiple cytosolic and organellar sources for ROS production including leakage from the mitochondrial electron transport chain, cytosolic/caveolar, sarcoplasmic reticulum and mitochondrial NOS, NADPH oxidase and xanthine oxidase. For example, superoxide ($O_2^{\bullet-}$) is generated in the cytosol via NADPH oxidases and xanthine oxidase, however mitochondria consume >90% of oxygen and therefore can become a major site of $O_2^{\bullet-}$ generation in response to a variety of cellular stresses. The IMM is impermeable to $O_2^{\bullet-}$, making the mitochondria a separate cellular compartment for $O_2^{\bullet-}$. The multiple ROS sources make ROS measurements challenging, particularly the identification of the subcellular origin. The most suitable fluorescent dye for mitochondrial $O_2^{\bullet-}$ detection in intact cardiomyocytes is MitoSox Red, a hydroethidium (HE) derivative which has been covalently modified with a hexyl linker to a triphenylphosphonium cation to form Mito-HE [179–181]. A positive charge and the lipophilic nature of the triphenylphosphonium (TPP) cation allow Mito-HE to accumulate preferentially in mitochondria [179, 180]. During cellular oxidative stress Mito-HE is oxidized by $O_2^{\bullet-}$ to form a hydroxylated product (HO-Mito-Ethidium⁺) that becomes fluorescent upon irreversible binding to the mitochondrial DNA (excitation at 510 nm and emission at 580 nm)[179]. Ethidium becomes highly fluorescent when intercalated into DNA, double stranded RNA or DNA/RNA complexes [180]. It is recommended to load myocytes with lower concentrations (0.3–0.5 μ M) of MitoSox Red for 30 min at 37°C. Higher concentrations of Mito-HE are toxic to mitochondria and can lead to rupture of the IMM and redistribution of fluorescence to the nucleus [179, 180]. Duration of light exposure should be minimized in order to prevent photo-oxidation of Mito-HE. Endogenous ROS species can oxidize HE resulting in multiple fluorescent compounds that complicate the measurement of $O_2^{\bullet-}$ with HE, however only $O_2^{\bullet-}$ - dependent oxidation generates a hydroxylated product (2-OH-E) that has a unique excitation at 396 nm in addition to the typical HE excitation at 510 nm [179]. Results obtained exclusively with HE excitation at 510 nm must be interpreted with caution as they likely overestimate the contributions of $O_2^{\bullet-}$ while underestimating contributions from other ROS [182].

The fluorescent membrane-permeable indicators 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA , also known as dichlorofluorescein diacetate (DCF)) and its chloromethyl ($CM-H_2DCFDA$) or carboxyl (carboxy- H_2DCFDA) derivatives that exhibit much better retention in live cells, are the most commonly used reagents for detecting intracellular ROS. Oxidation of the dye by ROS results in formation of a highly fluorescent DCF product which can be detected at 530 nm upon excitation at 488 nm [183]. While DCFs are mostly used for hydrogen peroxide (H_2O_2) detection (Figure 5), they also react with a wide variety of ROS species including, but not limited to peroxy ($ROO\bullet$) and hydroxyl ($HO\bullet$) radicals and the peroxynitrite anion ($ONOO^-$). In addition to the lack of specificity, DCF and its derivatives have limitations and disadvantages that include photooxidation and generation of ROS upon light exposure (which results in artificial signal amplification) and the irreversible nature of the binding reaction that generates the fluorescent molecule (which precludes the measurement of decreases of ROS levels).

To avoid some of these disadvantages, specific H_2O_2 sensitive fluorescent probes that do not depend on probe oxidation were created [184], including Peroxy Green 1 (PG1) and Peroxy Crimson 1 (PC1) which have been successfully used to measure cellular hydrogen peroxide (H_2O_2) levels. Shortcomings of these probes are the relative slow and irreversible nature of their response [183, 185].

Recent developments generated genetically encoded fluorescent sensors that can be targeted to specific subcellular locations and compartments [186]. The probe pHyPer-dMito is based on the yellow fluorescent protein (YFP) inserted into the regulatory domain of *E. coli*

protein OxyR (OxyR-RD) [187] and is capable of detection of mitochondrial H_2O_2 [188]. pHyPer-dMito has submicromolar affinity to H_2O_2 , is insensitive to other oxidants tested such as $\text{O}_2^{\bullet-}$, oxidized glutathione, nitric oxide and peroxynitrite [188], and other than MitoSox Red and DCF, the fluorescent signal is reversible which allows dynamic real-time measurements of ROS changes in living cells. Indeed, using a similar circularly permuted YFP (cpYFP) bursts of $\text{O}_2^{\bullet-}$ generation, termed ‘superoxide flashes’, could be demonstrated in individual mitochondria of adult myocytes [189]. In contrast to pHyPer-dMito, cpYFP emission was unchanged by H_2O_2 (0.1–10 mM) and peroxynitrite, and was decreased by hydroxyl radical and nitric oxide. Physiological levels of Ca^{2+} , ATP, ADP, NAD(P)^+ , and NAD(P)H , and (unlike GFP-based redox biosensors [165, 190]) variations in redox potential had no significant effects on cpYFP fluorescence [189].

SNAP-tag (Covalys, Bioscience, Switzerland) is a genetically encoded fluorescent probe specific for H_2O_2 [191] that can be targeted to the nucleus, to the sarcoplasmic reticulum and to mitochondria by fusing SNAP-tag to the carboxyl terminus of cytochrome c oxidase subunit 8 (SNAP-Cox8A) [183]. SNAP-tag allows to monitor H_2O_2 changes with subcellular resolution, however shares the disadvantages of all irreversible probes.

Most recently, a ratiometric mass spectrometry probe has been developed [192] to assess mitochondrial matrix H_2O_2 levels in vivo. The probe MitoB comprises a TPP cation (driving mitochondrial accumulation) conjugated to arylboronic acid that reacts with H_2O_2 to form the phenol MitoP. Quantifying the MitoP/MitoB ratio by liquid chromatography-tandem mass spectrometry enables the measurement of average mitochondrial H_2O_2 .

Measurement of the mitochondrial nitric oxide (NO) production

Despite growing evidence that mitochondrial Ca^{2+} uptake stimulates NO production inside mitochondria (see [26, 42, 48–50, 193] for more details), the direct measurement of mitochondrial NO production ($[\text{NO}]_m$) in intact cells has remained difficult due to the coexistence of multiple sources of NO (mitochondria, caveolae, SR) and the high diffusibility of NO. The most direct evidence for mitochondrial NO generation in cardiomyocytes stems from experiments in membrane-permeabilized (digitonin) cardiomyocytes using the NO-sensitive fluorescent dyes DAF-2 DA (4,5-diaminofluorescein diacetate) or DAF-FM DA (4-Amino-5-methylamino-2',7'-difluorofluorescein) [42]. Activation of mitochondrial Ca^{2+} uptake in the presence of the NOS substrate L-arginine caused an increase in DAF-2 fluorescence (Figure 6), presumably due to activation mtNOS. Caveolar and SR NOS could be excluded as the source of NO in these experiments and NO production was abolished after inhibition of mitochondrial Ca^{2+} uptake pinpointing mtNOS as the source of NO [42]. The peak level of NO produced by a single cardiac mitochondrion upon stimulation of mitochondrial Ca^{2+} uptake was estimated to be ~15–28 nM [42, 48]. Although the question of existence and nature of mtNOS is highly controversial (see [26, 43–46] for reviews) and the molecular identity of mtNOS remains to be determined, genetic [48] and pharmacological [42] approaches identified mtNOS as a Ca^{2+} /calmodulin-dependent nNOS isoform. When mtNOS was deprived from L-arginine and the cofactor tetrahydrobiopterin (BH_4) mtNOS became a source of ROS production that significantly increased the propensity for opening of the mPTP (Figure 2B). The DAF-type NO indicators are currently the best choice for fluorescent NO measurements at the cellular and organellar level, despite the disadvantage of the irreversibility of the reaction inducing fluorescence and concerns that DAF dyes not only react with NO but also with ROS species [194].

Detection of the mitochondrial permeability transition pore opening

The mitochondrial permeability transition defines a sudden increase in the permeability of the IMM to ions and solutes with molecular weights up to 1500 Da [41]. This process is

attributed to the opening of a voltage- and Ca^{2+} -dependent, cyclosporin A (CsA)-sensitive, high-conductance channel that is termed mitochondrial permeability transition pore (mPTP) [34, 195]. The molecular identity of mPTP is largely unknown (see [34, 195, 196] for recent reviews) and the original model which proposed a complex that includes the adenine nucleotide translocase (ANT; cf. Fig. 1) in the IMM, the VDAC of the OMM, and the regulatory matrix protein cyclophilin D (CypD), has been challenged by recent genetic studies [197, 198]. In the heart [199, 200] the mPTP plays a crucial role in myocyte injury following oxidative stress. In isolated mitochondria, opening of the mPTP causes matrix swelling with subsequent distension and local disruption of the OMM (whose surface is smaller than that of the IMM because of crista formation), release of soluble products from the IMS, dissipation of $\Delta\Psi_m$, and release of small molecules from the matrix [201, 202]. Therefore, in isolated mitochondria, measurements of matrix swelling [203], Ca^{2+} fluxes [203], $\Delta\Psi_m$ or fluorescent dye loading [37] (cf. Figure 2) have successfully been employed to monitor mPTP activity. In IMM preparations (mitoblots) ion currents through the pore could be measured with the patch clamp technique [202, 204]. In intact cells, however, these methods are either unsuitable or complicated by concomitant cytosolic and surface membrane processes. Nonetheless, mitochondrial swelling in conjunction with mPTP activity has also been assessed in intact cardiomyocytes [205]. Since mPTP opening is typically accompanied with IMM depolarization, $\Delta\Psi_m$ monitoring (as described above) in intact cells can provide an indirect estimate of the mPTP activity as evidenced by the sensitivity to the mPTP inhibitor cyclosporin A and facilitation by high Ca^{2+} and decreased pH [90]. A more direct approach probes the release of mitochondria-entrapped fluorescent dyes calcein (MW~620 Da) [42, 206] (Figure 2) or calcein red-orange (MW~790) [207] and can be employed in intact myocytes, however the approach may require elimination of cytosolic dye by cytosolic perfusion through a patch pipette, plasma membrane permeabilization [42, 47] or Co^{2+} quenching [36]. This method has been validated in isolated mitochondria [37] and the decline in mitochondrial calcein fluorescence should be tested for CsA-sensitivity to ensure the involvement of the mPTP. In perfused hearts, 2-deoxy[^3H]glucose entrapment technique [208–210] has been successfully used to monitor mPTP activity, based on the fact that 2-deoxy[^3H]glucose will only enter mitochondria when the mPTP is open.

Methods for ATP measurement in single living cells

Adenosine-5'-triphosphate (ATP) fuels numerous energy-requiring cellular processes and the failure of maintaining adequate cytosolic ATP levels ($[\text{ATP}]_i$) are at the heart of the changes that take place during metabolic distress. $[\text{ATP}]_i$ levels can be measured in whole hearts by noninvasive P^{31} NMR, however the technique allows to measure only slow changes of total ATP [211, 212]. To follow the fast changes in intracellular ATP ($[\text{ATP}]_i$), an indirect $[\text{ATP}]_i$ detection technique based on measurements of free magnesium ($[\text{Mg}^{2+}]_i$) with fluorescent indicators was proposed [213, 214]. This method is based on the fact that a large portion of the intracellular Mg^{2+} pool is bound to ATP and present as MgATP . Since free $[\text{Mg}^{2+}]_i$ is kept constant within a rather narrow range, ATP hydrolysis leads to a concomitant increase in free $[\text{Mg}^{2+}]_i$ as measured with fluorescent Mg^{2+} indicators such as mag-indo [213], magnesium Green [214], magFura-2 [215], and mag-Fluo-4 [216].

To distinguish between cytosolic and mitochondrial compartmentalized ATP, a luciferase-based adenoviral transfection method was developed [112, 217] which allows to measure ATP in pH- and Ca^{2+} -independent manner [218, 219] and with the possibility of targeting the enzyme to individual organelles. This can be achieved by fusing of the luciferase gene with a cDNA encoding a localization sequence such as the mitochondrial import sequence of cytochrome c oxidase subunit VIII. Using either untargeted (cytosolic) or mitochondrially targeted luciferase, it has been demonstrated that no changes in ATP occurred during the

contractile cycle with or without beta-adrenergic stimulation, indicating that even if Ca^{2+} enters the mitochondria on a beat-to-beat basis [111, 112, 116], this is not translated into ATP changes on this timescale. Only large workloads led to an initial drop in ATP concentration followed by a recovery with an overshoot increase in ATP above basal level [112, 217]. Furthermore, mitochondrial NADH or NAD(P)H measurements [220–222] can be utilized as indirect indicators of the energetic state of mitochondria, and therefore reflect changes in ATP concentration.

Conclusions

Mitochondria are essential for normal cardiac function as the dominant source of ATP but also because of the crucial role in Ca^{2+} homeostasis, programmed cell death, and ROS signaling. An understanding of the mitochondrial function in normal and pathophysiological states is critical for the development of new therapeutical tools for treatment of mitochondria-related diseases. Here we briefly summarized the current state of experimental approaches to study mitochondria of intact cardiac cells under normal and pathological conditions.

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Highlights

- Mitochondria are organelles referred to as the ‘power plants’ for cellular energy demands.
- Mitochondria participate in a wide range of cellular processes.
- Mitochondria have been implicated in human diseases including cardiac dysfunction.
- Understanding mitochondrial functions leads to new therapies for cardiac diseases.
- This review describes methods to study mitochondrial function in intact cardiomyocytes.

Figure 1. Schematic overview of mitochondrial structure and transport mechanisms
For details and abbreviations see text.

Figure 2. Measurements of $\Delta\Psi_m$ and mPTP activity in permeabilized ventricular myocytes and isolated mitochondria

A, $\Delta\Psi_m$ monitored with the membrane potential sensitive dye TMRM in permeabilized rabbit ventricular myocytes upon inhibition of ETC complex I with rotenone and subsequent exposure to ETC complex II substrate succinate. The mitochondrial uncoupler FCCP completely depolarized Ψ_m . **B**, mPTP activity monitored with mitochondria entrapped calcein (620 Da) in permeabilized cat ventricular myocytes. Opening of mPTP was induced by enhanced ROS formation (prevented by superoxide and peroxynitrite scavenger MnTBAP, 50 μM) resulting from Ca^{2+} -induced stimulation of mtNOS in the absence of L-arginine. Opening of mPTP resulted in the loss of calcein and a decrease of fluorescence. The pore forming antibiotic alamethicin induced maximum calcein release. Cyclosporine A (CsA, 5 μM) blocked mPTP opening. **C**, mPTP openings recorded from isolated mitochondria with TMRE. PTP opening can occur as brief incomplete (arrows) and prolonged maximal depolarizations. **D**, simultaneous monitoring of mPTP activity as changes in $\Delta\Psi_m$ (TMRE) and loss of calcein in isolated mitochondria. Brief incomplete depolarizations (arrow) did not cause loss of calcein, however prolonged and complete depolarizations were accompanied by loss of calcein. The decline of calcein fluorescence was preceded by a transient increase (asterisk) caused by calcein dequenching. (Panel B: from Dedkova and Blatter, *The Journal of Physiology*, 2009, with permission; panels C and D: from Hüser, Rechenmacher and Blatter, *Biophysical Journal*, 1998, with permission).

Figure 3. Measurements of $[Ca^{2+}]_m$ with compartmentalized fluo-3 in single permeabilized ventricular myocytes

A, top: changes in fluo-3 fluorescence following membrane permeabilization with digitonin and increase in extramitochondrial $[Ca^{2+}]$ ($[Ca^{2+}]_{em}$). Images a to c (bottom) were taken at times indicated by arrows. **B**, changes of $[Ca^{2+}]_m$ in response to rapid cytosolic Ca^{2+} transients. **a** and **b**, technique used to generate rapid changes in $[Ca^{2+}]_{em}$ to simulate cytosolic Ca^{2+} transients. Cells were placed in the laminar flow of zero- Ca^{2+} solution (1 mM EGTA) and computer controlled pressure ejections of 10 μM Ca^{2+} -containing solution from a glass micropipette. **c**, changes of $[Ca^{2+}]_m$ in a permeabilized myocyte in response to stimulation with Ca^{2+} pulses applied at 0.5 Hz (pulse duration = 0.5 s; $[Na^+]_{em} = 40$ mM; $[Ca^{2+}]_{pip} = 10$ μM). (From Sedova, Dedkova & Blatter, Am. J. Physiol. Cell Physiol., 2006; Am. Physiol. Soc., used with permission)

Figure 4. Monitoring mitochondrial redox potential in intact ventricular myocytes by measuring FAD autofluorescence

Top, from left to right: confocal images of ventricular myocyte FAD autofluorescence (excitation 488 nm, emission 510–525 nm) before (cntrl) and after exposure to ETC complex II substrate methyl succinate (Met-Succinate), ETC complex IV inhibitor sodium cyanide (NaCN), and mitochondrial uncoupler FCCP. **Bottom**, time course of changes in FAD/FADH₂ fluorescence recorded from the regions of interest shown on top. Exposure to NaCN represents maximal reduction of FAD (i.e., 0% fluorescence from FAD with maximal formation of FADH₂), while exposure to FCCP reflects maximal oxidation of FAD.

Figure 5. Measurements of the mitochondrial reactive oxygen species (ROS) generation with DCF in intact ventricular myocytes

Top, from left to right: confocal images of ventricular myocytes loaded with ROS-sensitive dye DCF (excitation 488 nm, emission 510–525 nm) before (cntrl) and after exposure to hydrogen peroxide (H_2O_2 ; 1 – 100 nM). **Bottom**, time course of DCF fluorescence in response to 1, 10 and 100 nM H_2O_2 .

Figure 6. Measurements of mitochondrial NO production with DAF-2

A, from left to right: confocal images of a ventricular myocyte loaded simultaneously with DAF-2 (green, emission 510–525 nm) before (cntrl) and after cell permeabilization with digitonin, and MitoTracker Red (red, emission >590 nm). The right panel shows colocalization of DAF-2 and MitoTracker Red represented by shades of yellow. **B**, confocal images of a permeabilized DAF-2 loaded cell under unstimulated conditions ($[Ca^{2+}]_{em} = 0.1 \mu M$), after an increase in $[Ca^{2+}]_{em}$ to $2 \mu M$, and after subsequent addition of the exogenous NO donor Sper/NO. Insets reveal NO signals from individual mitochondria. **C**, time course of DAF-2 fluorescence recorded from a subcellular region of interest ($\leq 40 \mu m^2$) under conditions shown in A and B. Treatment with the NO scavenger hemoglobin (Hb, $10 \mu M$) prevented the Ca^{2+} - and Sper/NO-induced increase in DAF-2 fluorescence. (From: Dedkova and Blatter, *The Journal of Physiology* 2009; with permission).