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Assessment of mitochondrial dysfunction and implications in cardiovascular disorders

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Abstract

Mitochondria play a pivotal role in cellular function, not only acting as the powerhouse of the cell, but also regulating ATP synthesis, reactive oxygen species (ROS) production, intracellular Ca^{2+} cycling, and apoptosis. During the past decade, extensive progress has been made in the technology to assess mitochondrial functions and accumulating evidences have shown that mitochondrial dysfunction is a key pathophysiological mechanism for many diseases including cardiovascular disorders, such as ischemic heart disease, cardiomyopathy, hypertension, atherosclerosis, and hemorrhagic shock. The advances in methodology have been accelerating our understanding of mitochondrial molecular structure and function, biogenesis and ROS and energy production, which facilitates new drug target identification and therapeutic strategy development for mitochondrial dysfunction-related disorders. This review will focus on the assessment of methodologies currently used for mitochondrial research and discuss their advantages, limitations and the implications of mitochondrial dysfunction in cardiovascular disorders.

Keywords: Mitochondrial dysfunction; Mitochondrial dynamics; Oxidative stress; Mitochondrial energy metabolism; Ca^{2+} signaling; Mitochondrial permeability transition pore

1. Introduction

Mitochondria are known as the powerhouse of the cell. Keeping mitochondrial function normal is necessary in the tissues and organs that are of high energy demand [1]. The maintenance of mitochondrial structural integrity, biogenesis and function is essential to the cells, since mitochondrial abnormalities can lead to a progressive mitochondrial dysfunction including the defects in the respiratory chain, uncoupling of the electron transport chain, ATP synthesis, reactive oxygen species (ROS) production, and in turn resulting in the disturbances of energy metabolism [2]. Such abnormalities can trigger the signaling pathways of necrotic or apoptotic cell death, which is a hallmark of various diseases.

In the last decades, a variety of *in vitro* methods have been developed to assess the disruption of mitochondrial function, including the detection of superoxides, mitochondrial membrane permeability, membrane potential, mitochondrial calcium, oxygen consumption and heat production. However, crosstalk between mitochondria and mitochondrial surrounding environment plays a crucial role in the regulation of mitochondria homeostasis, it is therefore important to assess mitochondrial function *in vivo* settings without isolating them from the natural cellular environment, such as freshly isolated mitochondria. Recently, a new approach to restore frozen biological samples to measure mitochondrial function seems to be plausible, which could enable to assess the integrated mitochondrial function using tissues in tissue biobanks retrospectively [3]. In addition, imaging of mitochondrial membrane potential *in vivo* using a voltage-sensitive positron emission tomography (PET) radiotracer might provide a valuable methodology for the evaluation of mitochondrial activity in physiology and diseases [4]. Effective approaches to assess mitochondrial function *in vitro* or *in vivo* are crucial for studying mitochondrial dysfunction in diseases. This review is aimed to address recent advances in the methodology of mitochondrial function assessment and the implications of mitochondrial dysfunction in the pathogenesis of cardiovascular diseases.

2. Overview of mitochondrial structure

Mitochondria exist in the cytoplasm of nearly all eukaryotic cells except for mature mammalian red blood cells. The shape and number of mitochondria in a cell vary considerably depending on the cell type or functional status with a length of 0.75–3 μm [5]. Cells with intensive oxidative capacity usually have more mitochondria with large numbers of cristae, whereas oxidative stress can cause mitochondrial swelling and loss of cristae [6].

Mitochondria are enclosed by two membranes, i.e., the outer mitochondrial membrane (OMM)

and the inner mitochondrial membrane (IMM) (**Figure1**). The OMM is composed of a phospholipid bilayer embedded with integral proteins porin (or called voltage-dependent anion channels, VDAC). Ions, nutrients, small molecules and proteins (<10kDa) can move freely into the intermembrane space. Disruption of the OMM leads to proteins in the intermembrane space to leak into the cytosol, especially some caspase activators including cytochrome *c* (Cyt *c*) release through the OMM, which could trigger cell apoptosis [7, 8]. By contrast, the inner membrane is a tight permeable barrier to all ions and molecules, which mediate highly selective diffusion of oxygen, carbon dioxide, and water. As a result of its selective permeability, an electrochemical membrane potential of about -180 mV builds up across the IMM. The IMM is extensively folded and compartmentalized. It comprises critical membrane proteins and enzymes, including those from electron transport chain (ETC), metabolite transporters such as the adenine nucleotide translocase (ANT), the mitochondrial calcium uniporter (MCU), and ATP synthase essential for cell survival. The matrix is the space enclosed by the inner membrane and contains specialized mitochondrial ribosomes, tRNA, DNA and soluble proteins and enzymes involved in the oxidation of pyruvate and fatty acids and the Krebs cycle [7]. The mitochondrial permeability transition pore (mPTP) is a supramolecular entity assembled at the interface of the IMM and spans across the OMM.

Mitochondria can be visualized under a phase contrast light microscopy, but their ultrastructure can only be seen by electron microscopy. The conventional transmission electron microscopy (EM) has been an indispensable technology in cell biology ever since it was first introduced in the early 1940s [9]. In addition, electron tomography (ET), Cryo-ET, and the newly emerging Cryo-EM have provided powerful tools to get insights into the 3D structures of mitochondrial transporters and multiprotein complexes architecture at near-atomic resolution [10]. These techniques have provided valuable structural information of mitochondrial enzymes and transporters.

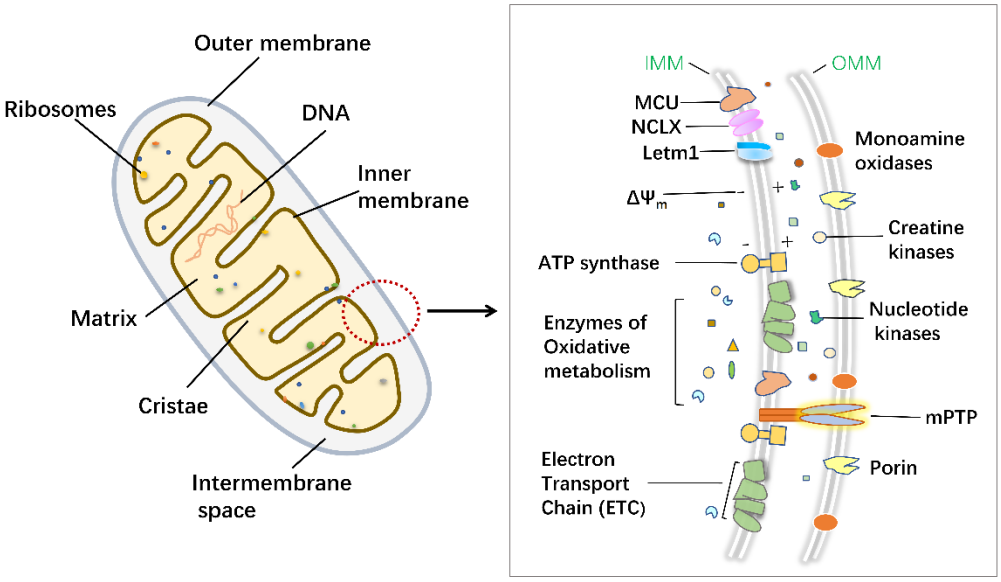

Figure 1. **Schematic diagram of mitochondrial structure.** Ion channels and enzymes in the outer mitochondrial membrane (OMM) including porins, monoamine oxidases, creatine kinases, nucleotide kinases, etc. The mitochondrial permeability transition pore (mPTP) spanning across the inner and outer membrane. The inner mitochondrial membrane (IMM) holds transporters (MCU, NCLX, Letm1), ATP synthase and proteins in electron transport chain (ETC). Mitochondrial matrix contains a mixture of enzymes for Krebs cycle, ribosomes, mitochondrial DNA and small soluble proteins and ions.

3. Assessment of mitochondrial dysfunction

3.1. mPTP and functional assessment

The inner membrane of mitochondria contains a high-conductance nonspecific channel, known as mPTP. Since the first biochemical description of the mitochondrial permeability transition in 1976 [11], many proposals have been made about the protein constituents of the mPTP. However, the precise molecular structure of mPTP still remains putative, although cyclophilin D (CyPD), the ADP/ATP translocase, the F1-FO-ATP synthase, and spastic paraplegia 7(SPG7) are key players for its function [12, 13].

The mPTP may operate under two distinct modes: low- and high-conductance modes [14, 15]. Under physiological conditions, the mPTP may serve as low-conductance state, open and close transiently and participate in the regulation of mitochondrial Ca^{2+} homeostasis [15], which is characterized by very limited permeability (cutoff, <300 Da) and permits the diffusion of small ions such as H^+, Ca^{2+} and K^+ but does not trigger detectable mitochondrial swelling (**Figure 2**). The functional coupling between transient mPTP opening and ETC may stimulate mitochondrial superoxide production, i.e., so-called “superoxide flashes” [16, 17]. Under pathophysiological conditions, mPTP shows high-conductance state (1-1.3 nS) and allows free movement of molecules with a molecular mass up to 1.5 kDa across the inner membrane and results in mitochondrial matrix swelling. The sustained opening of mPTP is potentiated by elevation of mitochondrial matrix [Ca^{2+}], especially when accompanied by oxidative stress or high phosphate [18, 19], resulting in uncoupling of oxidative phosphorylation and potentially cell death [20]. Therefore, the opening of mPTP is critical for initiating cell apoptosis and necrosis, which may act as a drug target [21].

Ca^{2+} retention capacity assay and Ca^{2+}-triggered mitochondria swelling assay are commonly used to assess the mitochondrial permeability transition using freshly isolated mitochondria [22]. Ca^{2+} uptake and release from mitochondria can be monitored using calcium-sensitive fluorescent probes, and the mitochondria swelling can be detected by a luminescence spectrometer to assess the light scattering intensity. . Therefore, several approaches have been developed to monitor the permeability of mPTP *in situ* or *in vivo*, which can be grouped as indirect pharmacological approaches and direct assays using fluorescent indicators or radioactive tracer. Application of cyclosporin A (CsA) to inhibit mPTP opening is the pharmacological approach, which can be used for cultured cells or freshly isolated cells. The inhibition of mPTP by CsA is mediated via the binding to mitochondrial CyPD [23]; however, CsA may also bind to cyclophilin-A and result in the inhibition of calcineurin-dependent signaling pathway [24].

The Co^{2+}-calcein assay has been introduced to assess mPTP opening in living cells *in vitro* since 1990s. Calcein-AM is a membrane permeable non-fluorescent hydrophobic probe. Calcein-AM accumulates in cytosolic compartments and mitochondria after loading and converted to green fluorescence calcein after hydrolysis by intracellular esterases. Co^{2+} causes quenching of cytosolic and nuclear calcein fluorescence, but it is impermeable to the normal IMM. However, once the pores of mPTP open, Co^{2+} can enter mitochondrial matrix and result mitochondrial fluorescence quenching. The intensity of mitochondrial fluorescence can be monitored by fluorescence microscopy or flow cytometry [25, 26]. This approach can be applied in different cell types and useful for the assessment of mPTP activity. The limitation of Co^{2+}-calcein approach is that Co^{2+}, a heavy metal, may exert toxic effects on cells such as by inhibiting respiration chain and Ca^{2+} uptake [27, 28].

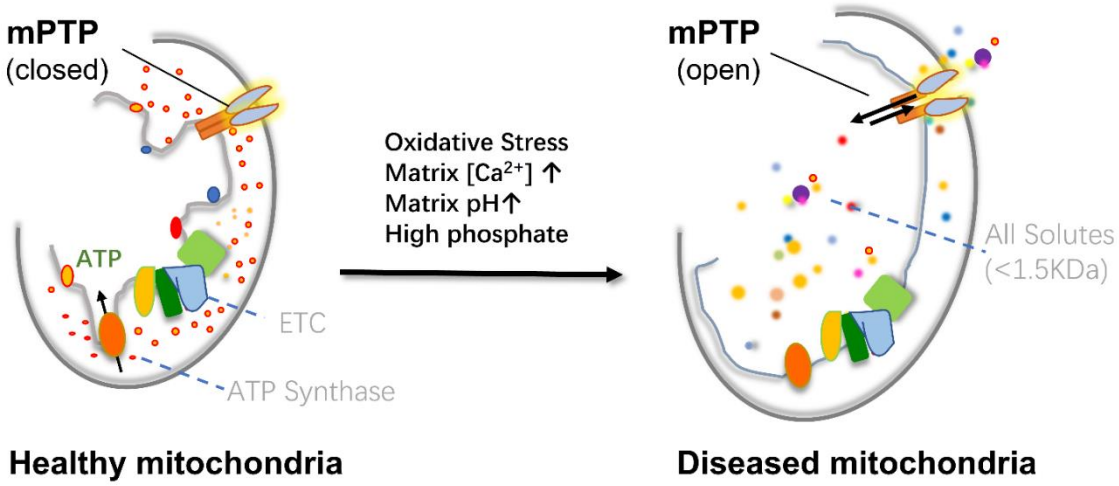

Figure 2. Mitochondrial permeability transition pore (mPTP) under healthy and diseased conditions. mPTP in normal or healthy condition remains closed or transient opening, which serves as a physiological role by allowing a quick exchange of ions and small molecules (<300 Da) in the matrix. Under the diseased status, numerous factors such as oxidative stress, increased matrix Ca^{2+} concentration, elevated phosphate concentrations and pH increases, can facilitate the persistent opening of mPTP, which leads to unselective diffusion of <1500 Da solutes and water across the IMM. Long-lasting opening of mPTP results in mitochondrial depolarization, ATP depletion, ROS overproduction, impaired cellular Ca^{2+} homeostasis, mitochondrial swelling and eventually cell death.

The permeability of mPTP in situ or in vivo may be different from that in the isolated mitochondria preparations. Radioactive tracer with 2-deoxy [^{3}H] glucose ([^{3}H]-DOG) is an *in vivo* approach to monitor mPTP activity. [^{3}H]DOG is phosphorylated to 2-deoxyglucose 6-phosphate inside a cell and accumulates in the cytosol, but not able to pass through the IMM until the mPTP pores are open. Therefore, measurement of mitochondrial [^{3}H] DOG entrapment could indicate the opening of mPTP. This approach can be used for *in vivo* organ or animal studies, such as ischemia/reperfusion (I/R) injury in isolated heart [29, 30]. However, [^{3}H] DOG approach is not suitable for research using single cells. Due to the requirement of special facility and the potential contamination issues of radioactive substances, the radioactive tracer approach is not popular for research laboratories and gradually replaced by the fluorescent probes [31].

3.2 Assessment of mitochondrial dynamics

Mitochondria are dynamic organelles showing membrane fusion and fission constantly. The cellular process of fusion and fission is called mitochondrial dynamics. Mitochondrial fission results in small and round mitochondria, while mitochondrial fusion leads to thin and elongated mitochondria with highly interconnected networks [32]. Fusion allows one mitochondrion to compensate another defected mitochondrion by sharing components, while fission is an important process for mitochondrial self-renewal, contributing to quantity by facilitating mitochondrial trafficking to form new mitochondria [33]. A series of GTPases is involved in the regulation of mitochondrial dynamics (**Figure 3**). Fission is driven by dynamic-related protein1 (DRP1) and mitochondrial fission 1 protein (FIS1) [34], whereas the fusion is regulated by mitofusin 1 and 2 (MFN1 and MFN2) and optic atrophy 1(OPA1) [35, 36]. Cytoskeleton is linked to focal adhesions and the mechanical force from extracellular matrix can be transmitted to the mitochondria and thus affect the mitochondrial dynamics [37]. Importantly, both cytoskeletal microfilaments and microtubules promote the recruitment of Drp1 to mitochondria anddrive mitochondrial fission [38]. Fission–fusion shifts can frequently occur under various stressful conditions, indicating an early event in mitochondria-dependent apoptosis or mitophagy [39, 40], which has been implicated in the pathophysiology of many diseases [41].

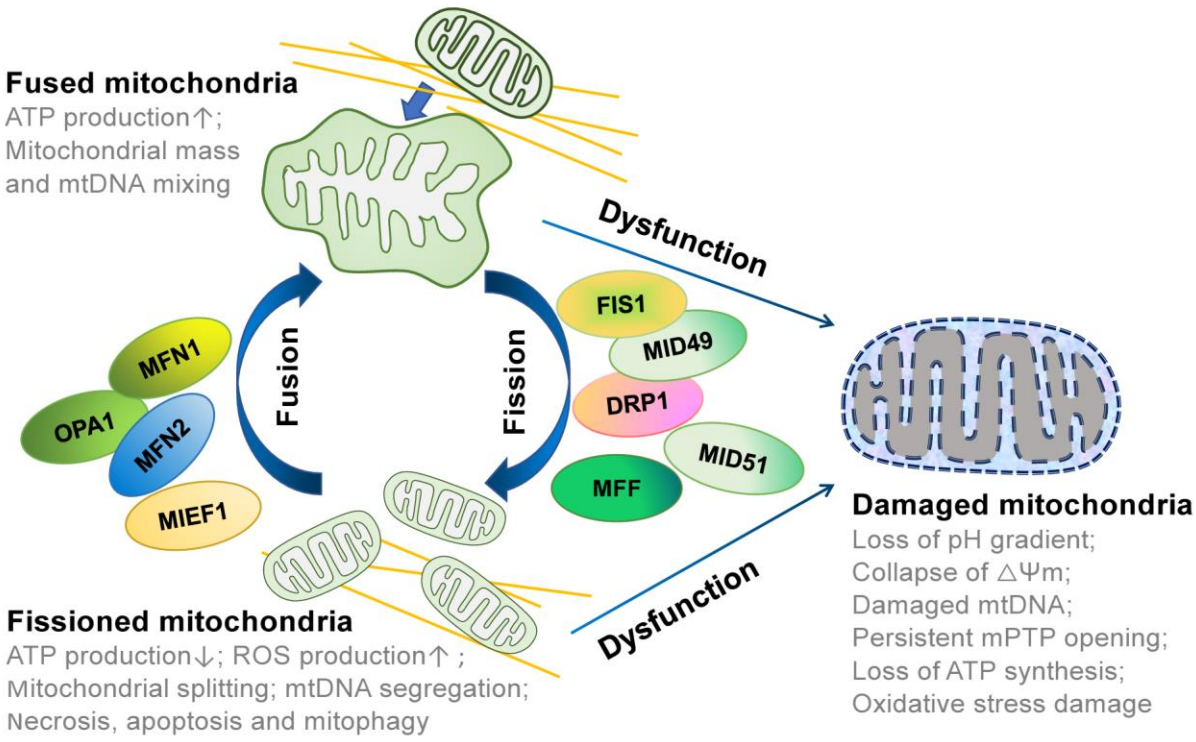

Figure 3. Mitochondrial fusion and fission cycle. Mitochondrial fusion joins two mitochondria together, while fission separates one into two. Mitochondrial fusion is facilitated by the proteins such as MFN1, MFN2 and OPA1, and mitochondrial fission is promoted by the multiple OMM-bound proteins (DRP1, FIS1, MFF, MIEF1/2 and MID51). Mitochondrial dysfunction results in the damaged mitochondria, which characterized by swelling and membrane leaks and then degraded by mitophagy. Yellow lines represent cytoskeleton, along which small mitochondrial clusters can travel with the aid of motor proteins.

The discovery of green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* in the early 1970s has made a breakthrough for dynamically monitor proteins and structural changes in a cell [42]. Mitochondrial proteins tagged with GFP in live cells can be visualized using a fluorescent microscope or laser confocal microscope. Mitochondrial network can also be assessed by the diffusion of mitochondria-targeted photo-activatable GFP (mt-PA-GFP) in live cells [43, 44]. For long-term imaging of mitochondrial dynamics in live cells, the progresses in super-resolution fluorescence microscopy development, such as stimulated emission depletion (STED), structured illumination microscopy (SIM) and stochastic optical reconstruction microscopy (STORM), combined with SNAP-tag fluorogenic probes, allow to sample high resolution live cell image with time lapse [45].

Mitochondrial dynamics can also be monitored in living cells by using highly sensitive photothermal microscopy without fluorescent labeling. Non-fluorescent molecules are usually less affected by photobleaching and thus make better imaging contrasts [46]. However, this technique may require a specialist to carry out such experiments and the facility is expensive, which limits its application. Instead, immunostaining of mitochondrial protein markers in fixed cells is a simple and inexpensive approach to monitor mitochondrial dynamics, which can also provide direct evidences of mitochondrial morphology and dynamics[47]. Additionally, transgenic mice such as alpha-MHC-MitoTimer mice [48], CAG-mito::mKate2 mice [49] with fluorescent reporter genes were generated for monitoring mitochondrial turnover or dynamics *in vivo*. Such *in vivo* approach development will give new insights into mitochondrial biology in health and disease.

3.3 Intracellular ATP measurement

Mitochondria are specialized organelles that generate ATP via ETC and oxidative phosphorylation system and maintain energy homeostasis in a cell [50]. ATP deficiency may come from mitochondrial dysfunction or the insufficient supply of oxygen or nutrients to the cells due to some pathological

conditions. Therefore, measurement of ATP as an index of mitochondrial dysfunction should consider other pathological factors affecting cell morphology and metabolism.

ATP in extracellular fluid or serum can be measured by biochemical endpoint assays, nuclear magnetic resonance spectroscopy (NMR), high-performance liquid chromatography (HPLC), and fluorescence-based biosensors or fluorescent ATP analogues [51, 52]. NMR is particularly useful in pathological situations where cytoplasmic ATP is mainly released into the circulation. The NMR method has good linearity in a range of 0.1–100 mM, but shows low temporal resolution (about 14 h). The usage cost for the equipment may also hinder its application [53]. HPLC is widely used to determine ATP, ADP, and AMP levels in different types of samples. The sensitivity of HPLC in detecting nucleotides is in the order of μM. Compared with NMR, HPLC is a fast (the retention time is about 20 min), automated, highly reproducible and accurate method to identify nucleotides in total cellular extracts [48, 54]. However, these approaches are hardly compatible with real-time analyses and do not provide a sufficient spatiotemporal resolution.

The most sensitive and reliable technique for detecting ATP levels in isolated living cells, subcellular level or serum samples is based on bioluminescent luciferin–luciferase reaction. Briefly, luciferase can oxidize luciferin into oxyluciferin, and the resulting luminescence is proportional to ATP concentration in the surrounding environment. Given that luciferase can be compartmentalized in different subcellular organelles, it could be used to measure ATP level in subcellular regions (mitochondria, cytoplasm, nucleus, etc.) [55, 56]. However, measurements of ATP by using luciferase or chromatography can only provide average ATP concentrations of the cell extracts. In addition, the extraction procedure might result in ATP loss or degradation. Attempts to monitor ATP levels in real-time using chemiluminescence have been reported; however, the luciferase concentration and activity, oxygen, luciferin as well as the consumption of ATP by luciferase itself may interfere intracellular ATP measurement [57]. Additionally, luciferase's bioluminescent output yields low photon fluxes and limits cellular-scale resolution imaging.

Recently, a series of alternative approaches have been reported for real-time detection of ATP production at the single cell level using fluorescent protein-based ATP sensors [58-60], including ATeam, iATPSnFRs, PercevalHR and GRABATP1.0 [61-63].These sensors display a range of ATP affinities, monitor ATP release and dynamics, being considered complementary to the use of luciferase-based ATP imaging approaches. Since fluorescence-based ATP sensors can be unlikely used for whole-rodent imaging, which is possible for luciferase-based sensors. Furthermore, the potential limitations including the pH sensitivity of fluorophores, autofluorescence artifacts and phototoxicity in long-term experiments could affect the results obtained with all fluorescent-based probe. Taken together, it is still challenging to determine absolute mitochondrial ATP concentrations in living cells.

3.4 Mitochondrial respiration

Mitochondrial respiration is the set of metabolic reactions to convert energy stored in macronutrients into ATP. Mitochondrial dysfunction affects mitochondrial bioenergetics and causes oxidative phosphorylation defects, and thus leads to altered cellular respiration and overproduction of ROS, thus inducing oxidative stress and cellular damage. Detection of the activity of respiratory enzymes is a straightforward approach for investigating mitochondrial dysfunction. There are several biochemical assays, such as cytochrome c oxidase (COX), succinate dehydrogenase (SDH) activity and Complex IV assays, can be used to measure mitochondrial dysfunction [64]. However, none of these assays can be performed in real time.

The high-resolution Clark electrodes (e.g., Oxygraph-2K) and the sensitive, high-throughput microplate reapirometry-based approach called Seahorse XF Analyzer have been developed to monitor mitochondrial bioenergetics [65]. The two systems enable the real time detection of the respiration rate of mitochondria. Both can be applied to monitor the mitochondrial respiratory function by simultaneously recording of oxygen consumption rate (OCR) and extracellular proton flux on isolated mitochondria as well as in cultured cells and tissues [66, 67]. Furthermore, OCR measurements in the isolated mitochondria allow to determine the direct effects of pharmacological agents on mitochondrial respiration. However, the limitation by using Oxygraph-2K is due to high labor

intensity to obtain optimal signal intensity and low throughput (only two samples can be performed at any one time) as well as incapable of recording extracellular pH levels. For the Seahorse XF analyzer, the disposable fluorescent plates are expensive. In addition, the injectable compounds may potentially interfere with sensor fluorescence and cause artifacts [66]. Moreover, the substrate-specific analysis is usually performed under non-physiological conditions, so such results may not accurately reflect the native physiological environment [68].

The main restriction for oxygen consumption measurements is that fresh tissue or cells are required, which prevents researchers from carrying out large-scale studies to examine the mitochondrial function both in health and disease groups, because freeze-thaw samples may impair the ETC activity. Recently, an approach named Respirometry In Frozen Samples (RIFS) has been reported with 90–95% recovery of the maximal respiratory capacity using frozen samples [3], which may open a new avenue for future clinical application.

3.5 Mitochondrial membrane potential measurement

The mitochondrial membrane potential (ΔΨm) generated by proton pumps (complexes I, III and IV) is a key indicator of mitochondrial activity. It reflects the process of electron transport and oxidative phosphorylation. The electrochemical gradient across the IMM couples ATP synthesis, the stability of ATP and ΔΨm is thought to be a requisite for normal cell function [69]. The occurrence of inner membrane ion leaks could significantly affect the magnitude of ΔΨm. Furthermore, at a high ΔΨm level, the mitochondrial respiratory chain leads to decreased energy production and increased production of ROS [70] .

The microelectrode recording was initially introduced to directly measure ΔΨm [71]. However, this technique is especially difficult. The integrity of the cells may be quickly damaged by the microelectrode and could only provide limited information related to the *in vivo* situations.

Due to the difficulties of direct ΔΨm recording, organic fluorescent probes based on cationic, lipophilic dyes have been developed and becoming the most common technique to monitor ΔΨm [72, 73]. For example, JC-1, Rhodamine 123 (Rhod123), Tetramethyl rhodamine ethyl (TMRE) or methyl (TMRM) ester. The intensity of such fluorescent probes shows a linear correlation to Δψm, which can be measured by flow cytometry, fluorescence microscope or plate reader [74]. Among probes, JC-1 is a classical ratio probe with the ability to discriminate low and high membrane potential in mitochondria [75]. The JC-1 exhibits potential-dependent accumulation in mitochondria and forms J-aggregates and emits red fluorescence (590 nm) at normal ΔΨm level, whereas emits green fluorescence (520 nm) in unhealthy or apoptotic cells. Decrease in the red/green fluorescence ratio of JC-1 indicates mitochondrial depolarization. However， labelling thick tissue slices with these fluorescent indicators poses limitations in requirement of increased dye concentration and loading time, resulting in non-specificity and concentration-dependent artifacts. A newly developed method by utilizing a low resistance glass pipette attached to a pressure injector shows highly precise fluorescent dye labelling of mitochondria, thereby enhancing signal to noise ratio [76]. Some nanomaterials, such as a fluorescent carbon dot [77], could be designed to combine ΔΨm fluorescent probes to enhance contrast and photostability for precise and long-term mitochondrial tracking.

The emergence of the target-switchable fluorescent probes provides a guide to the development of multi-mode probes, both the space and colors could indicate the variation of ΔΨm [78]. The two-photon and near-infrared fluorescent probes (KMG-501) have a huge potential for detecting ΔΨm in tissues and living animals due to their low fluorescent signal background and ultra-high tissue penetration [79, 80]. Furthermore, an *in vivo* non-invasive approach using a voltage sensitive PET tracer, 4-[18F]fluorobenzyl triphenylphosphonium (18FBnTP), has been developed to functionally image the mitochondrial membrane potential in live tumors [4, 81, 82].

3.6 Detection of ROS homeostasis

Mitochondrial ROS play important roles in cell signaling, homeostasis, and apoptosis [83]. ROS are natural byproducts of highly reactive oxygen metabolites in the mitochondria, comprise a number of oxygen-containing molecules such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), peroxynitrite

($ONOO^{\bullet-}$), hydroxyl radicals (•OH), and singlet oxygen ($^1O_2$). These radicals accumulate over time in living system, stimulate distinct cell signaling pathways and lead to diverse outcomes depending on their amount and subcellular localization [84]. Due to their high reactivity and numerous clearance mechanisms, ROS exist *in vivo* in either picomolar or nanomolar steady-state concentration. Therefore, determination of mitoROS in biological systems requires probes that react very rapidly with ROS to compete with antioxidants and produce stable products, which can be quantified [85].

A series of redox-active fluorescent probes such as Amplex Red or CellROX Deep Red, Dihydroethidium (DHE), MitoSOX Red reagent have been developed for the detection of different species ($O_2^{\bullet-}$ or H_2O_2). Amplex Red is a highly specific and sensitive probe for quantitative analysis of H_2O_2 released from isolated mitochondria [86]. Amplex Red is a colourless and nonfluorescent compound. It can convert into the highly fluorescent product resofurin when oxidized by H_2O_2. Resorufin is a stable product that allows detection of H_2O_2 both in oxidative and reductive conditions [87]. Another cell permeant reagent dihydroethidium (DHE) is a fluorogenic dye commonly used for the detection of $O_2^{\bullet-}$. However, these fluorophores are prone to autoxidation during sample treatment [88]. MitoSOX Red is a mitochondria-target form of DHE, its reaction with superoxide anions is very similar to that of DHE but with faster kinetics [89]. The dynamics of mitoSOX Red can be monitored using FlexStation [90]. Whereas a caveat to use of mitoSOX is that high concentrations can overload the mitochondria and compromise mitochondrial specificity for $O_2^{\bullet-}$ detection [91]. Additionally, the probe is susceptible to photobleaching and prone to photo-oxidation, and high concentrations of mitoSOX significantly interfere with and even impair mitochondrial function [92]. Similar to MitoSOX, another membrane permeable fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH-DA), routinely used to detect H_2O_2 and oxidative stress in cells or mitochondria, is also susceptible to photobleaching. Different conditions of dye loading could potentially influence the subcellular compartmentalization of DCFH-DA, which may result in a mixed signal from cytosol, mitochondria, and other organelles [92].

For the *in vivo* measurement of ROS with low levels and transient lifetimes , the choice of an assay for a particular application may vary greatly [84, 93]A variety of genetically encoded fluorescent protein-based ROS indicators targeting mitochondria opened a new era in redox biology research. These methods do not introduce artifacts caused by sample preparation. The most widely used *in vivo* redox probes belong to HyPer and the redox-sensitive green FP (roGFP) families. HyPer is the first fully genetically encoded ratiometric fluorescent indicator capable of monitoring H_2O_2 in live cells [94]. But all HyPer family probes have the disadvantage of being pH sensitive, and thus pH changes in mitochondria could lead to misinterpretation. Another group of H_2O_2 probe is based on reduction–oxidation-sensitive green fluorescent proteins (roGFPs), which have similar sensitivity but slower responsiveness compared to HyPer. However, H_2O_2 probes are not so sensitive to detect the basal intracellular H_2O_2 concentrations in the sub-nanomolar to low nanomolar range. HyPer7, a recently new discovered ultrasensitive and ultrafast indicator, is stable to pH variations, and functional in different organisms [95]. The limitation of Hyper7 is due to its green fluorescence, which means that its spectrum is not optimal for deep-tissue imaging in relatively large non-transparent organisms like rodents [96]. Given the limitations of each assay, it is advisable to use more than two methods to ensure the accuracy and specificity for *in vivo* ROS measurement [89, 92].

3.7 Mitochondrial calcium monitoring

Ca^{2+} is a ubiquitous cellular signal. Changes in intracellular Ca^{2+} concentration not only stimulate a variety of intracellular events but also trigger cell apoptosis or necrosis. Mitochondrial Ca^{2+} uniporter (MCU), Na^+/Ca^{2+} exchanger (NCLX) ， Ca^{2+}/H^+ antiporter (Letm1), and mPTP tightly maintain cellular Ca^{2+} homeostasis and regulate physiological Ca^{2+}-dependent processes [97, 98]. Uptake and release of Ca^{2+} by mitochondria serve to buffer and shape intracellular Ca^{2+} transient, and also to maintain intracellular Ca^{2+} homeostasis, regulate energy metabolism and cell death [99]. Critical processes such as mitochondrial fission and fusion, ATP generation, ROS homeostasis, and mPTP opening, depend directly on the dynamic changes of mitochondrial Ca^{2+}[99]. Thus, precise determination of influx and

efflux of Ca^{2+} from mitochondria is crucial for understanding mitochondrial Ca^{2+} handling in these processes.

Numerous methods have been developed to measure mitochondrial Ca^{2+} concentrations ($[Ca^{2+}]_m$). These methods are essentially classified into two major groups: fluorescent dyes and genetically encoded Ca^{2+} indicators (GECIs). A number of fluorescent indicators like the Fura-2 and Indo-1 families are currently available, and most of them can be easily loaded into the cytosol with the help of non-invasive acetoxymethyl (AM) ester loading technique. To further promote the mitochondrial accumulation, a positively charged Ca^{2+} sensitive dye Rhod-2/AM and Fura2-FF AM results in Δψm-driven uptake into the mitochondrial matrix, eliminating cytosolic dye contamination and thus more reliable mitochondrial Ca^{2+} can be monitored [100]. In some studies, Fura-2 was usually used in combination with Rhod-2, allowing simultaneous measurement of cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_c$) and $[Ca^{2+}]_m$, respectively.

The discovery of GDCIs targeting to mitochondrial matrix, such as R-CEPIA3mt and R-CEPIA4mt, has enabled monitoring mitochondrial Ca^{2+} dynamics with high spatiotemporal resolution in parallel with the use of green fluorescent probes and optogenetic tools [101]. Advancements in imaging and genetically encoded sensor technologies enable visualization of mitochondrial Ca^{2+} dynamics with high spatiotemporal resolution, and provide the opportunity to visualize mitochondrial Ca^{2+} transients in live mice.

3.8 Mitochondrial thermo indicators

Mitochondria are major actors in maintaining body temperature in warm-blooded animals. It has been demonstrated that only part of the energy (67%) in brain is used to synthesize ATP, the rest 33% of this energy is immediately dissipated into heat [102]. Therefore, mitochondrial temperature is a critical indicator of cell metabolism.

Using a temperature-sensitive fluorescent probe Mito-Thermo-Yellow (MTY), Chrétien and colleagues first reported that mitochondria are physiologically maintained at close to 50˚C. The fluorescence of MTY falls by around 2.7% for each 1 °C rise in temperature in aqueous solution. When the mitochondrial respiration was inhibited or mitochondrial DNA was depleted, the differential between mitochondria and external temperature will be abolished [103].

Many factors could affect the measurement using fluorescence thermo indicator. For example, the loading of thermo indicator in the mitochondria could be affected by pH, oxygen tension, superoxide production, and membrane potential [104]. Recently, a breakthrough has been made in the quantitative visualization of mitochondrial temperature [105, 106]. As the first fixable, fluorescent molecular thermometer, Mito-TEM is based on a positively charged rhodamine B fluorophore that has the tendency of being attracted into mitochondria, and can be used to visualize the temperature changes quantitatively in live cells [107]. In addition, a thermosensitive rhodamine B (RhB)-derived fluorogenic probe (RhBIV) was also reported, it has a long half-life (t1/2) and enables fluorescent labeling of cell mitochondria at concentrations as low as 1 μM. This unique thermosensitive probe offers a simple, nondestructive method for longitudinal monitoring of mitochondrial temperature both *in vitro* and *in vivo* [108]. Furthermore, a new generation of ratiometric fluorescence probe Mito-TEM 2.0 exhibits excellent linear correlation and temperature sensitivity, indicating that the fluorescent thermo probe could be a reliable tool for monitoring mitochondrial temperature for a long time *in vivo* experiment [105].

4. Mitochondrial dysfunction in cardiovascular diseases

Many studies have demonstrated that mitochondrial dysfunction is closely related to the development of cardiovascular diseases, including ischemic heart disease, cardiomyopathy, atherosclerosis, hypertension, and severe hemorrhagic shock [109]. Mitochondrial abnormalities in cardiovascular diseases include impaired mitochondrial electron transport chain activity, increased ROS production, disturbed energy metabolism, aberrant mitochondrial dynamics, and abnormality of Ca^{2+} homeostasis. Interventional procedures to improve mitochondrial dysfunction seems to be beneficial [110]. For example, the reduction of mitochondrial ROS production significantly improved

the endothelial function and also associated with better metabolic control [111]. Alterations of proteins responsible for mitochondrial fission and fusion have been linked to the progression of cardiovascular diseases [112]. Identification of mitochondrial dysfunction biomarkers in blood may enable the selection of patients who could benefit from therapies targeting mitochondria [113]. Therefore, restoration of normal mitochondrial function may be considered as a new therapeutic strategy for cardiovascular diseases.

4.1 Ischemic heart disease and cardiomyopathy

Cardiomyocytes are highly dependent on aerobic metabolism to meet their energy requirements. Mitochondrial quality is critical in the pathological process of ischemic heart disease [114]. Excessive ROS production during ischemic myocardial reperfusion damages the mitochondrial membrane system, which impairs the $\Delta\Psi$ and mitochondrial ATP synthesis, and causes oxidative stress, Ca^{2+} overload, and sustained mPTP opening [115]. In addition, disturbance of Ca^{2+} homeostasis in mitochondria contribute to abnormal cardiac excitation–contraction coupling [116]. Accumulating studies have demonstrated that a series of molecular mechanisms are involved in promoting the transition of mitochondria in heart from energy-producing to death-initiating functions [117, 118], but their role in cardiac hypertrophy awaits further investigations. The morphology and function of mitochondria is responsive to changes in cardiomyocytes during the development of pathological cardiac hypertrophy [119]. For example, cardiac hypertrophy secondary to aortic constriction in rats is associated with aberrant electron transport chain activity, loss of mitochondrial membrane potential, altered mPTP opening, and ultimately swelling of the mitochondria. Moreover, impaired mitochondrial dynamics, reduced production of ATP, incapability of the mitochondrial network to regulate Ca^{2+} homeostasis can alter cardiac function [120, 121]. Furthermore, fission–fusion shifts can frequently occur under various stress conditions, representing an early event in the mitochondria-dependent cell apoptosis, which eventually leads to chronic myocardial damage [122].

4.2 Atherosclerosis

Atherosclerosis is a chronic inflammatory condition and the underlying pathological basis of cardiovascular disease. A growing number of studies have demonstrated that mitochondrial function is required for normal vascular cell growth and function. Vascular smooth muscle cells are the main components of the blood vessel wall and plaques. Accumulation of mitochondrial DNA (mtDNA) mutations and damage, increased production of ROS, and progressive respiratory chain dysfunction lead to the dedifferentiation and abnormal proliferation of vascular smooth muscle cells, promote the development of atherosclerosis and plaque vulnerability [123]. Besides, accelerated accumulation of oxidized low-density lipoprotein (ox-LDL) in arterial wall can inhibit the activity of mitochondrial respiratory enzymes or mitochondrial dynamics, induce alterations in mitochondrial membrane potential, lead to prolonged opening of the mPTP and increase the generation of MitoROS, thus forming a vicious circle and promoting endothelial apoptosis and atherosclerosis [124, 125]. Furthermore, mitochondrial dysfunction may promote the activation of NLRP3 inflammasomes through the excessive generation of ROS, eventually leading to the occurrence and development of atherosclerosis [126]. Moreover, mitochondrial dysfunction in macrophages or pericytes may also contribute to unstable plaque development through maintaining chronic inflammatory conditions, favoring foam cell formation or plaque rupture [127]. Therefore, mitochondrial dysfunction could affect endothelial NO bioavailability, vascular smooth muscle proliferation and macrophage polarization, which play a key role in the initiation and progression of atherosclerosis.

4.3 Hypertension

Many studies have suggested that mitochondria-derived superoxide anion can oxidize the NO released by endothelial cells, decrease the endothelium-dependent vasodilation, and contribute to vascular dysfunction and remodeling in hypertension. Moreover, ROS induce mutations in mtDNA, which result in a marked loss of complex-I activity and ATPase synthase subunits and thus decrease electron-transport chain activity and ATP synthesis, which is implicated to the development and progression of hypertension and its complications [128].

In addition, numerous studies have demonstrated that mitochondrial metabolic disorders and oxidative stress can be mediated by hyperacetylation of mitochondrial proteins (such as sirt3, SOD2 or CyPD), promoting vascular dysfunction and hypertension [129-131]. For example, Cyclophilin D (CypD), an essential structural component of mPTP, promotes mitochondrial impairment and oxidative stress due to being activated by S-glutathionylation and acetylation, which contribute to vascular dysfunction and hypertension, targeting CyPD decreases mitochondrial O2•− and reduces hypertension[132, 133]. Alterations in both mitochondrial dynamics and ROS production have been associated with endothelial dysfunction, development of inflammation and hypertension [134]. Therefore, the effective agents targeting mitochondrial dysfunction may be useful for the treatment or prevention of hypertension.

4.4 Hemorrhagic shock

Hemorrhagic shock is characterized by profound hemodynamic alterations, microcirculatory dysfunction associated with multiple organ dysfunction. Our previous data [135, 136] and others [137, 138] have demonstrated that mitochondrial dysfunction occurred in different organs and cell types, which is a common pathway involving in the cell injury and organ failure of severe shock. Mitochondria in severe shock appeared spherical or irregularly shaped, apparently swollen with poorly defined cristae. The mitochondrial structure， the opening of mPTP, membrane potential and ATP production in shock were partially protected by mitochondrial protectors such as ciclosporin A, resveratrol and polydatin. The morphological damage is fundamental for the assessment of mitochondrial dysfunction in severe shock. In addition, the abnormal opening of mPTP, the reduced mitochondrial membrane potential (mitochondrial depolarization), the increased intracellular lipid hydroperoxide (LPO), and the reduced intracellular ATP content were observed in severe hemorrhagic shock and all of which indicated the mitochondrial dysfunction [136]. The treatment to prevent mitochondrial dysfunction has become a new potential management for severe shock, which includes following approaches: provision of mitochondrial substrate or mitochondrial cofactor; reduction of mitochondrial oxidative stress; inhibition of mPTP opening; and activation of SIRT1/3 [135].

5. Conclusion

Mitochondrial dysfunction is currently recognized as an important therapeutic target for treatment of many chronic diseases. Considering that mitochondria are dynamic and highly compartmentalized, approaches that allow to simultaneously visualize mitochondrial structure and function will certainly be of particular interest in the future. Recent advances in biological technology and development of instrumentation and reagents have facilitated investigations into the mitochondrial function both *in vivo* and in *vitro*. Each method *in vitro* provides a unique set of advantages as well as limitations. For example, isolation of mitochondria is the pure way to assess mitochondrial function free from other factors, but the isolation procedure requires relatively large tissue samples and may cause some disruption of the mitochondrial structure and morphology. However, it has been suggested recently combining multiple approaches may yield significant insight into the regulation of mitochondrial bioenergetics in cardiovascular diseases. Since each method has potential pitfalls and advantages, it is clear that a variety of experimental parameters and/or approaches must be carefully considered and combined when selecting and conducting studies on mitochondrial function. The mitochondrial dysfunction targets and assessment methods are summarized in **Table 1**. Given the molecular mechanisms of mitochondrial dysfunction and their relationship with diseases require further clarification, proper assessment of mitochondrial functions in different pathological conditions will facilitate the development and design of pharmacological therapies to target mitochondria and promise benefits in the treatment of cardiometabolic diseases.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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Table 1. Mitochondrial dysfunction and major assessment methods

Targets	Assessment methods		Advantages and limitations	Ref
Morphology	*In vitro*	Transmission electron microscopy (EM); Electron tomography (ET), Cryo-ET, and Cryo-EM	3D structures with superresolution highly sophisticated but expensive for such facilities.	10
mPTP	*In vitro*	Ca^{2+} retention capacity assay Ca^{2+}-triggered mitochondria swelling assay Pharmacological approach: mPTP inhibitor-CsA Fluorescent probe: Co^{2+}-calcein assay	Simple and effective for Ca^{2+} and swelling assays. Potential inhibition of **calcineurin**-dependent signaling by CsA. Co^{2+} may exert toxic effects.	22,23 25,26
	In vivo	Radioactive tracer: 2-deoxy [3H] glucose ([3H]-DOG	Special facility requirement; Radioactive contamination.	29,30
Mitochondrial dynamics	*In vitro*	Fluorescent probe: mt-PA-GFP Non-fluorescent labeling: photothermal microscopy Imaging of mitochondrial protein markers	Kinetic data acquisition from multiple cells in parallel; Simple and less affected by photobleaching. Live cell imaging facilities required.	43-47
	In vivo	Transgenic mice with fluorescent reporter gene: MitoTimer mice; CAG-mito::mKate2 mice	*In vivo* imaging	48,49
Mitochondrial ATP	*In vitro*	NMR, HPLC Luciferase-based sensors	NMR with good linearity but low temporal resolution; HPLC is fast and highly reproducible; Luciferase sensors can be for real-time.	51-54
	In vivo	Fluorescent probes ATeam, iATPSnFRs, PercevalHR, GRABATP1.0	Fluorescent probes can be used for *in vivo* studies.	55-63
Mitochondrial respiration	*In vitro*	Biochemical assays: COX, SDH or Complex IV assays High-resolution Clark electrodes (e.g., OxygraB) Seahorse XF Analyzer Respirometry In Frozen Samples (RIFS)	Biochemical assays are simple and inexpensive. Unstable signals and high background noise for OxygraB. Real-time monitoring for mitochondrial function using Seahorse. RIFS with frozen biological specimens.	3, 64-68
Mitochondrial membrane potential (ΔΨm)	*In vitro*	Microelectrode recording Fluorescent probes: JC-1, Rhod123, TMRE or TMRM. Nanomaterials: fluorescent carbon dot	Technical difficult to use microelectrode. Δψm sensitive, pH sensitive, potential respiratory inhibition Enhanced contrast and photostability	71 72-75, 77
	In vivo	Two-photon and near-infrared fluorescent probes (KMG-501) PET tracer: 18FBnTP	Low background, ultra-high tissue penetration PET imaging with 18FBnTP and expensive	79,80 81,82
ROS	*In vitro*	Fluorescent probes: Amplex Red, Dihydroethidium, MitoSOX Red	Detection of H_2O_2 , specific and sensitive Detection of $O_2^{\bullet-}$, photobleaching and photo-oxidation	86-92
	In vivo	Genetically encoded fluorescent protein-based ROS indicators: HyPer, HyPer7 and roGFP	Not optimal for deep-tissue imaging	94-96
Mitochondrial calcium	In vitro	Fluorescent dye: Fura-2 AM, Indo-1AM; Fura2-FF AM Rhod-2/AM	cell-permeable, atiometric and UV light—excitable high specificity, wide dynamic range, and low pH sensitivity	100
	In vivo	Genetically encoded Ca2+ indicators (GECIs): R-CEPIA3mt and R-CEPIA4mt	high spatiotemporal resolution	101
Mitochondrial temperature	*In vitro*	Temperature-sensitive fluorescent probe: Mito-Thermo-Yellow (MTY); Mito-TEM	sensitivity but high endogenous background permanent immobilization but poor quantitative accuracy.	103-107
	In vivo	Rhodamine B (RhB)-derived fluorogenic probe (RhBIV); Mito-TEM 2.0	brightness, light stability, and sensitivity ratiometric imaging, sensitivity, and specific immobilization	105,108