Common methods in mitochondrial research (Review)

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Abstract. Mitochondrial abnormalities are primarily seen in morphology, structure and function. They can cause damage to organs, including the heart, brain and muscle, by various mechanisms, such as oxidative stress, abnormal energy metabolism, or genetic mutations. Identifying and detecting pathophysiological alterations in mitochondria is the principal means of studying mitochondrial abnormalities. The present study reviewed methods in mitochondrial research and focused on three aspects: Mitochondrial extraction and purification, morphology and structure and function. In addition to classical methods, such as electron microscopy and mitochondrial membrane potential monitoring, newly developed methods, such as mitochondrial ultrastructural determination, mtDNA mutation assays, metabolomics and analyses of regulatory mechanisms, have also been utilized in recent years. These approaches enable the accurate detection of mitochondrial abnormalities and provide guidance for the diagnosis and treatment of related diseases.

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1. Introduction

Mitochondria are semi-autonomous organelles found in most eukaryotic cells with a bilayered structure consisting of an outer membrane, an intermembrane space and an inner membrane. They serve key roles in a variety of cellular processes, including cell metabolism, signal transduction and the regulation of cell death. Mitochondria have numerous biological functions, including the production of ATP for cellular energy, regulation of the dynamic balance of intracellular Ca^{2+}, production of reactive oxygen species (ROS), the release of cytochrome c and regulation of intracellular environmental homeostasis. As an important signaling hub in cells, the mitochondrion serves a key role in diseases such as aging and obesity. Mitochondrial biogenesis and mitochondrial homeostasis require the expression of nuclear genes and mitochondria-nuclear signaling pathways to be regulated (1). On the one hand, it depends on the regulatory pathways of nuclear gene transcription and anterograde signaling. Mitochondria, on the other hand, pass intracellular signaling molecules, such as Ca^{2+}, mitochondrial DNA (mtDNA), reactive oxygen species (ROS), adenosine triphosphate (ATP), coenzyme Q (CoQ) and nicotinamide adenine dinucleotide (NAD) and then present mitochondrial abnormalities and cellular metabolic change signals to the nucleus (retrograde signaling). This triggers the nucleus to activate important signaling pathways by mobilizing a series of nuclear transcription factors (2-5), mitochondrial transcription and mitochondrial biosynthesis. Among them, the activation of signaling pathways is closely related to inflammation and tumorigenesis (6). During cellular stress and virus infection, mtDNA and ROS are released from abnormal mitochondria and retrogradely presented to the nucleus as danger signals. The nucleus can promote the expression of PTEN-induced kinase 1 (PINK1) and then upregulate mitophagy to clear abnormal mitochondria and maintain a stable intracellular environment. When too many abnormal mitochondria cannot be completely removed, mtDNA can activate Toll-like receptor 9 (TLR9) and its downstream inflammatory pathways and lead to inflammation. Excessive ROS can cause DNA damage by oxidizing nucleic acid bases, which is closely related to tumorigenesis. Abnormalities in mitochondrial structure and function can lead to a variety of intracellular signaling cascades, oxidative stress and the initiation of programmed cell death, thereby contributing to the development and progression of nearly all diseases. Therefore, the detection of mitochondrial abnormalities is crucial and various mitochondrial assays (Fig. 1) developed

in the last century have contributed substantially to the differential diagnosis of mitochondrial diseases. The present study reviewed common experimental methods (Table I) in mitochondrial research. In particular, it discussed a wide range of imaging and detection techniques for i) extraction and purification, ii) analyses of morphology and structure and iii) analyses of function, with a focus on the clinical implications for disease detection and treatment.

2. Extraction and purification of mitochondria

A suitable method is needed to extract purified mitochondria from various tissues and cells (7). The basic extraction method mainly relies on differential centrifugation, while purification mainly depends on density gradient centrifugation. The specificity of tissue cells determines the details of the method (8-10).

Extraction of mitochondria. When extracting mitochondria, because the homogenization process can heat the sample locally, resulting in protein denaturation and aggregation, the equipment must be pre-cooled and the temperature kept low throughout the process (11). Tissue or cell homogenization is followed by continuous differential centrifugation. Unlysed cells, cell debris and nuclei are first removed by low-speed centrifugation (600 x g or 1,000 x g) (12-15). As mitochondria can remain in flaky precipitates generated by low-speed centrifugation, resuspending the pellet and centrifuging it again at low speed increases mitochondrial yield. The supernatant obtained by two low-speed centrifugation steps is collected for high-speed centrifugation (3,500 x g or 10,000 x g) (12-15), resulting in a coarse-lifted mitochondria precipitate (16). The purity of these crudely extracted mitochondria can meet some applications, including the analysis of the activity of known mitochondrial proteins, the detection of mitochondrial morphology and mitochondrial apoptosis; however, they often contain a certain amount of peroxisomes, endoplasmic reticulum and microsomes. Mitochondrial purity is low; thus, mitochondrial purification and reduction of membrane fouling are required when analyzing proteins present in multiple cells or determining the localization of a protein (17). Furthermore, although the mitochondrial extraction method is suitable for most tissues and cells, the extraction efficiency and quantity of mitochondria in different tissues and cells are significantly different. This is determined by the number of mitochondria in the tissue or cell and the energy consumption of muscles and liver; larger tissues contain more mitochondria, so these tissues and cells have higher mitochondrial extraction efficiency than other tissues, such as the lungs (18-20).

Purification of mitochondria. Purified mitochondria are the prerequisites for mitochondrial proteomics research. Density gradient centrifugation emerged in the 1950s and has become a common method for separating extracts owing to its ease of operation and low cost (21). For example, sucrose density gradient centrifugation suspends the cell or a homogeneous tissue slurry in a uniform suspension medium according to the density of each cell component and is separated by differential centrifugation (22-24). The buffered sucrose solution, the most commonly used suspension medium, is relatively close to the dispersion phase of the cytoplasm and can maintain the structure of various organelles and the activity of enzymes to a certain extent (25-28).

Sucrose density gradient centrifugation is a classic method for extracting mitochondria by separating cellular fractions of different densities (29). It involves three main processes: Tissue homogenization, fractionation and analysis (30-32). Homogenization refers to the disruption of cells or tissues in a homogenizer by adding sucrose at a low temperature to form a homogenate containing various organelles and other substances (33). Fractionation is the sequential settling of particles of different densities and sizes in the sample by centrifugation at different speeds. Analysis refers to the use of biochemical methods to identify the morphological function of the separated components; it is conducted using the Janus green live dyeing method, which is easy to operate and stable in performance. However, at high concentrations, sucrose has a high viscosity and high osmotic pressure, which can easily cause repeated shrinkage and mitochondrial expansion. Compared with sucrose, the price of commonly used density gradient media (including Percoll, Nycodenz and OptiPrep) is generally higher, but the morphology of the extracted mitochondria is generally complete. Percoll has a low diffusion constant, the gradient formed is very stable and it does not penetrate the biofilm; as such, it minimizes organelle rupture and is often used to isolate platelet mitochondria (12,34,35). Nycodenz is increasingly widely used owing to its high density, low viscosity and lack of effect on osmotic pressure (36-38). The yield of intact mitochondria is significantly higher in Nycodenz gradients containing sorbitol as an osmotic stabilizer instead of sucrose (37,38). As a dimer of Nycodenz, OptiPrep has the advantage of forming automatic gradients in a short period of time (39-42). Additionally, some researchers use streptavidin magnetic beads to separate *Arabidopsis* mitochondria. After the tissues are lysed, they are mixed with anti-mitochondrial outer membrane protein 22 (TOM22) magnetic beads and the mixed samples placed in the sorting column. Only mitochondria remain on the sorting column after washing, followed by elution, isolating the complete mitochondria in less than 30 min with a success rate, purity and integrity significantly higher than the density gradient centrifugation (43-47). Therefore, the magnetic bead method can be used to extract mitochondria in tissues with fewer mitochondria. As such, this approach will probably become increasingly common in mitochondrial extraction and purification (48-50). In conclusion, among the current mitochondrial extraction and purification methods, the magnetic bead method has the best effect on eliminating impurities such as microsomes and peroxisomes and the mitochondrial purity obtained by the differential centrifugation method is the lowest and the effect on eliminating these impurities is the worst.

3. Determination of mitochondrial morphology and structure

Mitochondria are organelles with a complex bi-membrane structure that regulate the entry and output of proteins, lipids, solutes and metabolite products and protect the cytoplasm from harmful mitochondrial products (51-53). Mitochondria can engulf abnormal mitochondria and remove excess harmful mitochondrial products to protect the body. This process is called mitophagy (54-56). Most mitochondria are spherical, rod-shaped, or tubular; however, mitochondrial morphology varies widely among tissues and cells depending on the energy

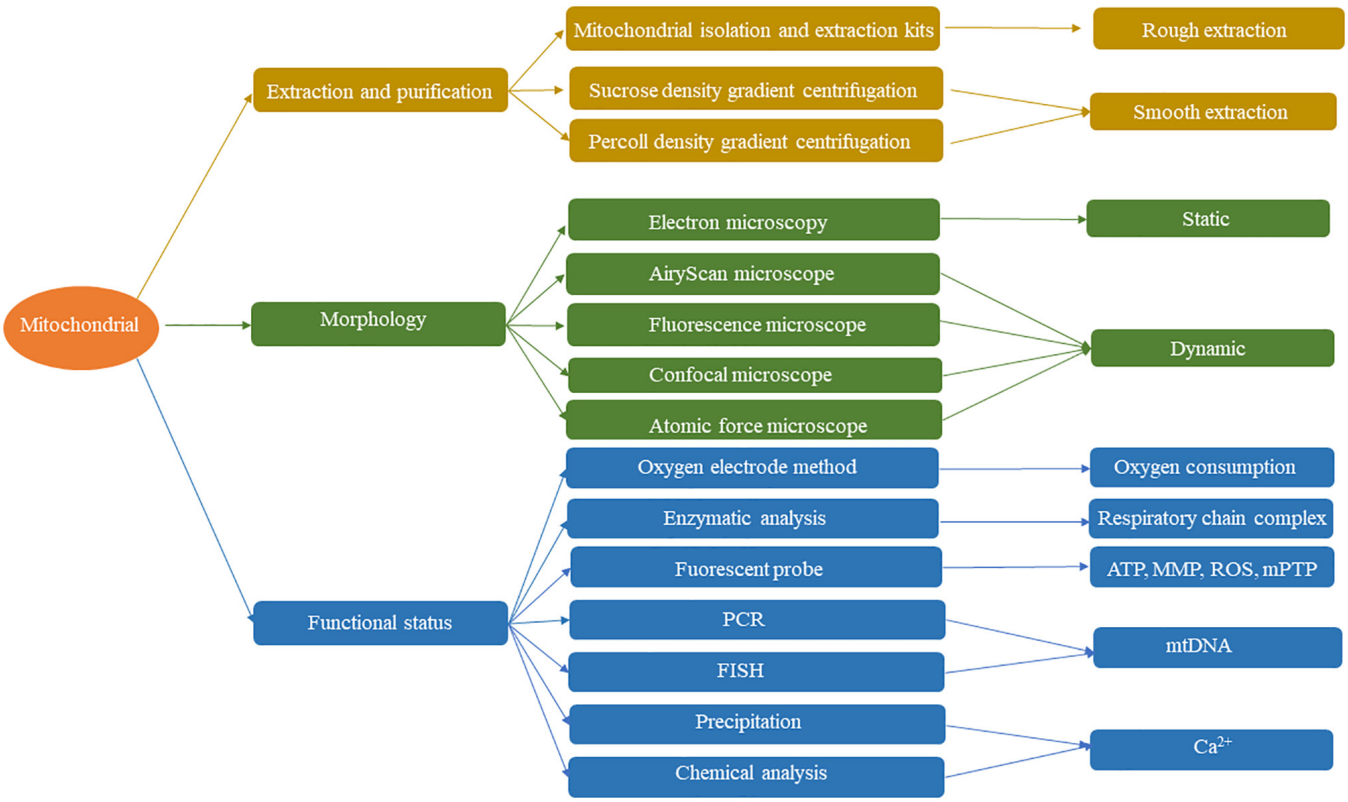

Figure 1. Commonly used research methods for mitochondria.

requirements of cells and the location of mitochondria within the cell (53,57). For example, mitochondria are spherical at synaptic terminals, whereas they appear as highly elongated rods in axons. In senescent and functionally impaired cells, mitochondrial morphology is significantly different from that in normal cells and they can be irregularly shaped (53,58,59). Therefore, morphological changes can be used in the initial assessment of mitochondrial function.

After over 50 years since its development, electron microscopy (EM) has become the central tool for observing organelles in eukaryotic cells and is the gold standard for observing mitochondrial structure (60). It can reveal mitochondrial swelling, rupture and other abnormalities of damaged mitochondria. However, it cannot clearly distinguish mitochondria from other membranous structures and is occasionally confusing. In the 1980s, atomic force microscopy, as an emerging observation method, could study the surface structure and properties of substances by detecting the extremely weak interatomic interaction between the surface of the sample to be tested and a miniature force-sensitive element. Due to the characteristics of resolution and real-time imaging, changes such as the formation of mitochondrial swelling can also be observed under liquid conditions but are significantly affected by the probe; thus, the application range is small (61-64)

The recently developed AiryScan microscope (Zeiss AG) can acquire images at high speed with high sensitivity to effectively observe the kinetic processes of mitochondrial fission, fusion and autophagy (65-67). In addition, both wide-field fluorescence microscopy and high-resolution confocal laser scanning microscopy can be used for imaging analyses of morphological changes in mitochondria with higher specificity than that of EM, but the dynamic changes of the mitochondria cannot be observed (68-76).

In most cases, microscopy can be used to observe and analyze two-dimensional mitochondrial morphologies and quantities. However, although this method is suitable for analyzing adherent cells with flat morphology, it is not suitable for thicker cells (77-83). Three-dimensional confocal microscopy can be used to observe mitochondrial morphology by observing specifically labeled mitochondrial proteins at the 3D level (84-87). In addition, after labeling mitochondria with specific dyes, mitochondrial morphology can be visualized using a combination of immunofluorescent staining and computer images (58,88,89).

4. Determination of mitochondrial function

Determination of mitochondrial membrane potential. Mitochondrial membrane potential (MMP) refers to the negative potential difference between the two sides of the inner mitochondrial membrane. It is a sensitive indicator for evaluating mitochondrial function (90-93). It is closely associated with cellular homeostasis and is most commonly used to determine the metabolic state of mitochondria (93-98).

Fluorescent dye probes used for flow cytometry are now commonly used in MMP assays. For example, rhodamine 123, a specific stain developed in the 1980s, is widely used in flow cytometry and MMP assays. In normal cells, rhodamine 123 can selectively enter the mitochondrial matrix depending on MMP and can emit bright yellow-green fluorescence; when cells undergo apoptosis or necrosis, the mitochondrial membrane permeability transition pore (mPTP) is abnormally opened and

Table I. Summary of mitochondrial research methods.

Area of research	Methods		Scope of application	Advantages	Drawbacks
Extraction and purification of mitochondria	Differential centrifugation extraction		Tissues and cells	Detect mitochondrial morphological structure	Low mitochondrial purity
	Density gradient centrifugation purification (different media)	Sucrose		Low cost and wide application	Poor mitochondrial morphological integrity
		Peroll		Isolate platelet mitochondria	Higher cost compared to sucrose
		Nycodenz		Compared to sucrose, higher density and lower viscosity without affecting osmotic pressure	
		Optiprep		Automatic gradients can be formed in a short time	
	Magnetic bead method		Tissues and cells	Mitochondrial purity and integrity superior to other methods	Not yet widely used
Mitochondrial morphology	Electron microscope			Gold standard	Cannot clearly distinguish mitochondria from other membranous structures
	AiryScan microscope			Observable mitochondrial dynamics	Not yet widely used
	Atomic force microscope			Observation of mitochondrial swelling and mitochondrial dynamics	
	3D Confocal Microscopy		Thicker cells	For thicker cells	
Mitochondrial function	Mitochondrial membrane potential	Rhodamine 123, JC-1	Tissues and cells	Intuitively reflect changes in MMP	High cytotoxicity
		TMRM, TMRE		Low cytotoxicity for quantitative analysis of MMPs	Not yet widely used
		TRR-CY		Extremely sensitive to detect minute changes in MMP	
		FRET		Monitoring dynamic changes of MMP	
	Mitochondrial oxygen consumption	Oxygen electrode polarography	Tissues and cells	Low cost, detection of respiratory control rate	Poor specificity
		Hippocampus analyzer		Comprehensive analysis of mitochondria by measuring oxygen consumption rate	Can be affected by chemicals such as phenol red
	Mitochondrial Ca^{2+} Detection	Electrochemical analysis		Suitable for experiments with low sensitivity, unable to distinguish mitochondrial Ca^{2+} from total Ca^{2+}	Poor specificity

Table I. Continued.

Area of research	Methods		Scope of application	Advantages	Drawbacks
		Calcium-Rhodamine 123	Tissues and cells	High specificity, suitable for the detection of mitochondrial Ca^{2+} in various living cells	Inability to distinguish between different cellular sources of Ca^{2+}
		Fluo-3		Distinguish mitochondrial Ca^{2+} from Ca^{2+} in other intracellular organelles	
	Mitochondrial membrane permeability transition pore	Fully automatic patch clamp	Suspension cells	Can be used for detection of suspension cells	Small scope of application
		Calcein-AM	Tissues and cells	Strong specificity, can reflect the opening degree of mPTP in real time	Easy to be quenched, timely observation is required
	Mitochondrial ATP	High pressure liquid chromatography	Tissues and cells	Can detect differences in cellular energy substances in different states	Requires a larger sample size
		Enzymatic analysis		It is greatly affected by the absorbance of the tested sample	Susceptible to redox reactions
		Fluorescence analysis		The amount of luminescence is proportional to ATP	Easy to quench
		Mito-Rh		Can specifically recognize ATP in mitochondria	
Mitochondrial function	Mitochondrial respiratory chain complex	Spectrophotometry	Tissues and cells	Wide range of applications, but less accurate	Vulnerable to external biochemical interference
		NIR spectroscopy non-invasive measurements		Less affected by the outside world, high accuracy	Requires a very large sample size
	ROS	Chemical reaction selective electrode method	Tissues and cells	High sensitivity, cheap and easy to operate, but poor specificity and unstable results	Poor specificity and unstable results
		Spectrophotometry		High sensitivity and specificity, but cannot perform localization analysis of oxygen free radicals	Unable to perform localization analysis of oxygen free radicals
		Reagent test kit		Strong specificity, easy operation, low background, large detection range, easy quenching	Easy to quench

Table I. Continued.

Area of research	Methods		Scope of application	Advantages	Drawbacks
		Electron spin resonance		The most direct and effective, expensive and complicated operation	Expensive and complicated to operate
	Mitochondrial DNA	PCR	Tissues and cells	Detectable mtDNA deletions	The presence of mtDNA heterogeneity in the primer binding region
		FISH		Visually detectable under a fluorescence microscope	Poor specificity and insufficient hybridization
		Sequencing		Gold standard for detecting heterogeneity	Limited to small scale projects
		Probe method		Detect mtDNA dynamic changes	Need real-time observation

TMRM, tetramethyl rhodamine methyl ester; TMRE, tetramethyl rhodamine ethyl ester; FRET, fluorescence resonance energy transfer; FISH, fluorescence *in situ* hybridization; MMP, mitochondrial membrane potential; mtDNA, mitochondrial DNA.

MMP is unbalanced. Rhodamine 123 is released from mitochondria, resulting in a significant decrease in the yellow-green fluorescence intensity in mitochondria, which reflects the changes in MMP (50,99-102). 5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) has higher sensitivity than that of rhodamine 123. At low MMP levels, JC-1 exists as a monomer and produces green fluorescence; at high MMP levels, JC-1 aggregates in the mitochondrial matrix and forms polymeric JC-1. This can be used for qualitative and quantitative analyses of MMP by fluorescence microscopy or flow cytometry (50,96,101,103-108). Tetramethyl rhodamine methyl ester (TMRM) and tetramethyl rhodamine ethyl ester (TMRE), like JC-1, are specific dyes that have recently become common tools for measuring MMP (109-112). TMRM can be excited at 488 nm, showing red-orange fluorescence and its fluorescence intensity has a linear relationship with MMP. Compared to rhodamine 123 and JC-1, these two dyes are very soluble, have short loading times (15-20 min) and have extremely low cytotoxicity, requiring micromolar inhibition of mitochondrial function. With staining concentrations in the range of 0.5-30 nM (the concentration of JC-1 needs to be >0.1 μM), the accumulation in mitochondria is limited to the change of membrane potential and the sensitivity is extremely high; this is markedly suitable for quantitative analysis of mitochondrial membrane potential and quantitative flow cytometry (113-118). However, in quantitative flow cytometry studies, the data must be corrected for the signal of MitoTracker Green FM, a dye that is not dependent on mitochondrial membrane potential. It is worth noting that the above fluorescent probes for measuring MMP are applicable to most tissues and cells, including plant cells and bacteria.

Fluorescence resonance energy transfer (FRET) is a non-radiative energy transition that transfers energy from the excited state of the donor to the excited state of the acceptor through intermolecular electric dipole interactions (119,120). This process does not involve photons, so it is non-radiative. This analytical method has the advantages of rapidity, sensitivity and simplicity. Fluorescence resonance energy transfer molecular pairs (FRET Pairs) have been designed and synthesized to monitor MMPs (121). The FRET donor molecule (FixD) is constructed by attaching a benzyl chloride group to a fluorophore with green fluorescence emission. FixD can be attached to and fixed in mitochondria by sulfhydryl groups of mitochondrial proteins. The FRET acceptor (LA) is a mitochondrial membrane potential-dependent probe with green absorption and deep red fluorescence emission. When MMP is at a normal level, both FixD and LA target mitochondria. When FixD has an excitation wavelength of 405 nm, FRET occurs between FixD and LA, allowing green fluorescence to be detected but not deep red LA fluorescence emissions. When MMP is gradually reduced, LA will gradually fall off from mitochondria. While FixD is still fixed in mitochondria, the distance between the molecules gradually blocks the occurrence of FRET between FixD and LA molecules, allowing deep red fluorescence emission to be detected gradually. The decrease and the gradual increase of green fluorescence emission can be used to monitor the dynamic changes of MMPs (122), providing new ideas for the development of novel MMP fluorescent probes and real-time *in situ* studies of MMPs in living organisms, tissues and cells (123,124).

MMP varies greatly among sites on the mitochondrial membrane; therefore, accurate measurement of MMP requires further study (125). In recent years, low concentrations of a hemicyanine derivative (TPP-CY) have been used to monitor trace changes in MMP at the subcellular level during apoptosis with very high sensitivity (125). This approach is a potentially useful tool for evaluating cell health.

Determination of mitochondrial oxygen consumption. Among organelles, mitochondria consume the most oxygen in cells and this oxygen consumption often reflects mitochondrial function (126-128). In the heart, mitochondrial oxygen consumption can be measured to determine cardiac mitochondrial function, providing an indicator of cardiac function (129-131). In children, mitochondrial dysfunction causes mitochondrial heart disease with hypertrophic myocardial infarction as the primary symptom; however, the exact mechanism and etiology remain to be investigated (129,132).

Oxygen electrode polarography is a common method for determining mitochondrial oxygen consumption and refers to the incubation of mitochondria in an oxygen-consuming medium in a magnetically stirred incubator at 30˚C. Briefly, rotenone is used to inhibit complex I in the electron transport chain, followed by the addition of succinate to measure mitochondrial state IV respiration (non-phosphorylating respiration). State III respiration is measured by incubating mitochondria in the presence of succinate and adenosine diphosphate. The respiratory control ratio (RCR) is the ratio of the state III respiration rate to state IV respiration rate, with a normal value of 3-10 (133-135). A low RCR indicates impaired mitochondrial ATP synthesis and mitochondrial dysfunction and a high RCR indicates vigorous cellular activity and accelerated metabolism (127,136,137).

In addition, the hippocampal analyzer can measure the changes in oxygen and pH levels through sensors and then automatically calculate the rate and detect the cellular oxygen consumption rate (OCR) and extracellular phosphorylation rate (ECAR) in real time to characterize the metabolic status of cells. Where OCR is caused by mitochondrial electron transfer, ECAR is derived from lactic acid fermentation (glycolytic acidification) and carbon dioxide produced by mitochondria (mitochondrial acidification) (138-140).

OCR is used to study mitochondrial oxidative phosphorylation function, with pMoles/min as the readout type (141). Generally, basal respiration in a normal state is measured first and then oligomycin is added to inhibit ATP synthase. This is a significant decrease in OCR, leaving only proton leakage (142). The oxygen consumption rate is caused by proton leakage and the reduced section is the oxygen consumption rate (ATP production) of oxidative phosphorylation. With the addition of the uncoupling agent FCCP, electron transport loses the constraints of the proton gradient and proceeds at a maximum rate (143). Therefore, the OCR increases sharply, reaching the maximum oxygen consumption (maximal respiration); the difference between this value and the basal respiration is termed the spare respiratory capacity. Finally, adding an electron transport inhibitor, such as antimycin A, completely inhibits electron transport and reduces the oxygen consumption rate to a minimum (144).

ECAR is often used to study metabolic conditions such as glycolysis, with mpH/min as the readout type (139,140,142). The basal value before adding glucose is non-catalytic acid production, such as mitochondrial acidification caused by carbon dioxide produced by mitochondrial respiration. Glucose is then added and the elevated value represents glycolysis. After the addition of oligomycin, the production of acid increases because oxidative phosphorylation is inhibited and the cells are forced to use lactic acid fermentation for energy. The value at this time is called glycolytic capacity and the difference from glycolysis is termed glycolytic reserve (140,142,143). Last added is 2-deoxyglucose, a competitive hexokinase inhibitor that can block glycolysis, so the curve should return to the basic value following its addition (144-146).

However, the direct measurement of glycolysis by ECAR is somewhat biased since the addition of glucose enhances glycolysis and oxidative phosphorylation. This will lead to increased mitochondrial acidification, causing the calculated amount of glycolysis to be high (147-149).

It is worth noting that during the measurement process of the hippocampal analyzer, the interference of phenol red should be avoided because it causes errors in the measurement results (141,150,151), but the specific reasons remain to be elucidated. In conclusion, the hippocampal analyzer can monitor OCR and ECAR to obtain multiple other parameters in a single analysis, including basal respiration, ATP-related respiration, maximal respiration, spare respiratory capacity and non-mitochondrial oxygen consumption, all of which can provide information on the mechanism of mitochondrial dysfunction (152,153).

Determination of mitochondrial Ca^{2+}. Intracellular Ca^{2+} is primarily stored in organelles, such as the mitochondria and endoplasmic reticulum, and serves an important role in biological processes such as signal transduction, blood coagulation, transmembrane ion transport and cell division (154-156). Mitochondrial Ca^{2+} is a central regulator of oxidative phosphorylation and serves a key role in the control of ATP synthesis (157). A Ca^{2+} imbalance can cause abnormal mitochondrial function and even cell damage and death, leading to pathological changes and affecting organismal health (158,159). The accumulation of mitochondrial Ca^{2+} promotes ATP synthesis in mitochondria; conversely, decreased mitochondrial Ca^{2+} leads to a decrease in mitochondrial ATP. Impaired ATP synthesis further leads to a Ca^{2+} imbalance (157,159), which in turn leads to endocrine dysfunction and numerous diseases, such as mitochondrial diabetes mellitus (160-165).

Methods for the determination of mitochondrial Ca^{2+} include precipitation, electrochemical analysis, EDTA chelation titration, flame photometry and atomic absorption spectroscopy, among which electrochemical analysis is the most convenient (87,88,156,166-168). First developed in the 19th century, the electrochemical analysis applies electrochemical principles and techniques to a class of analytical methods that take advantage of the electrochemical properties of chemical cells in solution and their changes. It can be used for the detection of both organic and inorganic substances and is simple in operation. It can be both qualitative and quantitative, but is susceptible to interference by sodium,

potassium, phosphate and sulfate. It is suitable for real-time detection and experiments with low optical sensitivity requirements (132,169). In addition, FRET can also detect Ca^{2+}; cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) are the most widely used FRET pairs in protein-protein interaction studies. The emission spectrum of CFP is similar to that of YFP. The absorption spectra of CFP overlap and when the distance between the two proteins is in the range of 5-10 nm, the fluorescence emitted by CFP can be absorbed by YFP and YFP is excited to emit yellow fluorescence. Whether the two proteins interact was determined by measuring the loss of CFP fluorescence intensity. The closer the two proteins are, the more fluorescence emitted by CFP is received by YFP and the less fluorescence is received by the detector. CFP and YFP are fused to calmodulin and calmodulin-binding peptide, respectively and expressed in the same cell (170-175). When the intracellular Ca^{2+} concentration is high, the combination of calmodulin and the calmodulin-binding peptide can induce FRET and the receptor protein YFP emits yellow fluorescence, so the cells appear yellow. When the intracellular Ca^{2+} concentration is low, FRET hardly occurs, so CFP is excited and emits green fluorescence during detection and the cells appear green (170,171,175). FRET can detect intracellular Ca^{2+}, but cannot specifically detect mitochondrial Ca^{2+}. A number of fluorescent probes have recently been used for the measurement of Ca^{2+} levels, including Quin-2AM, fluo-3AM, indo-1AM, Rhod-2, Fluo-4, Mag-fluo-4 and calcium-rhodamine 123 (rhodamine 123) (158,176-178). Quin-2AM, fluo-3AM, indo-1AM, Fluo-4 are cytosolic Ca^{2+} indicators. Mag-fluo-4 is an ER Ca^{2+} indicator. The rhodamine 123 complex assay is suitable for the determination of mitochondrial Ca^{2+} concentrations in various living cells owing to its simple operation and stable performance. It can be quantified by fluorescence spectrophotometry to detect aggregation in mitochondria and thereby to measure the Ca^{2+} content (179-184). At present, Fluo-3 is a widely used typical single-wavelength fluorescent indicator with an excitation wavelength in the visible light range (185,186). The maximum absorption peak and maximum emission wavelength are located at 506 and 526 nm, respectively. The fluorescence intensity of Fluo-3 combined with Ca^{2+} is ~40 times higher than that of free cells, thus avoiding the fluorescence interference of the cells themselves (185,187). As a long-wavelength indicator, Fluo-3 can be used in confocal laser imaging studies that can analyze the distribution of Ca^{2+} in individual intact living cells and distinguish mitochondrial Ca^{2+} from Ca^{2+} in other organelles within the cell; this method is suitable for mitochondrial Ca^{2+} in various living cells and is easy to operate, stable in performance and highly specific (155,187). However, the current mitochondrial Ca^{2+} fluorescent probes cannot distinguish mitochondrial Ca^{2+} from different cells.

Detection of mitochondrial permeability transition pores. mPTP is a class of protein complexes between the inner and outer mitochondrial membranes that permit the passage of substances with a molecular weight of <1.5 kDa and serve as the structural basis for transitions in mitochondrial permeability (188-191). Additionally, mPTP is very sensitive to changes in intracellular and extracellular ion concentrations and serves an important role in signal transduction systems. It is currently hypothesized that the abnormal opening of mPTP is closely associated with abnormal changes in Ca^{2+} concentrations, oxidative stress and mitochondrial DNA (mtDNA) mutations (154,188,189,192,193). By contrast, MMP and mitochondrial Ca^{2+} concentrations are the principal drivers of mPTP opening, resulting in the release of cytochrome c and other substances associated with cell death into the cytosol (191,192,194-197). This leads to mitochondrial swelling and reduced mitochondrial respiratory chain activity, which can cause various diseases, such as neurodegenerative diseases and cancers (190,198-200). Furthermore, studies have shown that PINK1 can inhibit mPTP opening by downregulating intracellular ROS levels, suggesting that mitochondrial autophagy serves a regulatory role in mPTP opening (191-193). Various methods have been developed for detecting mPTP, such as the patch-clamp, spectrophotometric and active substance labeling methods. The patch-clamp method is the earliest, originating in 1976 and can reflect ion channel activity by recording ion channel currents to evaluate mitochondrial function (188,189,201). As the magnification of AFM is as high as 1 billion times, the opening of mPTP can be directly observed, which can serve a guiding role in the abnormal opening of mPTP (202-205). Fully automated patch-clamp techniques have recently emerged; these are simple in operation and have greatly improved efficiency but are only applicable to the detection of cells in suspension. Compared to the active substance labeling and patch-clamp methods, spectrophotometry is simpler and more commonly used.

The calcein-cobalt fluorescent probe technique is an emerging technique for the detection of mPTP and is simple in operation and highly sensitive (Fig. 2). Calcein-AM (190,194, 198,206,207), in which the acetylmethoxy methyl ester (AM) group enhances the hydrophobicity of the stain for easy penetration of the living cell membrane, is used to fluorescently label living cells. Next, calcein-AM is cleaved by intracellular esterases to yield highly fluorescent and polar calcein (208-210). When cells are incubated with calcein and Co^{2+}, both enter the cytoplasm; however, calcein is further captured by mitochondria (211,212). Calcein that accumulates in the mitochondria exhibits fluorescent staining, whereas calcein remaining in the cytoplasm or released from the mitochondria into the cytoplasm is rapidly quenched by Co^{2+} (213-219). Under normal physiological conditions, mPTP opens transiently and calcein that enters the cytoplasm from the mitochondria is rapidly quenched. In pathological states, such as calcium overload and oxidative stress, mPTP can appear to be continuously open and Co^{2+} in the cytoplasm can enter the mitochondria to quench the calcein fluorescence, resulting in a gradual decrease in fluorescence intensity in the mitochondria, thus indicating the degree of mPTP opening (195,196,220-222).

Determination of mitochondrial ATP. ATP is often considered the primary energy currency of cells and is primarily derived from the mitochondria (137,223-228). It serves major roles in material transport, energy conversion and information transfer. Mitochondria are sensitive to external environmental stimuli, such as hypoxia, oxidative stress, toxic substances and high glucose. Once mitochondria are damaged, ATP production decreases and free radical production increases, which

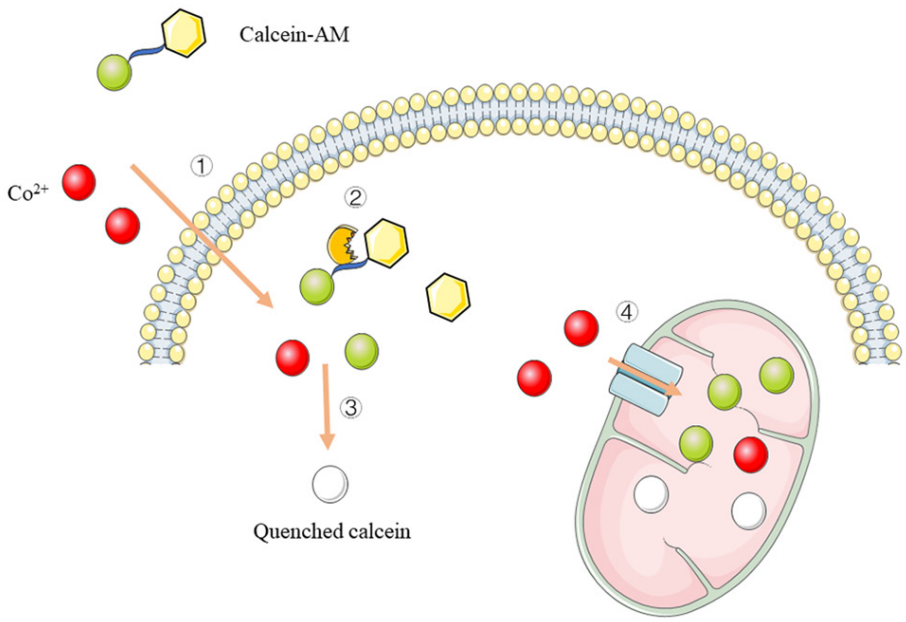

Figure 2. The working mechanism of calcein-AM probe when mPTP is abnormally opened: ① Calcein-AM and Co^{2+} enter the cell, ② Calcein-AM is then cleaved by intracellular esterase, ③ Calcein is quenched by Co^{2+} and ④ Co^{2+} quenches calcein through abnormally open mPTP. mPTP, mitochondrial membrane permeability transition pore.

affects a number of cellular processes and contributes to the development of a number of diseases, such as Parkinson's disease, cancer, cardiovascular disease and endocrine dysfunction (224-227). Therefore, ATP levels are a key indicator of the status of cellular energy metabolism and mitochondrial function.

Analyzing ATP levels requires freshly extracted mitochondria, as mitochondria must remain intact and in a coupled state (229). Several techniques are available to measure mitochondrial ATP levels, including chromatography, electrophoresis, high-performance liquid chromatography (HPLC) and enzymatic analysis (225,227,229-232). Chromatography and electrophoresis are chemical methods that were developed in the 18 and 19th centuries and have gradually improved. Classic liquid chromatography uses a large-diameter glass tube column and a difference in liquid levels at room temperature and atmospheric pressure to force the mobile phase (231,232). However, this technique has low column efficiency and is very time-consuming (often requiring several hours). HPLC was developed based on classic liquid chromatography following the introduction of gas chromatography theory in the late 1960s. The differences between HPLC and classic liquid chromatography include a faster analysis speed, smaller and more uniform particles as packing material and high column efficiency of the small particles. However, this causes high resistance and requires high pressure to force the mobile phase; therefore, this technique is also known as high-speed liquid chromatography (233-235). HPLC can be used to determine differences in cellular energy substances in different states, is easy to operate and has high sensitivity (233-236). The enzymatic method is based on spectrophotometry, where ADP production is assessed by measuring the absorbance of NAD^+ in phosphoenolpyruvate (237-240). Fluorescence analysis techniques have been improved in recent years and are commonly used to determine mitochondrial ATP synthesis activity (241-244). For example, in the luciferin-luciferase luminescence method, luciferin is rapidly oxidized under the action of luciferase, producing green fluorescence and the amount of luminescence is linearly correlated with the level of ATP (245,246). This is a fast and accurate method; however, fluorescein is an amphiphilic molecule whose carboxyl group is charged at physiological pH and thus does not easily cross the cell membrane (244-246). A novel synthetic fluorescent probe called Mito-Rh can specifically identify ATP in mitochondria with high sensitivity and a detection range of 0.1-10 mM. In another method, the level of ATP can be determined directly by measuring the amount of inorganic phosphate based on the principle that ATP gives rise to ADP and inorganic phosphate (225). In addition, FRET can also be used to detect the level of ATP synthesis after labeling the ATP synthase subunit. When CFP and YFP are labeled on ATP synthase subunits, when the ATP synthase activity is enhanced, the interaction between the subunits is enhanced, the shortened distance between the subunits brings CFP and YFP closer to each other and FRET occurs and CFP excites YFP to emit yellow fluorescence. The lower the green fluorescence intensity received by the detector, the higher the ATP synthase activity and the higher the ATP level. When ATP synthase activity is low, the interaction between subunits is weakened, FRET hardly occurs and CFP is excited at this time and the cell emits green light.

In addition, a multi-color ATP indicator has appeared in recent years. Different from the previous indicators that can only specifically detect intracellular ATP, the multi-color ATP indicator is based on a single fluorescent protein indicator with red, green and blue colors (247-249). Alternatively, it can simultaneously detect ATP in different organelles in the same cell and simultaneously detect ATP dynamics in the mitochondria of mammalian, plant and even worm cells and will have an assured role in promoting energy metabolism research in the future (225,226).

Detection of mitochondrial respiratory chain complexes. The mitochondrial respiratory chain, with functions in energy production, the regulation of cell death and calcium metabolism (183,250-253), is located on the inner mitochondrial membrane and consists of five complexes. Mitochondrial respiratory chain complex I (NADH oxidase) and mitochondrial respiratory chain complex II (succinate dehydrogenase) are the major elements for electrons entering the mitochondrial electron transport chain (ETC). Complex I oxidizes NADH and transfers electrons to coenzyme Q (254-257). Complex II transfers electrons from succinate to coenzyme Q, a process that does not involve proton transport (258-260). Mitochondrial respiratory chain complex III (cytochrome c reductase) is an essential protein for mitochondrial oxidative phosphorylation, the gatekeeper of the mitochondrial respiratory chain and a major source of third reactive oxygen species. Complex III transfers electrons from coenzyme Q to cytochrome c while using the released energy to pump protons into the intermembrane space. The mitochondrial respiratory chain complex IV (cytochrome c oxidase) is the terminal electron acceptor of the mitochondrial electron transport chain. Complex IV transfers electrons from cytochrome c to oxygen, half the number of protons is synthesized into water and the other half is pumped into the intermembrane space. Mitochondrial respiratory chain complex V and the above four complexes complete oxidative phosphorylation to generate ATP, which is called ATP synthase, also known as F1F0-ATPase (254,260-265). The energy released by complex V through the electron transport chain during respiration or photosynthesis is first converted into a transmembrane proton (H^+) gradient and the proton then flows along the proton gradient and passes through ATP synthase to enable ADP+Pi to synthesize ATP (266-269). It is also hypothesized that abnormalities in mitochondrial complexes are closely associated with mitochondrial encephalopathy, mitochondrial liver disease and mitochondrial nephropathy (265). It should be noted that the mitochondrial respiratory chain complex is closely related to the occurrence of tumors (251,270-272). Therefore, mitochondrial complex inhibitors may be used as a new treatment for tumors (252,253,260,273). Therefore, the accurate detection of mitochondrial complexes is essential and spectrophotometric assays remain the first-line technique for detecting the activity of mitochondrial respiratory chain complexes I-V (266,274,275).

Samples are generally selected from purified mitochondria and 4-40 μg of mitochondrial protein is required per respiratory chain complex assay (257,269,276-279). To compare the activity of mitochondrial respiratory chain complexes in different cells or tissues, the activity of citrate synthase in the Krebs cycle is measured simultaneously as a control and the reaction system is carried out at 30˚C in a volume of 200 μl or 1 ml. The activity of complexes I and V is directly proportional to, and can be determined by measuring, the oxidation rate of NADH, which is measured as the decrease in absorbance at 340 nm (280). In the oxidation of succinate catalyzed by complex II, 2,6-dichlorophenolindophenol (DCPIP) is used as a dye and absorbance at 600 nm decreases as DCPIP decreases (259,281,282), which is used to measure the activity of complex II (283-287). The activity of complexes III and IV can be determined by measuring cytochrome activity (absorbance at 550 nm) (268,288-294). However, the spectrophotometric method is susceptible to external biochemical interference that can lead to changes in enzyme kinetics (chemicals in the liquid or gas phase react with the sample resulting in a change in the absorbance of the sample), which can have serious effects on the sensitivity and accuracy of the assay (255,258,280,295-298). In addition, western blotting can directly reflect the expression level of respiratory chain complexes I-V in the band by using the specific antibody reaction of the complex, which has been widely used in experiments related to mitochondrial research (274,296,297). However, the protein expression level and protein activity are occasionally not correlated and spectrophotometry is still the preferred method for detecting mitochondrial respiratory chain complexes. In recent years, great progress has been made in the non-invasive measurement of mitochondrial complexes using near-infrared spectroscopy. This method is similar to spectrophotometry in principle but is less affected by the external environment (265-268). The fundamental reason why near-infrared light can achieve non-invasive optical measurement is that in the near-infrared light region of 600-900 nm, biological tissue is relatively transparent because the absorption of water and hemoglobin in this wavelength region is very small. As an 'optical window', some studies have used it to detect the activity of complex IV to judge the severity of depression. Myoglobin is essential for oxygen metabolism in muscle tissue, including a group of blood cells similar to hemoglobin. The most important of which is complex IV, which has been used to detect the activity of complex IV to judge the severity of depression (299,300). However, due to the large amount of samples required for near-infrared spectroscopy and different instrument models, it has severe limitations and has not been widely used (183,250-253).

Mitochondrial respiratory chain function can also be determined by RCR, which reflects both mitochondrial integrity and mitochondrial oxidative respiratory chain function (256,265,267,301).

Measurement of ROS. As the central organelle for cellular oxidative phosphorylation, mitochondria are the principal site of ROS production (3,302-305). Under physiological conditions, the intracellular antioxidant defense system is in equilibrium with oxygen radicals. The levels of intracellular ROS, including superoxide radicals, hydrogen peroxide and its downstream products (peroxides and hydroxyl radicals), are maintained at low physiological ranges. Under pathological conditions, the balance between the intracellular antioxidant system and oxygen radicals is disrupted. When intracellular ROS levels are too high, mitochondrial structure and function are impaired and cytochrome c is released through mPTP, resulting in damage to mitochondrial enzymes, lipids and nucleic acids as well as oxidative stress (303,306-310). ROS can also attack mitochondrial DNA (mtDNA) to produce oxidative damage, resulting in reduced mitochondrial ATP synthesis and MMP damage. Therefore, the functional status of mitochondria can be determined by measuring ROS levels (311-313).

Common methods for detecting ROS include the chemical reaction method, selective electrode method, spectrophotometry and direct detection by kits. ROS shows high reactivity and

can react with different compounds to produce various products, which can be analyzed quantitatively or qualitatively. The chemical reaction method is characterized by high sensitivity, low cost and simple operation; however, it has poor specificity and measurement results are easily affected by some redox reactions or enzyme-catalyzed reactions. Tetranitromethane, nitrotetrazolium blue chloride (NBT), cytochrome c, epinephrine and reduced coenzyme I are commonly used for spectrophotometric methods; these react with superoxide anion radicals to produce ferrous cytochromes with a specific absorbance (detectable at a wavelength of 550 nm), which can be used to directly measure ROS levels (307,314-317). The NBT assay is highly sensitive and is commonly used for the histochemical localization of oxygen radicals; however, it is difficult to measure dynamic changes in oxygen radicals in cells or aqueous systems. Cytochrome c has oxidative activity and can be used to detect the production of oxygen radicals. However, cytochrome c is easily reduced by other reducing agents and is therefore limited for the accurate localization of oxygen radicals. In the last decade, a number of ROS kits have been developed to detect intracellular or mitochondrial ROS (mtROS) levels directly. Intracellular ROS are usually measured using the fluorescent probe DCHF-DA, which is non-fluorescent and can freely cross the cell membrane. After DCHF-DA enters cells, it is hydrolyzed by intracellular esterases to generate DCHF, which cannot enter or exit the cell membrane, thus allowing the probe to easily label the cell. In the presence of ROS, DCHF is oxidized to produce the fluorescent substance DCF, whose fluorescence intensity is directly proportional to intracellular ROS levels. mtROS is usually measured using the fluorescent probe MitoSOX, which is highly specific to mitochondrial ROS and is characterized by simple operation, low background signals, wide linear range and high detection efficiency; however, it requires the immediate imaging of assay results and protection from light to prevent fluorescence quenching. Prior to the widespread use of kits, ROS levels were indirectly measured by detecting products of oxidative damage. Levels of malondialdehyde (MDA) reflect the degree of lipid peroxidation in the body and can be measured using the thiobarbituric acid (TBA) chemical colorimetric method. Condensation under acidic conditions generates the MDA-TBA complex, a red product with a maximum absorption peak at 535 nm, which can be used to indirectly determine the MDA content by spectrophotometry, indicating ROS levels. However, this technique has poor sensitivity and is prone to contamination. Fluorescent protein-based ROS detection methods are designed by combining fluorescent proteins and prokaryotic redox-sensitive proteins (318,319). The recombinant proteins are introduced into cells via plasmids or adenoviruses and target organelles to detect intracellular redox status (320,321). Redox-dependent fluorescence spectral changes of recombinant proteins are achieved through structural changes of disulfide bonds and part of the backbone under oxidative conditions (319,321).

Electron spin resonance (ESR) technology has emerged in recent years. Also known as electron paramagnetic resonance (EPR), its principle is similar to nuclear magnetic resonance (322-325). The sample is controlled in a fixed frequency microwave and the applied magnetic field is then changed so that the electron energy level difference is the same as the microwave energy (326,327). Unpaired electrons can move between the two energy levels and the net absorption energy of the microwave can be measured to obtain the ESR spectrum. Due to the high reactivity and short lifespan of ROS, the ESR signal is not easy to detect directly. The combination of ESR and spin traps can make up for this defect. The spin-electron trapping agent reacts with free radicals to generate relatively stable free radical addition products that are easily detected by ESR, which is then determined by ESR technology. This powerful and reliable technique can unambiguously measure the presence of free radicals in biological samples. ROS is the most direct and effective method for detecting free radicals and is widely used in physics, chemistry and biomedicine (328-331).

Detection of mtDNA. Human mitochondria carry a small circular double-stranded genome of 16569 bp known as mtDNA, which encodes mitochondrial 16S and 12S ribosomal RNA, 22 mitochondrial tRNA molecules and 13 respiratory chain proteins. Each organism contains only one type of mtDNA and mutations such as the conversion, inversion, insertion, or deletion of one or several bases of mtDNA, resulting in more than one type of mtDNA within an individual, are referred to as mtDNA heterogeneity (332-335). Owing to the lack of protective histones and effective DNA repair systems, the mutation frequency of mtDNA is ~10 times higher than that of nuclear DNA (336-339). Moreover, mutated mtDNA gradually accumulates and can cause irreversible damage to the nervous, cardiovascular, respiratory and reproductive systems after reaching a certain threshold (60-80%). In addition to these diseases, studies have also shown that mtDNA mutations are closely associated with the development of infertility (308,339-342). mtDNA dysfunction can be both quantitative (e.g., mtDNA copy number variation and deletions) and qualitative (e.g., strand breaks, point mutations and oxidative damage) (343-345).

mtDNA can be released from the cell as circulating free mitochondrial DNA (CCF-mtDNA) via extracellular vesicles (EVs) (346,347). CCF-mtDNA can serve as a damage-associated molecular pattern leading to the activation of inflammatory pathways, a process closely associated with TLR9. Numerous reports have shown that elevated levels of CCF-mtDNA are associated with various TLR9-dependent pathologies, such as rheumatoid arthritis, atherosclerosis, hypertension, acute liver injury and nonalcoholic steatohepatitis (48,348).

mtDNA damage can be detected using PCR, fluorescence *in situ* hybridization (FISH), DNA sequencing technology and the probe method, among others. The principle of DNA sequencing is to use DNA polymerase to extend the primers bound to the template of the undetermined sequence until a chain-terminating nucleotide is incorporated. Termination of replication and detection with isotopic labeling is the gold standard for detecting heterogeneity, but speed is limited when working on large-scale projects. The speed of large-scale projects was not guaranteed until the advent of high-throughput sequencing. PCR, as a molecular biology technology that emerged in the 1980s, is a method for enzymatically synthesizing and amplifying specific nucleic acid fragments *in vitro* based on the semi-conservative replication mechanism of DNA. This can purposefully amplify target regions and

is especially suitable for enriching small-scale genomes such as mtDNA (349-353). However, mtDNA is present in primer-binding regions, but accuracy is not sufficient due to heterogeneity. Over time, reverse transcription-quantitative (RT-q) PCR is able to monitor the number of amplified DNA molecules in real time, facilitating the determination of mtDNA in individual cells, along with the copy number and other impairments (deletions) (350-352). As a contemporaneous product of PCR, FISH is also a classic specific detection method. It uses fluorescently labeled specific nucleic acid probes to hybridize with corresponding target DNA or RNA molecules in cells. Fluorescent signaling with relatively poor specificity and insufficient hybridization compared to PCR is not the method of choice for the detection of mtDNA (149,354-362). Moreover, after the mitochondria are separated from cells or tissues, the DNA in the remaining material is extracted (kits can be used) and the DNA of the sample can be sequenced. qPCR or chromatin immunoprecipitation (ChIP) experimental methods can be used to detect the level of CCF-mtDNA, among which ChIP is often used to verify the binding of mtDNA to downstream signaling pathways, such as TLR9 inflammatory pathway or cGAS signaling pathway (335,363-371). As a DNA sensor in the cytoplasm, cGAS can recognize CCF-mtDNA and then catalyze the formation of the second messenger cGAMP (2'3'-cGAMP) to activate the interferon-stimulated gene-dependent signaling pathway. In addition, CCF-mtDNA containing unmethylated DNA (CpG DNA) fragments can be recognized by TLR9, causing TLR9 dimerization and activation of MyD88-mediated inflammatory pathway.

Unrepaired depurinated/depyrimidinated sites (AP sites) in mtDNA lead to the misbinding of nucleotides, which can have serious downstream effects (372-374). Therefore, the rapid and accurate quantification of AP sites in mtDNA is crucial for the real-time assessment of mtDNA oxidative damage. Researchers have used a specific fluorescent probe (BTBM-CN2) for the real-time detection of mtDNA (375-378). At ~20 sec after contact with AP sites, red fluorescence is detectable at 598 nm and after ~100 sec, green fluorescence is detectable at 480 nm. More AP sites result in green fluorescence with greater intensity and duration and the degree of mtDNA damage can be quantified based on the time of appearance and intensity of fluorescence at 480 nm. Doxorubicin (Dox), a common anticancer drug, not only causes damage to the nuclear DNA of cells but can also be rapidly inserted into the mtDNA of living cells, causing the aggregation of mtDNA nucleoids and changing the distribution of nuclear proteins (375-382). Therefore, after Dox induces mtDNA damage, morphological changes of mtDNA can be tracked in real time using the two-photon fluorescent probe CNQ, which emits red fluorescence and is localized to mtDNA. When incubated with Dox, dynamic changes in mtDNA can be observed, providing a new method for studying mtDNA damage in real time (383,384).

5. Treatment of mitochondrial diseases

In addition to primary mitochondrial disease caused by mtDNA damage, mitochondrial dysfunction occurs in a number of infectious and non-infectious diseases (262,385,386), such as inflammation, neurodegeneration, diabetes, obesity and cardiovascular disease and several therapies targeting mitochondria have been developed (Table II). Mitochondrial transplantation and mitochondrial replacement can fundamentally address the inadequate energy supply in pathological states and have been applied in clinical settings for the treatment of pediatric congenital heart disease (385).

Leber hereditary optic neuropathy (LHON), the most common primary mitochondrial disease, is a maternally-inherited bilateral-blinding optic neuropathy mainly caused by mtDNA mutations, including m.3460G>A (MT-ND1), m.11778G>A (MT-ND4) and m.14484T>C (MT-ND6), of which m.11778G>A is the most common mutation (387,388). These mutations can affect the mitochondrial respiratory chain complex I of retinal ganglion cells, impair mitochondrial function and increase the production of reactive oxygen species, leading to apoptosis and optic nerve degeneration and atrophy, which further leads to rapidly progressive loss of binocular vision (389-391). Treatment of LHON is mostly based on ectopic expression, that is, intravitreal injection of adeno-associated viral vectors with mitochondrial targeting sequences and then guiding the translated protein into mitochondria to restore mitochondrial function, which has been successfully and safely applied to cell models. Transplant into an inducible LHON animal model that preserves retinal ganglion cells and visual function (392,393).

The mitochondrial diseases associated with mtDNA deletion mainly include chronic progressive external ophthalmoplegia (CPEO), Kearns-Sayre syndrome (KSS) and Pearson syndrome. CPEO is mostly associated with m.3243A>G(MT-TL1) deletion, which manifests as progressive paralysis of the ocular muscles, resulting in ocular movement disorders and ptosis, which usually appear in late childhood or early adulthood (394,395). KSS is a more severe syndrome than CPEO and is mostly associated with m.8993T>G (APT6) deletion. Its main clinical manifestations are progressive external ophthalmoplegia and retinitis pigmentosa, usually occurring before the age of 20 (396-399). Other symptoms may include mild skeletal muscle weakness, hearing loss, cognitive impaired cognitive function and diabetes. Pearson's syndrome is a syndrome caused by sideroblastic anemia and pancreatic exocrine insufficiency. There are very few cases (~100 cases worldwide) that may be related to the deletion of ATPase 6 and 8. Most patients die during infancy; however, a minority of patients who survive into adulthood tend to develop symptoms of KSS syndrome. Due to the double-membrane structure of mitochondria and the inability of foreign nucleic acids to recombine on endogenous mtDNA (168,400,401), there is currently no effective method to directly import nucleic acids into mitochondria and the localization of proteins to mitochondria is a routine practice in the treatment of mitochondrial diseases. In principle, expression of mitochondrial-targeted DNases that specifically recognize mutated sequences can remove mutated mtDNA, or at least reduce its abundance in a heterogeneous background. Restriction endonucleases, zinc finger nucleases and transcription activator-like effector nucleases have been tested and proven effective; these specific enzymes can be used to eliminate aberrant mtDNA and thereby reduce the rate of aberrant mtDNA in cells (402-406).

In addition, mitochondrial neurogastrointestinal encephalomyopathy, a rare mitochondrial disease, is often associated with TYMP gene mutations, manifesting as

Table II. Treatment of mitochondrial diseases.

Author, year	Mitochondrial diseases	Treatment method	Representative intervention	Mechanism	Effect on mitochondria	Application status	(Refs.)
Feng *et al*, 2019	Primary mitochondrial disease	Edit mtDNA	AAV, CRISPR-Cas9	Reduce mtDNA damage	Protection	Pre-clinical	(379-415)
Dabravolski *et al*, 2022							
Hamel *et al*, 2021							
Karshovska *et al*, 2020							
Gao *et al*, 2019							
Grady *et al*, 2018							
Bozi *et al*, 2020							
Jing *et al*, 2019							
Amore *et al*, 2021							
Chen and Bhatti, 2021							
Mejia-Vergara *et al*, 2020							
Newman *et al*, 2021							
Stenton *et al*, 2021							
Wang *et al*, 2021							
Yu-Wai-Man *et al*, 2020							
Heighton *et al*, 2019							
Wu *et al*, 2019							
Del Monte *et al*, 2021							
Di Mambro *et al*, 2021							
Di Nora *et al*, 2019							
Nguyen *et al*, 2019							
Ashton *et al*, 2018							
Bonora *et al*, 2019							
Ni *et al*, 2018			MSC-EVs				
Porporato *et al*, 2018							
Qi *et al*, 2019							
Ramachandra *et al*, 2020							
Soukas *et al*, 2019							
Bonora *et al*, 2021							
D'Angelo *et al*, 2020							
Hirano *et al*, 2021							
Kripps *et al*, 2020							
Parés *et al*, 2021							
Jackson *et al*, 2020							

Table II. Continued.

Author, year	Mitochondrial diseases	Treatment method	Representative intervention	Mechanism	Effect on mitochondria	Application status	(Refs.)
Jiang and Shen, 2022 Mok *et al*, 2020 Ng *et al*, 2021 Fang *et al*, 2019 Gong *et al*, 2021 González *et al*, 2021 Gu *et al*, 2017							
Feng *et al*, 2019	Pediatric congenital heart disease	Mitochondrial renewal	Mitochondrial transplantation	Mitochondrial numbers	Protection	Clinical evaluation	(379)
Feng *et al*, 2019			Mitochondrial replacement				
Li *et al*, 2017 Bhatti *et al*, 2017	Metabolic disease, neurodegenerative disorder		Vitamin E				(55,168, 417-419)
Li *et al*, 2017 Bhatti *et al*, 2017			Ubiquinone				
Li *et al*, 2017 Bhatti *et al*, 2017			N-acetylcysteine	Oxidative stress	Protection	Have been approved	
Li *et al*, 2017 Bhatti *et al*, 2017			Glutathione				
Li *et al*, 2017 Bhatti *et al*, 2017			Melatonin				
Gong *et al*, 2021 González *et al*, 2021 Gu *et al*, 2017 He *et al*, 2019		Drugs	Tetracyclines, Actinomycins				
Gong *et al*, 2021 González *et al*, 2021 Gu *et al*, 2017			Creatine, Ursodeoxycholic acid				
Russell *et al*, 2020 Saeb-Parsy *et al*, 2021 Kelly and Pearce, 2020 Rahman and Rahman, 2018 Tabish and Narayan, 2021 Yuan *et al*, 2021 Ballarò *et al*, 2021	Heart and kidney disease, sepsis, diabetes		SS-31	Remove reactive oxygen species, protect and restore mitochondrial structure	Protection	Clinical evaluation	(437-451)
Bhatti *et al*, 2021			mitoTEMPO				

Table II. Continued.

Author, year	Mitochondrial diseases	Treatment method	Representative intervention	Mechanism	Effect on mitochondria	Application status	(Refs.)
Deng *et al*, 2021 Le Gal *et al*, 2021 Bhatti *et al*, 2021 Grosser *et al*, 2021 He *et al*, 2022 He *et al*, 2021 He *et al*, 2021						Pre-clinical	
Labarta *et al*, 2019 Wu *et al*, 2019 Del Monte *et al*, 2021 Nguyen *et al*, 2019	ATP deficiency		Resveratrol	Mitochondrial biogenesis	Protection	Have been approved	(54,395, 396,399, 400,435, 465-471)
Ashton *et al*, 2018 Roth *et al*, 2020			AICAR			Pre-clinical	
Del Monte *et al*, 2021 Nguyen *et al*, 2019 Gabandé-Rodríguez *et al*, 2019 Cho *et al*, 2020 Liu *et al*, 2021			Epicatechin			Have been approved	
Deng *et al*, 2020 Gao *et al*, 2020 Andrieux *et al*, 2021 Zeng *et al*, 2021			RTA-408			Pre-clinical	
Heighton *et al*, 2019 Wu *et al*, 2019 Del Monte *et al*, 2021 Di Mambro *et al*, 2021 Di Nora *et al*, 2019 Nguyen *et al*, 2019 Ashton *et al*, 2018	Cancers	Nanomaterials	TPP	Mitochondrial membrane potential	Protection	Pre-clinical	(394-400, 420-436)
He *et al*, 2019 He *et al*, 2020 He *et al*, 2020 Zhao *et al*, 2021 Macdonald *et al*, 2018 Tan *et al*, 2013			MPPs				

Table II. Continued.

Author, year	Mitochondrial diseases	Treatment method	Representative intervention	Mechanism	Effect on mitochondria	Application status	(Refs.)
Lee *et al*, 2019							
Wallace, 2018							
Strobbe and Campanella, 2018							
Wang *et al*, 2018							
Kim *et al*, 2017			Graphene				
Lleonart *et al*, 2017							
Tian *et al*, 2021							
Kim *et al*, 2017							
Chen *et al*, 2017							
Jung *et al*, 2017							
Roth *et al*, 2020							
Nash *et al*, 2021							

mtDNA, mitochondrial DNA; MSC-EVs, mesenchymal stem cell-derived extracellular vesicles; TPP, triphenylphosphine; MMP, mitochondrial membrane potential.

splanchnic neuropathy and marked motor impairment, often combined with CPEO, sensorimotor polyneuropathy and white matter encephalopathy (407-409). With advances in gene editing technology, CRISPR/Cas9 has been proposed for the treatment of mitochondrial diseases, aiming to eliminate abnormal mtDNA sequences through the principles of bacterial immunology (410,411).

To treat primary mitochondrial diseases, gene therapy based on ectopic expression is still the first choice; however, the application of viral vectors in live animals to correct any gene mutation still has the following significant problems: High cost (390,412-415), carcinogenicity and immunogenicity. Non-viral vector-mediated *in situ* mitochondrial gene therapy may be a promising approach to overcome the bottleneck of existing gene therapy LHON, such as liposome-based nanoparticles, which require further investigation (416-421).

Mesenchymal stem cell-derived EVs are a promising nanotherapeutic strategy to effectively attenuate mitochondrial damage and the inflammatory response by promoting mitochondrial transcription factor A expression and preventing mtDNA damage and leakage from target cells (422).

Oxidative stress caused by mitochondrial dysfunction is one of the etiologies of metabolic disease and is a potential target for the treatment of metabolic and neurodegenerative disorders (55,168,423-426). A number of antioxidants, such as vitamin E, ubiquinone, *N*-acetylcysteine, glutathione and melatonin, can effectively scavenge mitochondrial ROS and regulate redox processes, thus alleviating or curing disease. Antibiotics (e.g., tetracyclines and actinomycins), drugs (e.g., creatine and ursodeoxycholic acid) and exercise can significantly improve oxidative stress and balance mitochondrial fission and fusion, thus increasing the number of mitochondria, contributing to the treatment of cancer (400-406,426-442). SS31 and mitoTEMPO are novel mitochondrial-targeted antioxidants that have a scavenging effect on ROS (443-446). In addition, SS31 accumulates in the mitochondrial membrane to protect and restore the mitochondrial structure without affecting healthy mitochondria (162,447-453). Thus, SS31 and mitoTEMPO have protective effects on a variety of diseases, including heart and kidney-related diseases, as well as sepsis and diabetes, which have been demonstrated in a variety of animal models (454-457). The use of nanomaterials for mitochondrial targeting therapy has become a recent focus of research. Nanomaterials are materials with at least one of three spatial dimensions at the nanometer scale (1-100 nm). They are a new generation of materials composed of nanoparticles with sizes between atoms, molecules and macroscopic systems and are widely used in the medical field owing to their large specific surface area and excellent biocompatibility. Ideally, medical nanomaterials should remain quiescent in normal tissues but accumulate precisely and act in mitochondria under pathophysiological conditions (404,458,459). Delocalized lipophilic cations (DLCs), such as triphenylphosphine (TPP) and mitochondria-penetrating peptides (MPPs), serve a major role in mitochondria-targeted therapies. DLCs can accumulate specifically in the mitochondria of tumor cells and increase their MMP, leading to altered mitochondrial membrane permeability and inducing apoptosis (56,130,400,403,428,458-470). Studies have shown that

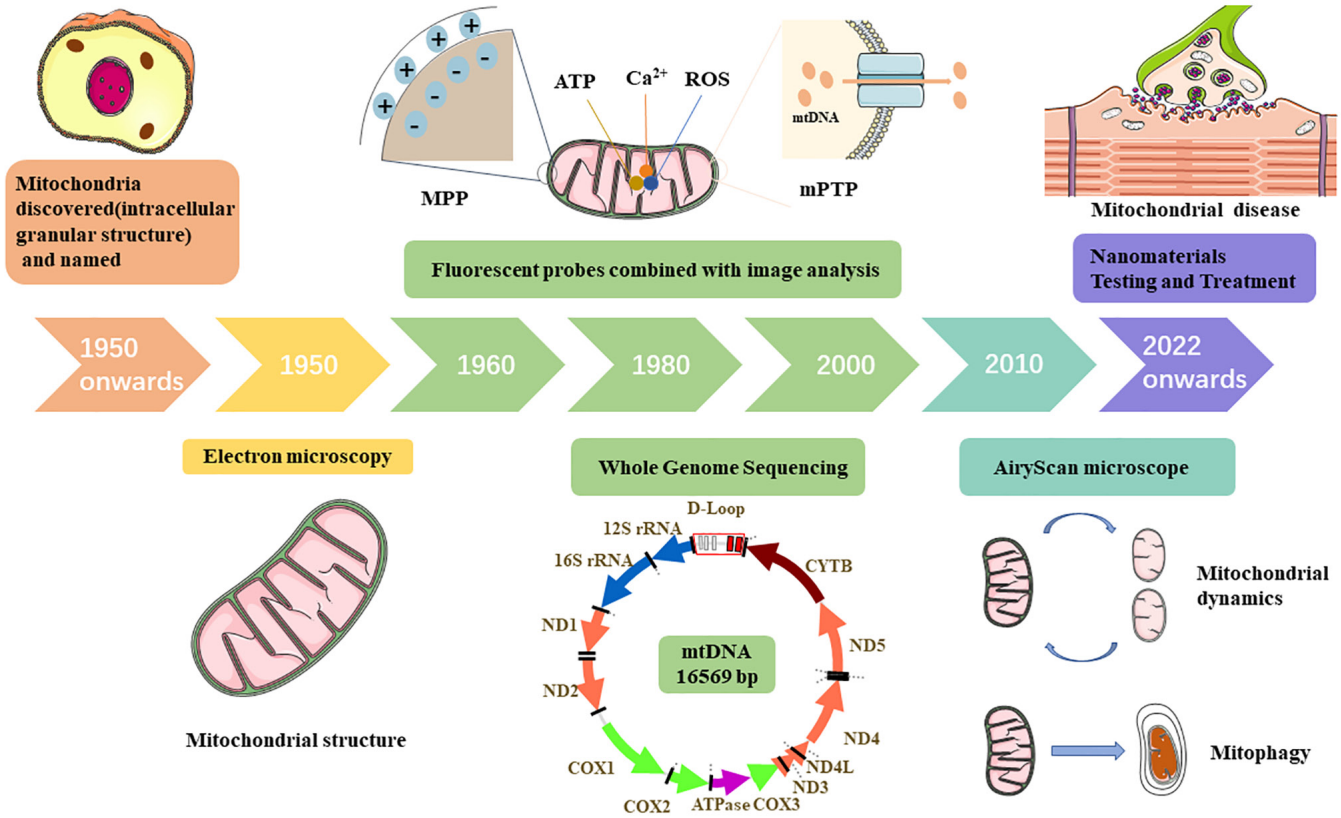

Figure 3. The development of mitochondrial research methods. MPP, mitochondria-penetrating peptides; mPTP, mitochondrial membrane permeability transition pore; ROS, reactive oxygen species.

graphene has a large specific surface area, good targeting and high biocompatibility, making it a promising nanodelivery system (441,471-473). Mitochondrial biogenesis is driven by PCG-1α, which can increase the number of mitochondria in the cell and thus meet the evolving energy demands of the cell, alleviating ATP deficiency in patients with mitochondrial diseases. Promoting mitochondrial biogenesis is also an important component of mitochondrial therapeutics (474). Resveratrol, 5-aminoimidazole-4-carboxamide riboside, epicatechin and RTA-408 have significant pro-mitochondrial biogenesis effects; the treatment of mice with these drugs enhances the expression of mitochondrial electron transport chain proteins and mitochondrial transcription factors and increases the abundance of mitochondrial cristae (54,401,402,405,406,441, 471-478).

6. Summary and outlook

As the powerhouses of the cell, mitochondria are at the center of cellular oxidative phosphorylation and are critical for growth and development as well as the development of a number of diseases. Mitochondrial abnormalities can cause disturbances in the intracellular environment and can lead to a variety of diseases, such as mitochondrial heart disease, mitochondrial encephalopathy, mitochondrial myopathy and even various pathologies of the reproductive and respiratory systems. Therefore, the accurate detection of mitochondrial abnormalities is essential for clinical guidance.

Since the beginning of the last century, a number of methods for mitochondrial research have been developed (Fig. 3), from the discovery of mitochondria as intracellular granular structures to the observation of mitochondrial microstructures via EM and the use of fluorescent probes to detect physiological indicators within mitochondria. The application of these methods has provided theoretical foundations for the detection and treatment of mitochondrial diseases. Accordingly, the treatment of mitochondrial diseases has gradually evolved from drug-based therapy to multidisciplinary combination therapies, such as the use of nanomaterials to precisely transport therapeutic drugs into mitochondria for targeted drug delivery, substantially improving therapeutic efficiency. However, the methods by which therapeutic efficacy is achieved still warrant investigation. The combined application of biomedicine and material science may be a promising means of detection and treatment. Notably, the specific molecular mechanism underlying the pathogenesis of the mitochondrial disease remains unclear. Current monitoring and treatment strategies cannot completely cure mitochondrial disease but only alleviate symptoms or slow disease progression. Therefore, methods for detection and treatment that are specific to the molecular mechanisms are needed. Using multi-omics and artificial intelligence, artificial mitochondrial models can be established through molecular co-assembly technology and mitochondria-targeted drugs can be screened to conduct in-depth discussions on abnormal mitochondria, which may elucidate the pathogenesis of mitochondrial diseases at the molecular level and provide new treatments for mitochondrial diseases.

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Availability of data and materials

Data sharing is not applicable to this article, as no data sets were generated or analyzed during the current study.

Authors' contributions

YY wrote the first draft of this review. HS provided valuable comments on this first draft. Both authors read and approved the final manuscript.

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Patient consent for publication

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Competing interests

The authors declare that they have no competing interests.

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