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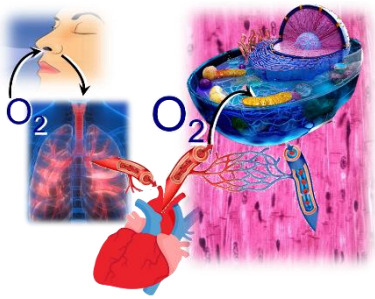
Introductory



Conflicts of interest
EG is an editor of the BEC Educational Series *Elements of mitochondrial physiology and bioenergetics* and had no influence on the review process.

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Mitochondrial respiratory function in living cells

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Summary

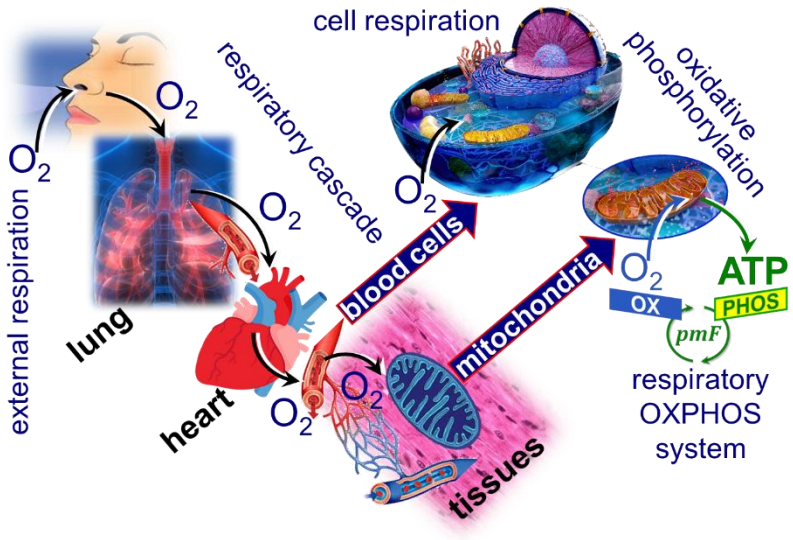
Breathing happens subconsciously, but every breath sets off a vital journey. Oxygen (O_2) enters through the nose and lungs, travels with the bloodstream, and reaches the brain, muscles, and every cell in the body. Deep within these tiny cells, oxygen kindles the fire of life in the mitochondria — microscopic structures comparable to bacteria. This pathway of oxygen links breathing (external respiration) to cell (internal) respiration. In the mitochondria, the energy of nutrients is converted into heat and a form of energy available for work. Mitochondria are electrochemical machines that consume oxygen and produce adenosine triphosphate (ATP), the cell's biochemical energy currency. Measuring cell respiration helps assess mitochondrial bioenergetic function to improve human performance, detect potential defects, and guide medical professionals in preserving their patient's aerobic capacity and vitality.

The following concepts on cell respiration are explained:

- Cellular routine respiration: controlled by the physiology of the living cell.
- Oxidative capacity: measured as maximal oxygen consumption decoupled from ATP production, in contrast to OXPHOS capacity — the capacity of oxidative phosphorylation.
- Leak respiration: idle respiration measured after inhibition of ATP production.
- Residual oxygen consumption: a small part of oxygen consumption that remains after fully inhibiting the oxidative capacity of the mitochondria.

Measurement of cell respiration in these experimentally controlled states and calculation of their relationships provides diagnostic information on mitochondrial fitness.

Introduction: Step up a flight of stairs and notice how your ventilatory rate increases. With each breath, you draw air into your lungs, inhaling oxygen (O_2) and exhaling carbon dioxide (CO_2). But why is breathing essential for survival? And what happens to O_2 once it enters the bloodstream?



O_2 makes up ~20 % of water vapour-saturated air we breathe. During external respiration, O_2 gas flows into the lungs, where it dissolves in the bloodstream and binds to hemoglobin in red blood cells. The heart then pumps oxygen-rich blood to tissues down the respiratory cascade. Microcirculation distributes O_2 molecules to individual cells. Oxygen diffuses into the cells when intracellular O_2 levels are lower than those in the blood.

Many cellular reactions consume oxygen, yet the most important oxidative reactions converting O_2 into water (H_2O) take place in the mitochondria. This fact explains why the O_2 concentration inside the cell is low. Cell respiration depends on external respiration and the continuous supply of O_2 through the respiratory cascade. If oxygen delivery stops, intracellular O_2 levels drop to zero, and mitochondria can no longer function. Conversely, external respiration alone cannot sustain life when mitochondria are damaged or mitochondrial content declines.

This contribution to the BEC Educational Series introduces cell respiration: Section 1 explains cell respiration studied under experimentally controlled conditions to determine respiratory rates in defined respiratory states. Section 2 illustrates the concept of cell respiration with an experimental example. Analysis of oxygen consumption rates helps us to learn more about specific functions of mitochondria in the cell.

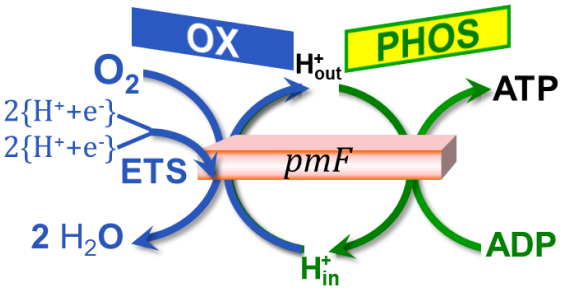
1. Cell respiration

Cell respiration powers life by transforming energy, and as a consequence makes it a cornerstone of bioenergetics. Energy is required in the cell to produce ATP in aerobic (oxygen-dependent) and anaerobic (oxygen-independent) processes. In

aerobic cell respiration, oxygen is essential for maintaining the 'fire of life' during the combustion of substrates used as fuel substrates. Cell respiration can be measured as oxygen consumption. In contrast, fermentation proceeds anaerobically without the involvement of oxygen. Glycolytic fermentation is studied by analysis of catabolic end products, such as ethanol in yeast or lactate in most animal cells. Catabolism is the breakdown of nutrients to smaller metabolites that are either discharged as waste products or utilized as building blocks required for biosynthesis (anabolism) and growth. Mitochondria are a metabolic hub connecting catabolism and anabolism.

Oxygen transported into the cell burns (oxidizes) fuel substrates derived from carbohydrates, fat, and protein. In the process of oxidation, the reduced fuel substrates (carbon molecules rich in hydrogen, H, such as pyruvate, $C_3H_4O_3$) transfer hydrogen ions (H^+) and electrons (e^-) to O_2 through a complex series of electron transfer reactions, stripping off all hydrogens $\{H^+ + e^-\}$ from the reduced carbon molecules and culminating in the formation of carbon dioxide (CO_2) and water (H_2O). This H^+-linked electron transfer gives the name to the electron transfer system (ETS) located in the mitochondria.

A primary role of mitochondrial (mt) respiration is oxidative phosphorylation (OXPHOS) — an electrically driven biochemical process that generates ATP, the cell's main energy currency. The driver of ATP production is the protonmotive force (pmF), which remains an enigmatic concept even for many specialists in bioenergetics [5]. In OXPHOS, the term *phosphorylation* (PHOS) relates to the binding of phosphate to adenosine diphosphate (ADP, with two phosphate groups), which yields adenosine triphosphate (ATP, with three phosphate groups). Before discussing PHOS further, some details are elaborated on *oxidation* (OX) and the associated process of *reduction*.



Mitochondria communicate through two membrane barriers — the inner and outer mt-membranes — with the other cellular compartments. Fuel substrates are transported into the mitochondria to be oxidized, acting as donors of $\{H^+ + e^-\}$. O_2 is reduced as the acceptor of $\{H^+ + e^-\}$. The chemical force from $\{H^+ + e^-\}$ donors to O_2 drives oxygen consumption, which generates the protonmotive force (pmF) by pumping protons (H^+) from the inner compartment of mitochondria (matrix space) across the mt-inner membrane. This can be visualized as charging the mitochondrial battery. Yet, the pmF is not only made up of an electric potential across the mt-inner membrane; it also includes a diffusive component, which arises from the pH difference between the two sides of the membrane [5].

The pmF drives ATP synthesis by pushing protons back into the matrix space through a molecular electrochemical generator localized in the mt-inner membrane. This molecular rotor is known as the ATP synthase. It can be compared with a wind

wheel or turbine that transforms kinetic into electric energy. ATP synthase converts the protonmotive energy of the mitochondrial battery into chemical energy in the form of ATP. ATP is essential for supporting cellular functions, sustaining health, and enabling growth or even controlled cell death. The coupled energy transformation in OXPHOS, however, can be decoupled by a short-circuit of the mitochondrial battery when the entire energy is wasted, when the capacity to perform work is lost. Pharmacological uncouplers increase the combustion of energy stores, and if you are overfed, help you lose excessive body mass, but they may exert detrimental effects on your health. Cell respiration is studied in different coupling states, which are experimentally controlled *in vitro* but must not be applied to living organisms for ethical reasons.

Notes on cell respiration

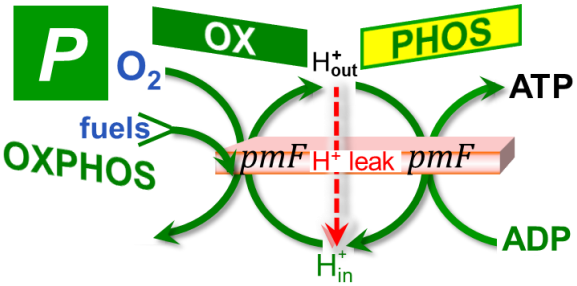
- Respiration and fermentation are distinguished as aerobic and anaerobic energy metabolism, respectively.
- In aerobic cell respiration, ADP/ATP and redox balance are maintained by oxygen as a H^+ and e^- acceptor.
- Fuel substrates are electron donors providing electrons e^- and hydrogen ions H^+. Oxygen becomes reduced and water is formed, $2\{H^+ + e^-\} + 0.5\ O_2 \rightarrow H_2O$.
- In OXPHOS, oxidation (OX) generates the protonmotive force (pmF) by pumping protons (H^+) across the mitochondrial inner membrane. Phosphorylation (PHOS) is the ATP production driven by the proton current downhill along the pmF.
- Protons H^+ play dual roles in coupling of oxidative phosphorylation, (*1*) as redox equivalents $\{H^+ + e^-\}$ in electron *transfer* during chemical oxidation [3], and (*2*) as charge mediators in compartmental proton *transport* by building up the protonmotive force [5].

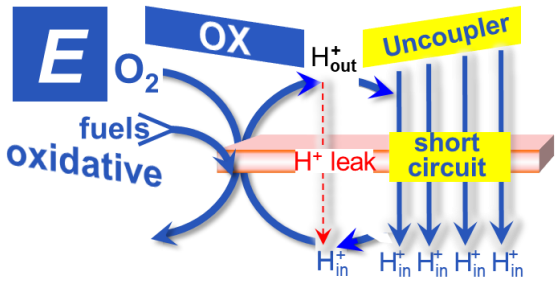
1.1. Oxidative capacity (electron transfer capacity)

Have you ever pushed yourself to maximal aerobic performance at a workout? You can do this on a treadmill or stationary bicycle by stepwise increasing the workload – either by running faster or adding resistance – until you max out. Work output is measured and controlled by an ergometer. The term *erg* comes from the Greek word for *work*, and work per unit of time is power. During such an ergometric test, your external respiration is monitored while breathing into a face mask to measure your maximum oxygen consumption (V_{O2max}). This combination of exercise and respirometric measurement is called *spiroergometry*.

Now, imagine putting living cells through a similar test — pushing them to their limits of maximal aerobic metabolic power. As they work harder, ATP would be broken down into ADP and inorganic phosphate at an increasing rate. To sustain activity, ADP is pushed ‘up’ to ATP by phosphorylation (PHOS), driven by the protonmotive force which, in turn, is depleted in an energetic ‘downwards’ direction. But to keep you running, the pmF must be continuously restored by recycling H^+ back ‘up’. This requires

an increasing rate of oxygen and fuel consumption (OX), pushing the system to its maximum OXPHOS capacity (*P*). Unfortunately, no such 'cell ergometer' exists. So how can we push cells to their limits in a different way?





The oxidative capacity *E* — in contrast to OXPHOS capacity *P* — of living cell is assessed by removing any control of PHOS, effectively short-circuiting the proton current by chemical uncouplers which collapse the protonmotive force, bypassing ATP synthesis by a short-circuit (decoupling), and forcing cells to respire at their maximal rate under the prevailing experimental conditions. Oxidative capacity (*E*) refers to the maximum oxygen consumption rate of cells when oxidative processes are decoupled from and, therefore, not limited by ATP generation. While code switching between the terms *oxidative capacity* and *electron transfer capacity* adds to the number of equivalent expressions, it may explain in a nutshell the nature of H^+-linked electron transfer. During oxidation, reducing equivalents $\{H^+ + e^-\}$ are transferred from fuel substrates to oxygen.

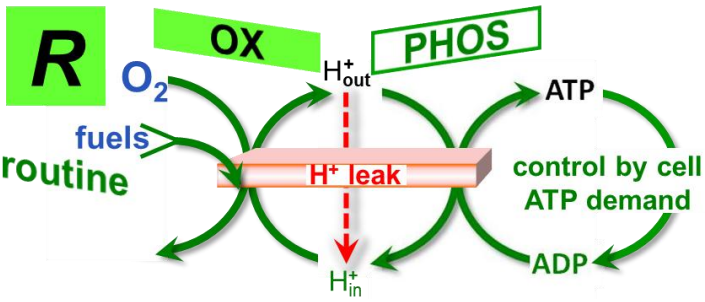
In several cell types, oxidative capacity (*E*) closely estimates mitochondrial work capacity, also known as OXPHOS capacity (*P*). However, oxidative capacity overestimates OXPHOS capacity in many other cases. Importantly, defects of the phosphorylation system responsible for ATP production compromise the ability to generate ATP, limiting *P* to a level which may be lower or even half of *E* in such conditions [1;2].

Notes on *E*

- Oxidative capacity (*E*) is obtained under non-physiological conditions when respiration is decoupled from ATP production.
- *E* is rarely, if ever, reached under physiological conditions in the cell.
- *E* overestimates OXPHOS capacity (*P*) and respiratory reserve of various cell types.
- Maximal respiration is an ambiguous term. Without further specification of the coupling state, it may refer to OXPHOS capacity, comparable to V_{O2max} measured as maximum aerobic capacity of external respiration in spiroergometry or V_{max} in enzyme kinetics. Oxidative capacity measured under given experimental conditions may be increased further by addition of external substrates or removal of inhibitory effects.
- A concept-driven terminology of mitochondrial respiratory states [7] is extended to respiratory physiology of living cells [2].

- The electron transfer system (ETS) is frequently referred to as the electron transport chain, obscuring the fundamental distinction between chemical *transfer* and compartmental *transport*. Is the ETS a chain? What is a chain?

1.2. Routine respiration

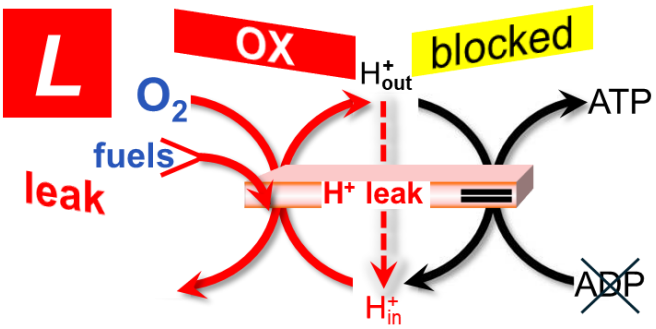


Routine respiration (*R*) is the oxygen consumption rate of living cells that meet their aerobic energy demands under physiological control. Routine respiratory activity varies depending on the nutrients available for energy conversion, the state of health, and is influenced by mitochondrial function. Increased ATP demand activates routine respiration if not compensated for by upregulated glycolytic (anaerobic) ATP production.

Notes on *R*

- Routine respiration is a bioenergetic parameter of living cells and cannot be measured in cells with permeabilized plasma membranes or isolated mitochondria.
- Routine respiration may differ in cells studied in respiration media with varying external substrates and ionic compositions.
- Basal respiration is ambiguous, since it is also used for leak respiration of isolated mitochondria and must not be confused with basal metabolic rate defined in organismic physiology.

1.3. Leak respiration

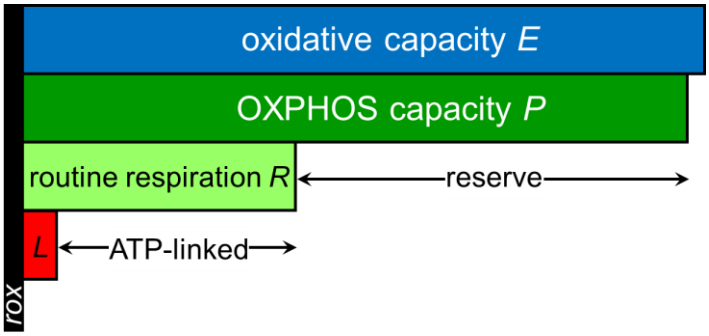


Leak respiration (*L*) is the mitochondrial oxygen consumption caused by a 'leak' in the mitochondrial inner membrane (H^+ leak). Leak respiration of living cells is measured after ATP production has been blocked. Instead of performing chemical work, idling mitochondria release energy in the form of heat which compromises efficiency [2]. Heat dissipation, however, is associated with oxygen consumption in any respiratory state and mainly regulated by respiratory rate. Leak respiration can modulate the protonmotive force and be diagnostically relevant as mitochondrial dysfunction.

Notes on *L*

- Proton leak must be distinguished from leak respiration since leak O_2 consumption compensates for but is not equivalent to proton current [7].
- Resting respiration is ambiguous since resting respiration in organisms supports ATP demand of body functions and low-effort activities beyond basal respiration.
- If leak respiration is taken as 'basal' in mt-preparations, then — to be consistent — leak respiration but not routine respiration would be 'basal' in living cells.
- The term State 4o or State 4Omy is traced back to the classical State 4 of isolated mitochondria. State 4 (a leak state) and State 2 are frequently confused [7]. Do not worry about these terms if you are not exposed to classical professional journals.

1.4. Residual oxygen consumption



Residual oxygen consumption (*rox*) is the cellular or mitochondrial oxygen consumption which remains after inhibition of respiratory enzymes and thus elimination of mitochondrial oxidative capacity. Mitochondrial respiration is corrected by subtraction of *rox* from total oxygen consumption. In this sense mitochondrial respiration is distinguished from cell respiration. The *rox* correction exerts the largest relative effect on leak respiration and becomes less prominent for routine respiration and oxidative capacity. Although *rox* is clearly set apart from production of reactive oxygen species (ROS), *rox* may be associated with ROS production, but a functional interpretation of *rox* is difficult.

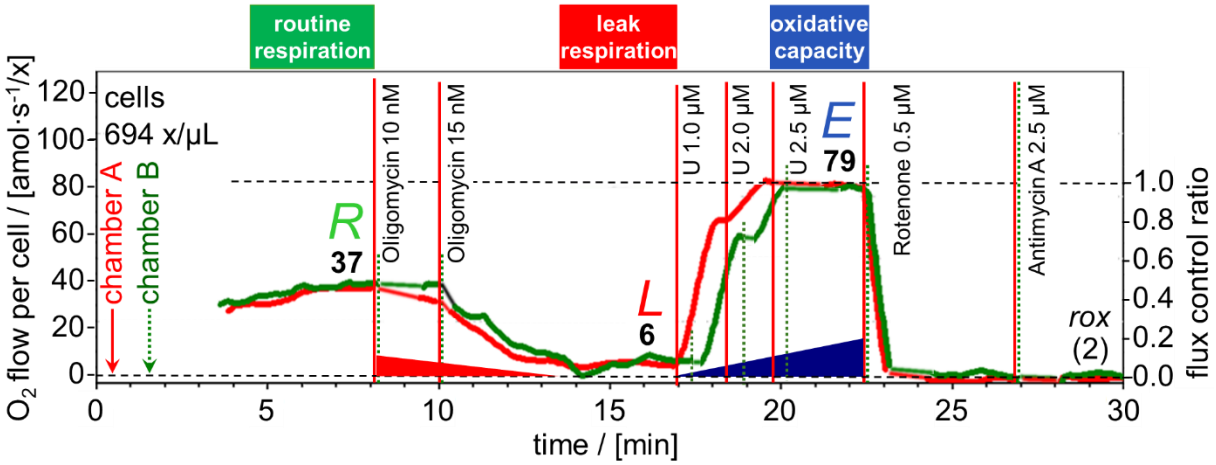
Notes on *rox*

- Mitochondrial respiration related to bioenergetic analyses is corrected for *rox.*
- A simple interpretation of the magnitude of *rox* is usually not possible.
- Non-mitochondrial respiration is an inaccurate term for *rox*, since oxygen consumption of isolated mitochondria may include mitochondrial *rox*.

2. Measurement of cell respiration

Should mitochondrial function be studied in isolated mitochondria, or is it better to examine it in living cells? To isolate mitochondria, researchers break the plasma membranes of cells, separating intact mitochondria from other structural and soluble cellular components. However, whether these isolated mitochondria accurately reflect

their function in living organisms has been debated, leading to a growing interest in studying mitochondrial physiology and bioenergetics in living cells [9;10]. Conversely, studies of isolated mitochondria provide bioenergetic information not easily obtained with living cells. Cell respiration is typically measured in small experimental chambers using thousands of blood cells obtained from liquid biopsies or cultured cells, such as fibroblasts. To ensure accurate comparisons, respiration rates are normalized based on cell count or mitochondrial markers.



The figure (modified after Zdrazilova et al 2022 [12]) shows respiration of human fibroblasts recorded simultaneously in two 0.5-mL chambers of the Oroboros O2k. The measurement is completed within 30 minutes (horizontal time axis). After adding the cell suspension (694 x/µL or 0.35 million cells in each chamber), it takes a few minutes for respiration to stabilize. A constant rate of routine respiration (*R*) is observed at 7 to 8 min of the experiment. Oligomycin added in two steps represses ATP production and thus lowers oxygen consumption to the level of leak respiration (*L*). Uncoupler titrations activate respiration stepwise to oxidative capacity (*E*). Sequential titrations of the respiratory inhibitors rotenone and antimycin A block oxidative capacity; residual oxygen consumption (*rox*) remains. The values on the left vertical axis are given in units of attomoles (10^{-18} moles) of O_2 consumed per second per single cell. The level of *rox* (2 amol·s^{-1}/x) is taken as zero. The averages of (*rox*-corrected) *R*, *L*, and *E* of all measurements are shown by numbers (from Table 4 in [12]). *R* of 37 amol·s^{-1}/x ($37 \cdot 10^{-18}$ mol·s^{-1}/x) appears like something small, but it actually means that a cell consumes 22 million molecules O_2 every second. Respiration per cell depends on cell size and mitochondrial density in the cell. To remove the effect of mitochondrial content on evaluation of mitochondrial respiration, cell respiration is expressed relative to *E* and shown as flux control ratio (right vertical axis).

Human red blood cells – they are red due to the oxygen-binding hemoglobin – do not contain mitochondria but rely on glycolytic ATP production. For cell respirometric studies, white blood cells are isolated from blood samples. These peripheral blood mononuclear cells (PBMCs) have important immunological functions. PBMCs are a heterogenous cell population consisting mainly of lymphocytes, typically 70 to 90 % in

human PBMCs. Less abundant are monocytes and dendritic cells. Contamination of the PBMC fraction by platelets must be avoided. Isolated platelets (thrombocytes) – without nucleus but containing mitochondria – can be used for cell respirometry. Blood cells are naturally suspended in the blood stream. In contrast, many cell types are cultured in a monolayer and detached for measurement in suspension, although other types can be cultured directly as cell suspensions. Fibroblast cell lines are applied in many studies of mitochondrial diseases [12].

Notes

- Manometric techniques for measuring mitochondrial and cell respiration were replaced by electrochemical methods 70 years ago [13].
- High-resolution respirometry (HRR; Oroboros, Innsbruck, Austria) on isolated mitochondria and living cells was introduced 30 years ago [6].
- Using HRR, the coupling control protocol was applied first in 2004 to human fibroblasts, distinguishing four respiratory states [8].
- This coupling control protocol is referred to as 'mitochondrial stress test' using multi-well platforms limited to four titration steps [11].
- OXPHOS capacity can be measured in mitochondrial preparations [2].
- Differences of respiratory rates, ratios, and respiratory control efficiencies facilitate robust bioenergetic interpretations [2;4;8;12]. A single mitochondrial health score does not reflect the complexity of mitochondrial respiratory functions and dysfunctions, and thus cannot provide sufficient diagnostic information [4].

Terms and symbols

ADP	adenosine diphosphate (di = 2)
ATP	adenosine triphosphate (tri = 3)
CO_2	carbon dioxide
e^-	electron, negative charge
E	oxidative capacity = electron transfer capacity (Section 1.1)
ETS	electron transfer system
H^+	hydrogen ion, positively charged
H_2O	water
L	leak rate of respiration (Section 1.3)
O_2	molecular oxygen, in the form of a gas in air or dissolved in solution
OX	oxidation, electron transfer, H^+-linked electron transfer (Section 1.1)
OXPHOS	oxidative phosphorylation
P	OXPHOS capacity (Section 1.1)
PHOS	phosphorylation of ADP to ATP, adding a phosphate group to ADP (diphosphate) with formation of ATP (triphosphate)
pmF	protonmotive force, coupling oxidation and phosphorylation in OXPHOS
R	routine respiration (Section 1.2)
rox	residual oxygen consumption (Section 1.4)

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Further reading

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