Abstract As the knowledge base and importance of mitochondrial physiology to human health expand, the necessity for harmonizing nomenclature concerning mitochondrial respiratory states and rates has become increasingly apparent. Clarity of concept and consistency of nomenclature are key trademarks of a research field. These trademarks facilitate effective transdisciplinary communication, education, and ultimately further discovery. Peter Mitchell's chemiosmotic theory establishes the link between vectorial and scalar energy transformation and coupling in oxidative phosphorylation. The unifying concept of the protonmotive force provides the framework for developing a consistent theory and nomenclature for mitochondrial physiology and bioenergetics. Herein, we follow IUPAC guidelines on general terms of physical chemistry, extended by considerations on open systems and irreversible thermodynamics. We align the nomenclature and symbols of classical bioenergetics with a concept-driven constructive terminology to express the meaning of each quantity clearly and consistently. In this position statement, in the frame of COST Action MitoEAGLE, we endeavour to provide a balanced view on mitochondrial respiratory control and a critical discussion on reporting data of mitochondrial respiration in terms of metabolic flows and fluxes. Uniform standards for evaluation of respiratory states and rates will ultimately support the development of databases of mitochondrial respiratory function in species, tissues, and cells.

Keywords: Mitochondrial respiratory control, coupling control, mitochondrial preparations, protonmotive force, oxidative phosphorylation, OXPHOS, efficiency, electron transfer, ET; proton leak, LEAK, residual oxygen consumption, ROX, State 2, State 3, State 4, normalization, flow, flux

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Executive summary

In preparation - see

http://www.mitoeagle.org/index.php/MitoEAGLE_preprint_2018-02-08#Executive_summary

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I think that the abstract is too difficult. I think it would be better to have a simple abstract to draw the readers in, now it seems written for the experts only and I think 'novice' in the field will be scared away. I suggest as follows:

Abstract

The key importance of mitochondrial physiology for human health is increasing realized, along with the rapid expansion of the mitochondrial knowledge base. Research efficiency as well as comparative and translational reliability would enormously benefit from a harmonized nomenclature of mitochondrial respiration states and rates. This is what we set out to do in the framework of the COST MitoEaGLE project and the result is presented here. IUPAC guidelines on general terms of physical chemistry are followed, extended with consideration on open systems and irreversible thermodynamics. We align the nomenclature and symbols of classical bioenergetics with a concept-driven constructive terminology to express the meaning of each quantity clearly and consistently. We define respiratory states. We provide a balanced view on mitochondrial respiratory control and critically discuss coupling and mitochondrial respiration in terms of metabolic rates, fluxes, regulation and control. We provide suggestions for appropriate normalization. Our proposal will contribute to uniform standards for respiratory states and rates and, consequently contribute the development of reference values and databases of mitochondrial function in species, tissues and cells.

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Box 1: In brief – Mitochondria and Bioblasts

Mitochondria are the oxygen-consuming electrochemical generators which evolved from endosymbiotic bacteria (Margulis 1970; Lane 2005). They were described by Richard Altmann (1894) as 'bioblasts', which include not only the mitochondria as presently defined, but also symbiotic and free-living bacteria. The word 'mitochondria' (Greek mitos: thread; chondros: granule) was introduced by Carl Benda (1898).

Mitochondrial dysfunction is associated with a wide variety of genetic and degenerative diseases. Robust mitochondrial function is supported by physical exercise and caloric balance, and is central for sustained metabolic health throughout life. Therefore, a more consistent presentation of mitochondrial physiology will improve our understanding of the etiology of disease, the diagnostic repertoire of mitochondrial medicine, with a focus on protective medicine, lifestyle and healthy aging.

We now recognize mitochondria as dynamic organelles with a double membrane that are contained within eukaryotic cells. The mitochondrial inner membrane (mtIM) shows dynamic tubular to disk-shaped cristae that separate the mitochondrial matrix, *i.e.*, the negatively charged internal mitochondrial compartment, and the intermembrane space; the latter being positively charged and enclosed by the mitochondrial outer membrane (mtOM). The mtIM contains the non-bilayer phospholipid cardiolipin, which is not present in any other eukaryotic cellular membrane. Cardiolipin promotes the formation of respiratory supercomplexes, which are supramolecular assemblies based upon specific, though dynamic, interactions between individual respiratory complexes (Greggio *et al.* 2017; Lenaz *et al.* 2017). Membrane fluidity is an important parameter influencing functional properties of proteins incorporated in the membranes (Waczulikova *et al.* 2007).

Mitochondria are the structural and functional elements of cell respiration. Cell respiration is the consumption of oxygen by electron transfer coupled to electrochemical proton translocation across the mtIM. In the process of oxidative phosphorylation (OXPHOS), the reduction of O_2 is electrochemically coupled to the transformation of energy in the form of adenosine triphosphate (ATP; Mitchell 1961, 2011). Mitochondria are the powerhouses of the cell which contain the machinery of the OXPHOS-pathways, including transmembrane respiratory complexes (*i.e.*, proton pumps with FMN, Fe-S and cytochrome *b*, *c*, aa_3 redox systems); alternative dehydrogenases and oxidases; the coenzyme ubiquinone (Q); F-ATPase or ATP synthase; the enzymes of the tricarboxylic acid cycle and the fatty acid oxidation enzymes; transporters of ions, metabolites and co-factors; and mitochondrial kinases related to energy transfer pathways. The mitochondrial proteome comprises over 1,200 proteins (Calvo *et al.* 2015; 2017), mostly encoded by nuclear DNA (nDNA), with a variety of functions, many of which are relatively well known (*e.g.* apoptosis-regulating proteins), while others are still under investigation, or need to be identified (*e.g.* alanine transporter).

in Figure 1 you use F_1F_o

There is a constant crosstalk between mitochondria and the other cellular components, maintaining cellular mitostasis through regulation at both the transcriptional and post-translational level, and through cell signalling including proteostatic (e.g. the ubiquitin-proteasome and autophagy-lysosome pathways) and genome stability modules throughout the cell cycle or even cell death, contributing to homeostatic regulation in response to varying energy demands and stress (Quiros *et al.* 2016). In addition to mitochondrial movement along the microtubules, mitochondrial morphology can change in response to energy requirements of the cell via processes known as fusion and fission, through which mitochondria communicate within a network, and in response to intracellular stress factors causing swelling and ultimately permeability transition.

Mitochondria typically maintain several copies of their own genome (hundred to thousands per cell; Cummins 1998), which is maternally inherited (White *et al.* 2008) and

known as mitochondrial DNA (mtDNA). One exception to strictly maternal inheritance in animals is found in bivalves (Breton *et al.* 2007). mtDNA is 16.5 kB in length, contains 13 protein-coding genes for subunits of the transmembrane respiratory Complexes CI, CIII, CIV and F-ATPase, and also encodes 22 tRNAs and the mitochondrial 16S and 12S rRNA. Additional gene content is encoded in the mitochondrial genome, *e.g.* microRNAs, piRNA, smithRNAs, repeat associated RNA, and even additional proteins (Duarte *et al.* 2014; Lee *et al.* 2015; Cobb *et al.* 2016). The mitochondrial genome is both regulated and supplemented by nuclear-encoded mitochondrial targeted proteins.

Abbreviation: mt, as generally used in mtDNA. Mitochondrion is singular and mitochondria is plural.

'*For the physiologist, mitochondria afforded the first opportunity for an experimental approach to structure-function relationships, in particular those involved in active transport, vectorial metabolism, and metabolic control mechanisms on a subcellular level*' (Ernster and Schatz 1981).

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1. Introduction

Mitochondria are the powerhouses of the cell with numerous physiological, molecular, and genetic functions (**Box 1**). Every study of mitochondrial function and disease is faced with **E**volution, **A**ge, **G**ender and sex, **L**ifestyle, and **E**nvironment (EAGLE) as essential background conditions intrinsic to the individual patient or subject, cohort, species, tissue and to some extent even cell line. As a large and highly coordinated group of laboratories and researchers, the mission of the global MitoEAGLE Network is to generate the necessary scale, type, and quality of consistent data sets and conditions to address this intrinsic complexity. Harmonization of experimental protocols and implementation of a quality control and data management system are required to interrelate results gathered across a spectrum of studies and to generate a rigorously monitored database focused on mitochondrial respiratory function. In this way, researchers within the same and across different disciplines will be positioned to compare findings across traditions and generations to an agreed upon set of clearly defined and accepted international standards.

Reliability and comparability of quantitative results depend on the accuracy of measurements under strictly-defined conditions. A conceptual framework is required to warrant meaningful interpretation and comparability of experimental outcomes carried out by research groups at different institutes. With an emphasis on quality of research, collected data can be useful far beyond the specific question of a particular experiment. Enabling meta-analytic studies is the most economic way of providing robust answers to biological questions (Cooper *et al.* 2009). Vague or ambiguous jargon can lead to confusion and may relegate valuable signals to wasteful noise. For this reason, measured values must be expressed in standardized units for each parameter used to define mitochondrial respiratory function. Standardization of nomenclature and definition of technical terms are essential to improve the awareness of the intricate meaning of current and past scientific vocabulary, for documentation and integration into databases in general, and quantitative modelling in particular (Beard 2005). The focus on coupling states and fluxes through metabolic pathways of aerobic energy transformation in mitochondrial preparations is a first step in the attempt to generate a harmonized and conceptually-oriented nomenclature in bioenergetics and mitochondrial physiology. Coupling states of intact cells, the protonmotive force, and respiratory control by fuel substrates and specific inhibitors of respiratory enzymes will be reviewed in subsequent communications.

(state) in which
, i.e ~~one~~ condition ~~where~~ energy is transferred ~~be~~ from one form to another,
need a definition. I find that most of my students do not understand the concept of coupling.

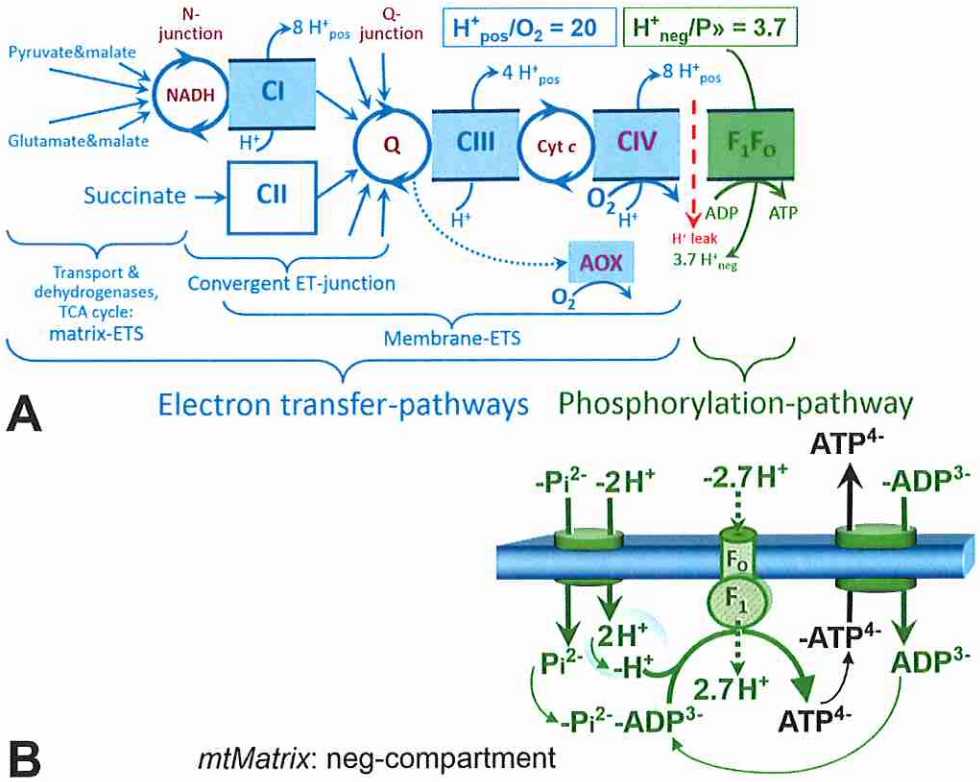

Fig. 1. The oxidative phosphorylation (OXPHOS) system. (A) The mitochondrial electron transfer system (ETS) is fuelled by diffusion and transport of substrates across the mtOM and mtIM and consists of the matrix-ETS and membrane-ETS. Electron transfer (ET) pathways are coupled to the phosphorylation-pathway. ET-pathways converge at the N-junction and Q-junction (additional arrows indicate electron entry into the Q-junction through electron transferring flavoprotein, glycerophosphate dehydrogenase, dihydro-orotate dehydrogenase, choline dehydrogenase, and sulfide-ubiquinone oxidoreductase). The dotted arrow indicates the branched pathway of oxygen consumption by alternative quinol oxidase (AOX). The H^+_{pos}/O_2 ratio is the outward proton flux from the matrix space to the positively (pos) charged compartment, divided by catabolic O_2 flux in the NADH-pathway. The H^+_{neg}/P» ratio is the inward proton flux from the inter-membrane space to the negatively (neg) charged matrix space, divided by the flux of phosphorylation of ADP to ATP (Eq. 1). Due to ion leaks and proton slip these are not fixed stoichiometries. **(B)** Phosphorylation-pathway catalyzed by the proton pump F_1F_O-ATPase, adenine nucleotide translocase, and inorganic phosphate transporter. The H^+_{neg}/P» stoichiometry is the sum of the coupling stoichiometry in the F-ATPase reaction (-2.7 H^+_{pos} from the positive intermembrane space, 2.7 H^+_{neg} to the matrix, *i.e.*, the negative compartment) and the proton balance in the translocation of ADP^{2-}, ATP^{3-} and P_i^{2-}. Modified from (A) Lemieux *et al.* (2017) and (B) Gnaiger (2014).

To provide a diagnostic reference for respiratory capacities of core energy metabolism, the capacity of *oxidative phosphorylation*, OXPHOS, is measured at kinetically-saturating concentrations of ADP and inorganic phosphate, P_i. The *oxidative* ET-capacity reveals the limitation of OXPHOS-capacity mediated by the *phosphorylation*-pathway. The ET- and phosphorylation-pathways comprise coupled segments of the OXPHOS-system. ET-capacity is measured as noncoupled respiration by application of *external uncouplers*. The contribution of *intrinsically uncoupled* oxygen consumption is most easily studied in the absence of ADP, *i.e.*, by not stimulating phosphorylation, or by inhibition of the phosphorylation-pathway. The corresponding states are collectively classified as LEAK-states, when oxygen consumption

compensates mainly for ion leaks including the proton leak (**Table 1**). Defined coupling states are induced by: (*1*) adding cation chelators such as EGTA, binding free Ca^{2+} and thus limiting cation cycling; (*2*) adding ADP and P_i; (*3*) inhibiting the phosphorylation-pathway; and (*4*) uncoupler titrations, while maintaining a defined ET-pathway state with constant fuel substrates and inhibitors of specific branches of the ET-pathway (**Fig. 1**).

Table 1. Coupling states and residual oxygen consumption in mitochondrial preparations in relation to respiration- and phosphorylation-rate, J_{kO_2} and $J_{P\text{»}}$, and protonmotive force, pmf. Coupling states are established at kinetically-saturating concentrations of fuel substrates and O_2.

State	J_{kO_2}	$J_{P\text{»}}$	pmf	Inducing factors	Limiting factors
LEAK	*L*; low, cation leak-dependent respiration	0	max.	proton leak, slip, and cation cycling	$J_{P\text{»}} = 0$: (*1*) without ADP, L_N; (*2*) max. ATP/ADP ratio, L_T; or (*3*) inhibition of the phosphorylation-pathway, L_{Omy}
OXPHOS	*P*; high, ADP-stimulated respiration	max.	high	kinetically-saturating [ADP] and [P_i]	$J_{P\text{»}}$ by phosphorylation-pathway; or J_{kO_2} by ET-capacity
ET	*E*; max., noncoupled respiration	0	low	optimal external uncoupler concentration for max. $J_{O_2,E}$	J_{kO_2} by ET-capacity
ROX	*Rox*; min., residual O_2 consumption	0	0	$J_{O_2,Rox}$ in non-ET-pathway oxidation reactions	full inhibition of ET-pathway; or absence of fuel substrates

Kinetic control: Coupling control states are established in the study of mitochondrial preparations to obtain reference values for various output variables. Physiological conditions *in vivo* deviate from these experimentally obtained states. Since kinetically-saturating concentrations, *e.g.* of ADP or oxygen, may not apply to physiological intracellular conditions, relevant information is obtained in studies of kinetic responses to conditions intermediate between the LEAK state at zero [ADP] and the OXPHOS-state at saturating [ADP], or of respiratory capacities in the range between kinetically-saturating [O_2] and anoxia (Gnaiger 2001).

The steady-state: Mitochondria represent a thermodynamically open system in non-equilibrium states of biochemical energy transformation. State variables (protonmotive force; redox states) and metabolic *rates* (fluxes) are measured in defined mitochondrial respiratory *states*. Strictly, steady states can be obtained only in open systems, in which changes by *internal* transformations, *e.g.*, O_2 consumption, are instantaneously compensated for by *external* fluxes, *e.g.*, O_2 supply, such that oxygen concentration does not change in the system (Gnaiger 1993b). Mitochondrial respiratory states monitored in closed systems satisfy the criteria of pseudo-steady states for limited periods of time, when changes in the system (concentrations of O_2, fuel substrates, ADP, P_i, H^+) do not exert significant effects on metabolic fluxes (respiration, phosphorylation). Such pseudo-steady states require respiratory media with sufficient buffering capacity and kinetically-saturating concentrations of substrates to be maintained, and thus depend on the kinetics of the processes under investigation.

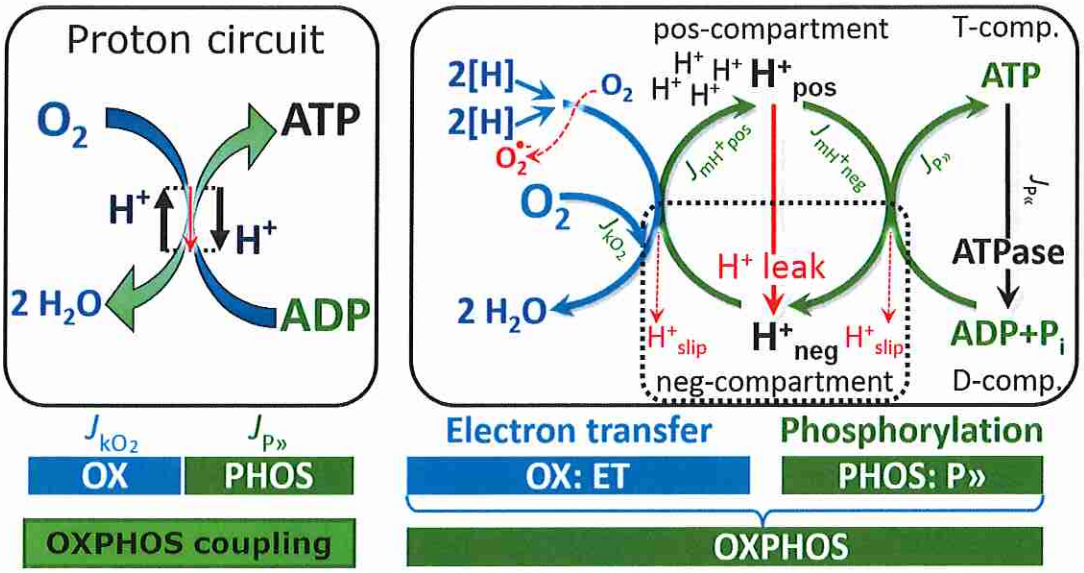

Fig. 2. The proton circuit and coupling in oxidative phosphorylation (OXPHOS). Oxygen flux, J_{kO_2}, through the catabolic ET-pathway, k, is coupled to flux through the phosphorylation-pathway of ADP to ATP, $J_{P»}$. The proton pumps of the ET-pathway drive proton flux into the positive (pos) compartment, J_{mH^+pos}, which generates the output protonmotive force. F-ATPase is coupled to inward proton current into the negative (neg) compartment, J_{mH^+neg}, to phosphorylate ADP+P_i to ATP. 2[H] indicates the reduced hydrogen equivalents of fuel substrates of the catabolic reaction k with oxygen. Fluxes are expressed per volume, V [m^3], of the system. The system defined by the boundaries (full black line) is not a black box, but is analysed as a compartmental system. The negative compartment (neg-compartment, enclosed by the dotted line) is the matrix space, separated by the mtIM from the positive compartment (pos-compartment). ADP+P_i and ATP are the substrate- and product-compartments (scalar ADP and ATP compartments, D-comp. and T-comp.), respectively. At steady-state proton turnover, $J_{\infty H^+}$, and ATP turnover, $J_{\infty P}$, maintain concentrations constant, when $J_{mH^+\infty} = J_{mH^+pos} = J_{mH^+neg}$, and $J_{P\infty} = J_{P»} = J_{P«}$. Modified from Gnaiger (2014).

(of mitochondrial respiration)

Uncoupling: Uncoupling is a general term comprising diverse mechanisms. Small differences of terms, *e.g.*, uncoupled *vs.* noncoupled, are easily overlooked, although they relate to different mechanisms of uncoupling (**Fig. 3**). An attempt at rigorous definition is required for clarification of concepts (**Table 2**).

1. Proton leak across the mtIM from the pos- to the neg-compartment (**Fig. 2**);
2. Cycling of other cations, strongly stimulated by permeability transition;
3. Proton slip in the proton pumps when protons are effectively not pumped (CI, CIII and CIV) or are not driving phosphorylation (F-ATPase);
4. Loss of compartmental integrity when electron transfer is acoupled;
5. Electron leak in the loosely coupled univalent reduction of oxygen (O_2; dioxygen) to superoxide anion radical ($O_2^{\bullet -}$).

uncoupling is considered here ~~as~~ only from the mitochondrial point of view. If we take this from the basal-metabolic-rate point of view (which in practice is measured as mitochondrial O_2 consumption) than any reaction where energy is lost constitutes uncoupling.

~~maybe~~ Either we should address this ~~or here~~, ~~and~~ and this may be easiest, specify that uncoupling of mitochondrial respiration discussed here (or respiratory uncoupling)

respiratory

503 **Table 2. Distinction of terms related to coupling and uncoupling (Fig. 3).**

Term	Respiration	P»/O$_2$	Note
acoupled		0	electron transfer in mitochondrial fragments without vectorial proton translocation
uncoupled	*L*	0	non-phosphorylating intrinsic LEAK-respiration, without added protonophore
uncoupled		0	component of LEAK-respiration, uncoupled *sui generis*, ion diffusion across the mtIM
decoupled		0	component of LEAK-respiration, proton slip
loosely coupled		0	component of LEAK-respiration, lower coupling due to superoxide anion radical formation and bypass of proton pumps
dyscoupled		0	pathologically, toxicologically, environmentally increased uncoupling, mitochondrial dysfunction
inducibly uncoupled	*E*	0	by UCP1 or cation (*e.g.* Ca^{2+}) cycling
noncoupled	*E*	0	non-phosphorylating respiration stimulated to maximum flux at optimum exogenous uncoupler concentration (**Fig. 6**)
well-coupled	*P*	high	phosphorylating respiration with an intrinsic LEAK component (**Fig. 5**)
fully coupled	*P* – *L*	max.	OXPHOS-capacity corrected for LEAK-respiration (**Fig. 7**)

504

505 **Proton leak and uncoupled respiration:** Proton leak is a leak current of protons. The
506 intrinsic proton leak is the *uncoupled* process in which protons diffuse across the mtIM in the
507 dissipative direction of the downhill protonmotive force without coupling to phosphorylation
508 (**Fig. 4**). The proton leak flux depends non-linearly on the protonmotive force (Garlid *et al.*
509 1989; Divakaruni and Brand 2011), is a property of the mtIM, and may be enhanced due to
510 possible contaminations by free fatty acids. Inducible uncoupling mediated by uncoupling
511 protein 1 (UCP1) is physiologically controlled, *e.g.*, in brown adipose tissue. UCP1 is a member
512 of the mitochondrial carrier family which is involved in the translocation of protons across the
513 mtIM (Klingenberg 2017). As a consequence of this effective short-circuit, the protonmotive
514 force diminishes, resulting in stimulation of electron transfer to O_2 and heat dissipation without
515 phosphorylation of ADP.
516 **Cation cycling:** There can be other cation contributors to leak current including calcium
517 and probably magnesium. Calcium current is balanced by mitochondrial Na^+/Ca^{2+} exchange,
518 which is balanced by Na^+/H^+ exchange or K^+/H^+ exchange. This is another effective uncoupling
519 mechanism different from proton leak.
520 **Proton slip and decoupled respiration:** Proton slip is the *decoupled* process in which
521 protons are only partially translocated by a proton pump of the ET-pathways and slip back to
522 the original compartment. The proton leak is the dominant contributor to the overall leak current
523 in mammalian mitochondria incubated under physiological conditions at 37 °C, whereas proton
524 slip is increased at lower experimental temperature (Canton *et al.* 1995). Proton slip can also
525 happen in association with the F-ATPase, in which case the proton slips downhill across the
526 pump to the matrix without contributing to ATP synthesis. In each case, proton slip is a property
527 of the proton pump and increases with the turnover rate of the pump.

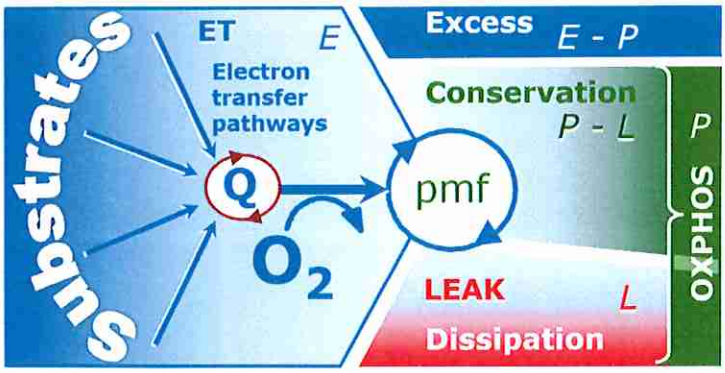

Fig. 7. Four-compartment model of oxidative phosphorylation. Respiratory states (ET, OXPHOS, LEAK) and corresponding rates (*E, P, L*) are connected by the protonmotive force, pmf. Electron transfer-capacity, *E*, is partitioned into (*1*) dissipative LEAK-respiration, *L*, when the Gibbs energy change of catabolic O_2 consumption is irreversibly lost, (*2*) net OXPHOS-capacity, *P-L*, with partial conservation of the capacity to perform work, and (*3*) the excess capacity, *E-P*. Modified from Gnaiger (2014).

E may exceed or be equal to *P*. $E > P$ is observed in many types of mitochondria, varying between species, tissues and cell types (Gnaiger 2009). *E-P* is the excess ET-capacity pushing the phosphorylation-flux (**Fig. 1B**) to the limit of its *capacity of utilizing* the protonmotive force. In addition, the magnitude of *E-P* depends on the tightness of coupling or degree of uncoupling, since an increase of *L* causes *P* to increase towards the limit of *E*. The *excess E-P* capacity, *E-P*, therefore, provides a sensitive diagnostic indicator of specific injuries of the phosphorylation-pathway, under conditions when *E* remains constant but *P* declines relative to controls (**Fig. 7**). Substrate cocktails supporting simultaneous convergent electron transfer to the Q-junction for reconstitution of tricarboxylic acid cycle (TCA cycle or Krebs cycle) function establish pathway control states with high ET-capacity, and consequently increase the sensitivity of the *E-P* assay.

E cannot theoretically be lower than *P*. $E < P$ must be discounted as an artefact, which may be caused experimentally by: (*1*) loss of oxidative capacity during the time course of the respirometric assay, since *E* is measured subsequently to *P*; (*2*) using insuffient uncoupler concentrations; (*3*) using high uncoupler concentrations which inhibit ET (Gnaiger 2008); (*4*) high oligomycin concentrations applied for measurement of *L* before titrations of uncoupler, when oligomycin exerts an inhibitory effect on *E*. On the other hand, the excess ET-capacity is overestimated if non-saturating [ADP] or [P_i] are used. See State 3 in the next section.

P»/O_2 ratio: The P»/O_2 ratio (P»/4 e^-) is two times the 'P/O' ratio (P»/2 e^-) of classical bioenergetics. P»/O_2 is a generalized symbol, independent of measurement of phosphorylation by determination of P_i consumption (P_i/O_2 flux ratio), ADP depletion (ADP/O_2 flux ratio), or ATP production (ATP/O_2 flux ratio).

The mechanistic P»/O_2 ratio, which may be referred to also as P»/O_2 stoichiometry, is calculated from the proton-to-oxygen and proton-to-phosphorylation coupling stoichiometries (**Fig. 1A**),

$$P\text{»}/O_2 = \frac{H^+_{pos}/O_2}{H^+_{neg}/P\text{»}} \quad (1)$$

The H^+_{pos}/O_2 *coupling stoichiometry* (referring to the full 4 electron reduction of O_2) depends on the ET-pathway control state which defines the relative involvement of the three coupling sites (CI, CIII and CIV) in the catabolic pathway of electrons to O_2. This varies with: (*1*) a bypass of CI by single or multiple electron input into the Q-junction; and (*2*) a bypass of CIV by involvement of AOX. H^+_{pos}/O_2 is 12 in the ET-pathways involving CIII and CIV as proton pumps, increasing to 20 for the NADH-pathway (**Fig. 1A**), but a general consensus on H^+_{pos}/O_2 stoichiometries remains to be reached (Hinkle 2005; Wikström and Hummer 2012; Sazanov

675 2015). The H^+_{neg}/P» coupling stoichiometry (3.7; **Fig. 1A**) is the sum of 2.7 H^+_{neg} required by
676 the F-ATPase of vertebrate and most invertebrate species (Watt *et al.* 2010) and the proton
677 balance in the translocation of ADP, ATP and P_i (**Fig. 1B**). Taken together, the mechanistic
678 P»/O_2 ratio is calculated at 5.4 and 3.3 for NADH- and succinate-linked respiration, respectively
679 (Eq. 1). The corresponding classical P»/O ratios (referring to the 2 electron reduction of 0.5 O_2)
680 are 2.7 and 1.6 (Watt *et al.* 2010), in direct agreement with the measured P»/O ratio for succinate
681 of 1.58 ± 0.02 (Gnaiger *et al.* 2000).
682 The effective P»/O_2 flux ratio ($Y_{P»/O_2} = J_{P»}/J_{kO_2}$) is diminished relative to the mechanistic
683 P»/O_2 ratio by intrinsic and extrinsic uncoupling and dyscoupling (**Fig. 3**). Such generalized
684 uncoupling is different from switching to mitochondrial pathways that involve fewer than three
685 proton pumps ('coupling sites': Complexes CI, CIII and CIV), bypassing CI through multiple
686 electron entries into the Q-junction, or CIII and CIV through AOX (**Fig. 1**). Reprogramming of
687 mitochondrial pathways may be considered as a switch of gears (changing the stoichiometry)
688 rather than uncoupling (loosening the stoichiometry). In addition, $Y_{P»/O_2}$ depends on several
689 experimental conditions of flux control, increasing as a hyperbolic function of [ADP] to a
690 maximum value (Gnaiger 2001).
691 The net OXPHOS-capacity is calculated by subtracting *L* from *P* (**Fig. 7**). Then the net
692 P»/O_2 equals P»/(*P-L*), wherein the dissipative LEAK component in the OXPHOS-state may
693 be overestimated. This can be avoided by measuring LEAK-respiration in a state when the
694 protonmotive force is adjusted to its slightly lower value in the OXPHOS-state, *e.g.*, by titration
695 of an ET inhibitor (Divakaruni and Brand 2011). Any turnover-dependent components of
696 proton leak and slip, however, are underestimated under these conditions (Garlid *et al.* 1993).
697 In general, it is inappropriate to use the term *ATP production* or *ATP turnover* for the difference
698 of oxygen consumption measured in states *P* and *L*. The difference *P-L* is the upper limit of the
699 part of OXPHOS-capacity that is freely available for ATP production (corrected for LEAK-
700 respiration) and is fully coupled to phosphorylation with a maximum mechanistic stoichiometry
701 (**Fig. 7**).
702 **Control and regulation:** The terms metabolic *control* and *regulation* are frequently used
703 synonymously, but are distinguished in metabolic control analysis: 'We could understand the
704 regulation as the mechanism that occurs when a system maintains some variable constant over
705 time, in spite of fluctuations in external conditions (homeostasis of the internal state). On the
706 other hand, metabolic control is the power to change the state of the metabolism in response to
707 an external signal' (Fell 1997). Respiratory control may be induced by experimental control
708 signals that *exert* an influence on: (*1*) ATP demand and ADP phosphorylation-rate; (*2*) fuel
709 substrate composition, pathway competition; (*3*) available amounts of substrates and oxygen,
710 *e.g.*, starvation and hypoxia; (*3*) the protonmotive force, redox states, flux-force relationships,
711 coupling and efficiency; (*4*) Ca^{2+} and other ions including H^+; (*5*) inhibitors, *e.g.*, nitric oxide
712 or intermediary metabolites, such as oxaloacetate; (*6*) signalling pathways and regulatory
713 proteins, *e.g.* insulin resistance, transcription factor HIF-1 or inhibitory factor 1. *Mechanisms*
714 of respiratory control and regulation include adjustments of: (*1*) enzyme activities by allosteric
715 mechanisms and phosphorylation; (*2*) enzyme content, concentrations of cofactors and
716 conserved moieties (such as adenylates, nicotinamide adenine dinucleotide [NAD^+/NADH],
717 coenzyme Q, cytochrome *c*); (*3*) metabolic channeling by supercomplexes; and *(4)*
718 mitochondrial density (enzyme concentrations and membrane area) and morphology (cristae
719 folding, fission and fusion). (*5*) Mitochondria are targeted directly by hormones, thereby
720 affecting their energy metabolism (Lee *et al.* 2013; Gerö and Szabo 2016; Price and Dai 2016;
721 Moreno *et al.* 2017). Evolutionary or acquired differences in the genetic and epigenetic basis
722 of mitochondrial function (or dysfunction) between subjects and gene therapy; age; gender,
723 biological sex, and hormone concentrations; life style including exercise and nutrition; and
724 environmental issues including thermal, atmospheric, toxicological and pharmacological

may be better to write in full hypoxia inducible factor 1 (I abbreviated use official nomenclature which is either (HIF1)

I am not sure what this refers to. →

mitochondria in the measuring chamber, in which case the nature of the interaction becomes an issue. Optimization of cell density and arrangement is generally important and particularly in experiments carried out in wells, considering the confluency of the cell monolayer or clumps of cells (Salabei *et al.* 2014).

Number concentration, C_{NX}: C_{NX} is the experimental *number concentration* of sample *X*. In the case of cells or animals, *e.g.*, nematodes, $C_{NX} = N_X/V$ [x·L^{-1}], where N_X is the number of cells or organisms in the chamber (**Table 4**).

Flow per object, I_{X,O_2}: A special case of normalization is encountered in respiratory studies with permeabilized (or intact) cells. If respiration is expressed per cell, the O_2 flow per measurement system is replaced by the O_2 flow per cell, I_{cell,O_2} (**Table 4**). O_2 flow can be calculated from volume-specific O_2 flux, J_{V,O_2} [nmol·s^{-1}·L^{-1}] (per V of the measurement chamber [L]), divided by the number concentration of cells, $C_{Nce} = N_{ce}/V$ [cell·L^{-1}], where N_{ce} is the number of cells in the chamber. Cellular O_2 flow can be compared between cells of identical size. To take into account changes and differences in cell size, further normalization is required to obtain cell size-specific or mitochondrial marker-specific O_2 flux (Renner *et al.* 2003).

The complexity changes when the sample is a whole organism studied as an experimental model. The well-established scaling law in respiratory physiology reveals a strong interaction of O_2 consumption and individual body mass of an organism, since *basal* metabolic rate (flow) does not increase linearly with body mass, whereas *maximum* mass-specific O_2 flux, $\dot{V}_{O2max}$ or $\dot{V}_{O2peak}$, is approximately constant across a large range of individual body mass (Weibel and Hoppeler 2005), with individuals, breeds, and certain species deviating substantially from this general relationship. $\dot{V}_{O2peak}$ of human endurance athletes is 60 to 80 mL O_2·min^{-1}·kg^{-1} body mass, converted to $J_{M,O2peak}$ of 45 to 60 nmol·s^{-1}·g^{-1} (Gnaiger 2014; **Table 6**).

3.4. Normalization for mitochondrial content

Tissues can contain multiple cell populations which may have distinct mitochondrial subtypes. Mitochondria undergo dynamic fission and fusion cycles, and can exist in multiple stages and sizes which may be altered by a range of factors. The isolation of mitochondria (often achieved through differential centrifugation) can therefore yield a subsample of the mitochondrial types present in a tissue, dependent on isolation protocols utilized (*e.g.* centrifugation speed). This possible artefact should be taken into account when planning experiments using isolated mitochondria. The tendency for mitochondria of specific sizes to be enriched at different centrifugation speeds also has the potential to allow the isolation of specific mitochondrial subpopulations and therefore the analysis of mitochondria from multiple cell lineages within a single tissue.

maybe it is better to use the word 'bias'

Part of the mitochondria from the tissue is lost during preparation of isolated mitochondria. The fraction of mitochondria obtained is expressed as mitochondrial recovery (**Fig. 9**). At a high mitochondrial recovery the sample of isolated mitochondria is more representative of the total mitochondrial population than in preparations characterized by low recovery. Determination of the mitochondrial recovery and yield is based on measurement of the concentration of a mitochondrial marker in the tissue homogenate, $C_{mtE,thom}$, which simultaneously provides information on the specific mitochondrial density in the sample (**Fig. 9**).

Normalization is a problematic subject and it is essential to consider the question of the study. If the study aims to compare tissue performance, such as the effects of a certain treatment on a specific tissue, then normalization can be successful, using tissue mass or protein content, for example. If the aim, however, is to find differences of mitochondrial function independent of mitochondrial density (**Table 4**), then normalization to a mitochondrial marker is imperative (**Fig. 10**). However, one cannot assume that quantitative changes in various markers such as mitochondrial proteins necessarily occur in parallel with one another. It is important to first

ET-capacity in human cell types including HEK 293, primary HUVEC and fibroblasts ranges from 50 to 180 amol·s^{-1}·cell^{-1}, measured in intact cells in the noncoupled state (see Gnaiger 2014). At 100 amol·s^{-1}·cell^{-1} corrected for *Rox*, the current across the mt-membranes, I_{eH+}, approximates 193 pA·cell^{-1} or 0.2 nA per cell. See Rich (2003) for an extension of quantitative bioenergetics from the molecular to the human scale, with a transmembrane proton flux equivalent to 520 A in an adult at a catabolic power of -110 W. Modelling approaches illustrate the link between protonmotive force and currents (Willis *et al.* 2016).

We consider isolated mitochondria as powerhouses and proton pumps as molecular machines to relate experimental results to energy metabolism of the intact cell. The cellular P»/O_2 based on oxidation of glycogen is increased by the glycolytic (fermentative) substrate-level phosphorylation of 3 P»/Glyc or 0.5 mol P» for each mol O_2 consumed in the complete oxidation of a mol glycosyl unit (Glyc). Adding 0.5 to the mitochondrial P»/O_2 ratio of 5.4 yields a bioenergetic cell physiological P»/O_2 ratio close to 6. Two NADH equivalents are formed during glycolysis and transported from the cytosol into the mitochondrial matrix, either by the malate-aspartate shuttle or by the glycerophosphate shuttle resulting in different theoretical yields of ATP generated by mitochondria, the energetic cost of which potentially must be taken into account. Considering also substrate-level phosphorylation in the TCA cycle, this high P»/O_2 ratio not only reflects proton translocation and OXPHOS studied in isolation, but integrates mitochondrial physiology with energy transformation in the living cell (Gnaiger 1993a).

4. Conclusions

MitoEAGLE can serve as a gateway to better diagnose mitochondrial respiratory defects linked to genetic variation, age-related health risks, sex-specific mitochondrial performance, lifestyle with its effects on degenerative diseases, and thermal and chemical environment. The present recommendations on coupling control states and rates, linked to the concept of the protonmotive force, are focused on studies with mitochondrial preparations. These will be extended in a series of reports on pathway control of mitochondrial respiration, respiratory states in intact cells, and harmonization of experimental procedures.

The optimal choice for expressing mitochondrial and cell respiration (**Box 3**) as O_2 flow per biological system, and normalization for specific tissue-markers (volume, mass, protein) and mitochondrial markers (volume, protein, content, mtDNA, activity of marker enzymes, respiratory reference state) is guided by the scientific question under study. Interpretation of the obtained data depends critically on appropriate normalization, and therefore reporting rates merely as nmol·s^{-1} is discouraged, since it restricts the analysis to intra-experimental comparison of relative (qualitative) differences. Expressing O_2 consumption per cell may not be possible when dealing with tissues. For studies with mitochondrial preparations, we recommend that normalizations be provided as far as possible: (*1*) on a per cell basis as O_2 flow (a biophysical normalization); (*2*) per g cell or tissue protein, or per cell or tissue mass as mass-specific O_2 flux (a cellular normalization); and (*3*) per mitochondrial marker as mt-specific flux (a mitochondrial normalization). With information on cell size and the use of multiple normalizations, maximum potential information is available (Renner *et al.* 2003; Wagner *et al.* 2011; Gnaiger 2014).

When using isolated mitochondria, total mitochondrial protein is a frequently applied mitochondrial marker, the use of which is restricted to isolated mitochondria. The mitochondrial recovery and yield, and experimental criteria for evaluation of purity versus integrity should be reported. Mitochondrial markers—such as citrate synthase activity as an enzymatic matrix marker—provide a link to the tissue of origin on the basis of calculating the