

1 **Mitochondrial respiratory states and rates:**
2 **Building blocks of mitochondrial physiology Part 1**
3

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5 Corresponding author: Gnaiger E

6 Co-authors:

7 Aasander Frostner E, Abumrad NA, Acuna-Castroviejo D, Ahn B, Ali SS, Alves MG, Amati
8 F, Aral C, Arandarčikaitė O, Bailey DM, Bajpeyi S, Bakker BM, Bastos Sant'Anna Silva AC,
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13 Pinna M, Cassina AM, Castro L, Cavalcanti-de-Albuquerque JP, Cervinkova Z, Chaurasia B,
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51 Tyrrell DJ, Urban T, Valentine JM, Velika B, Vendelin M, Vercesi AE, Victor VM, Vieyra A

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 55 Zaugg M, Zhang Y, Zhang YZ, Zischka H, Zorzano A

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59 Correspondence: Gnaiger E

60 *Chair COST Action CA15203 MitoEAGLE* – <http://www.mitoeagle.org>

61 *Department of Visceral, Transplant and Thoracic Surgery, D. Swarovski Research*
 62 *Laboratory, Medical University of Innsbruck, Innrain 66/4, A-6020 Innsbruck, Austria*

63 *Email: mitoeagle@i-med.ac.at; Tel: +43 512 566796, Fax: +43 512 566796 20*

64
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104 **Abstract** As the knowledge base and importance of mitochondrial physiology to human health
 105 expands, the necessity for harmonizing the terminology concerning mitochondrial respiratory
 106 states and rates has become increasingly apparent. The chemiosmotic theory establishes the
 107 mechanism of energy transformation and coupling in oxidative phosphorylation. The unifying
 108 concept of the protonmotive force provides the framework for developing a consistent
 109 theoretical foundation of mitochondrial physiology and bioenergetics. We follow IUPAC
 110 guidelines on terminology in physical chemistry, extended by considerations on open systems
 111 and irreversible thermodynamics. The concept-driven constructive terminology incorporates
 112 the meaning of each quantity and aligns concepts and symbols to the nomenclature of classical
 113 bioenergetics. In the frame of COST Action MitoEAGLE open to global bottom-up input, we
 114 endeavour to provide a balanced view on mitochondrial respiratory control and a critical
 115 discussion on reporting data of mitochondrial respiration in terms of metabolic flows and fluxes.
 116 Uniform standards for evaluation of respiratory states and rates will ultimately support the
 117 development of databases of mitochondrial respiratory function in species, tissues, and cells.
 118 Clarity of concept and consistency of nomenclature facilitate effective transdisciplinary
 119 communication, education, and ultimately further discovery.

120

121 *Keywords:* Mitochondrial respiratory control, coupling control, mitochondrial
 122 preparations, protonmotive force, uncoupling, oxidative phosphorylation, OXPHOS,
 123 efficiency, electron transfer, ET; proton leak, LEAK, residual oxygen consumption, ROX, State
 124 2, State 3, State 4, normalization, flow, flux, O₂

125

126

126 **Executive summary**

127

128 1. In view of the broad implications for health care, mitochondrial researchers face an
 129 increasing responsibility to disseminate their fundamental knowledge and novel
 130 discoveries to a wide range of stakeholders and scientists beyond the group of
 131 specialists. This requires implementation of a commonly accepted terminology
 132 within the discipline and standardization in the translational context. Authors,
 133 reviewers, journal editors, and lecturers are challenged to collaborate with the aim
 134 to harmonize the nomenclature in the growing field of mitochondrial physiology
 135 and bioenergetics, from evolutionary biology and comparative physiology to
 136 mitochondrial medicine.

137 2. Aerobic respiration depends on the coupling of phosphorylation (ADP → ATP) to O₂
 138 flux in catabolic reactions. Coupling in oxidative phosphorylation is mediated by
 139 translocation of protons across the inner mitochondrial membrane through proton
 140 pumps generating or utilizing the protonmotive force, that is measured between the
 141 mitochondrial matrix and intermembrane compartment or outer mitochondrial
 142 space. Compartmental coupling distinguishes vectorial oxidative phosphorylation
 143 from glycolytic fermentation as the counterpart of cellular core energy metabolism
 144 (**Figure 1**).

145 3. To exclude fermentation and other cytosolic interactions from exerting an effect on the
 146 analysis of mitochondrial metabolism, the barrier function of the plasma membrane
 147 must be disrupted. Selective removal or permeabilization of the plasma membrane
 148 yields mitochondrial preparations—including isolated mitochondria, tissue and
 149 cellular preparations—with structural and functional integrity. Then extra-
 150 mitochondrial concentrations of fuel substrates, ADP, ATP, inorganic phosphate,
 151 and cations including H⁺ can be controlled to determine mitochondrial function
 152 under a set of conditions defined as coupling control states. A concept-driven
 153 terminology of bioenergetics explicitly incorporates in its terms and symbols
 154 information on the nature of respiratory states that makes the technical terms readily
 155 recognized and more easy to understand.

156 **Figure 1. Mitochondrial respiration**
 157 **is the oxidation of fuel substrates**
 158 **(electron donors) and reduction of**
 159 **O₂ catalysed by the electron transfer**
 160 **system, ETS: (a) mitochondrial**
 161 **catabolic respiration; (b)**
 162 **mitochondrial and non-**
 163 **mitochondrial catabolic O₂**
 164 **consumption; O₂ balance of (c) total**
 165 **cellular O₂ consumption and (d)**
 166 **external respiration**

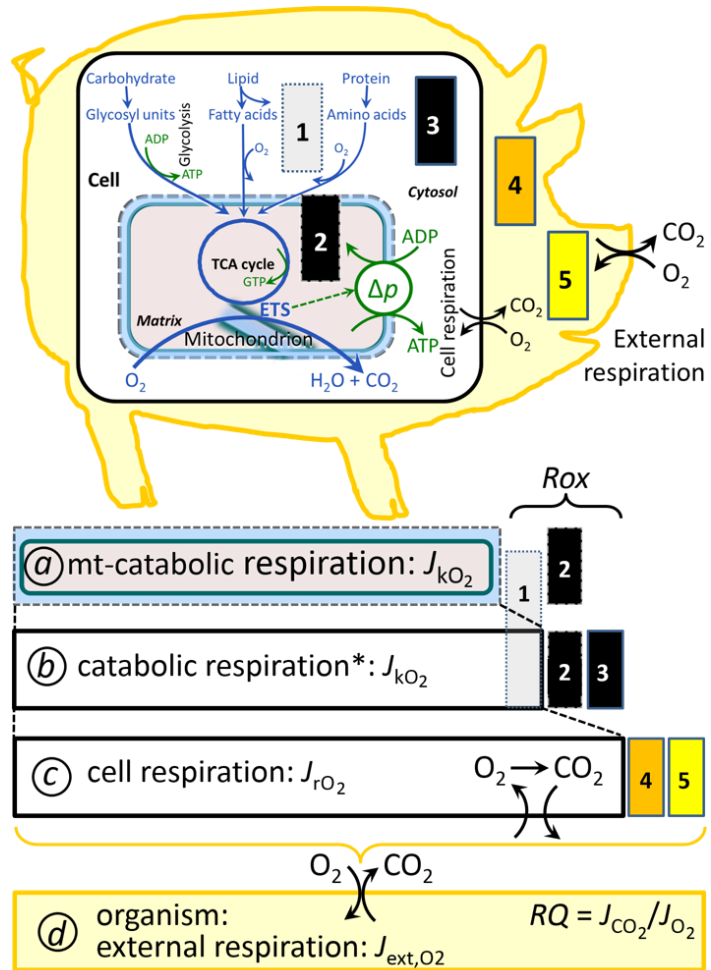
167 All chemical reactions, r , that
 168 consume O₂ in the cells of an
 169 organism, contribute to cell
 170 respiration, J_{RO_2} . ❶ Non-mitochondrial
 171 O₂ consumption by catabolic
 172 reactions, particularly peroxisomal
 173 oxidases and microsomal cytochrome
 174 P450 systems; ❷ mitochondrial
 175 residual oxygen consumption, R_{ox} ,
 176 after blocking the ETS; ❸ non-
 177 mitochondrial R_{ox} ; ❹ extracellular O₂
 178 consumption; ❺ aerobic microbial
 179 respiration. Bars are not at a
 180 quantitative scale.

181 **a Mitochondrial catabolic**
 182 **respiration**, J_{kO_2} , is the O₂
 183 consumption by the mitochondrial ETS maintaining the protonmotive force, Δp . J_{kO_2}
 184 excludes R_{ox} .

185 **b Catabolic respiration** is the O₂ consumption associated with catabolic pathways in the cell,
 186 including peroxisomal and microsomal oxidation reactions (❶) in addition to mitochondrial
 187 catabolism (* The reactions k have to be defined specifically for *a* and *b*.)

188 **c Cell respiration**, J_{RO_2} , takes into account internal O₂-consuming reactions, r , including
 189 catabolic respiration and R_{ox} . Internal respiration of an organism includes extracellular O₂
 190 consumption (❹) and aerobic respiration by the microbiome (❺). Respiration is
 191 distinguished from fermentation by: (1) External electron acceptors for the maintenance of
 192 redox balance, whereas fermentation is characterized by an internal electron acceptor
 193 produced in intermediary metabolism. In aerobic cell respiration, redox balance is
 194 maintained by O₂ as the electron acceptor. (2) Compartmental coupling in vectorial oxidative
 195 phosphorylation, in contrast to exclusively scalar substrate-level phosphorylation in
 196 fermentation.

197 **d External respiration** balances internal respiration at steady-state. O₂ is transported from the
 198 environment across the respiratory cascade (circulation between tissues and diffusion across
 199 cell membranes) to the intracellular compartment, while bicarbonate and CO₂ are transported
 200 in reverse to the extracellular milieu and the organismic environment. Hemoglobin provides
 201 the molecular paradigm for the combination of O₂ and CO₂ exchange, as do lungs and gills
 202 on the morphological level. The respiratory quotient, RQ , is the molar CO₂/O₂ exchange
 203 ratio; when combined with the respiratory nitrogen quotient, N/O₂ (mol N given off per mol
 204 O₂ consumed), the RQ reflects the proportion of carbohydrate, lipid and protein utilized in
 205 cell respiration during aerobically balanced steady-states.
 206



- 207 4. Mitochondrial coupling states are defined according to the control of respiratory oxygen
 208 flux by the protonmotive force. Capacities of oxidative phosphorylation and
 209 electron transfer are measured at kinetically saturating concentrations of fuel
 210 substrates, ADP and inorganic phosphate, and O₂, or at optimal uncoupler
 211 concentrations, respectively, in the absence of Complex IV inhibitors such as NO,
 212 CO, or H₂S. Respiratory capacity is a measure of the upper bound of the rate of
 213 respiration, depends on the substrate type undergoing oxidation, and provides
 214 reference values for the diagnosis of health and disease, and for evaluation of the
 215 effects of Evolutionary background, Age, Gender and sex, Lifestyle and
 216 Environment (EAGLE).
- 217 5. Incomplete tightness of coupling, *i.e.*, some degree of uncoupling relative to the
 218 substrate-dependent coupling stoichiometry, is a characteristic of energy-
 219 transformations across membranes. Uncoupling is caused by a variety of
 220 physiological, pathological, toxicological, pharmacological and environmental
 221 conditions that exert an influence not only on the proton leak and cation cycling,
 222 but also on proton slip within the proton pumps and the structural integrity of the
 223 mitochondria. A more loosely coupled state is induced by stimulation of
 224 mitochondrial superoxide formation and the bypass of proton pumps. In addition,
 225 uncoupling by application of protonophores represents an experimental
 226 intervention for the transition from a well-coupled to the noncoupled state of
 227 mitochondrial respiration.
- 228 6. Respiratory oxygen consumption rates have to be carefully normalized to enable meta-
 229 analytic studies beyond the specific question of a particular experiment. Therefore,
 230 all raw data should be published in a supplemental table or open access data
 231 repository. Normalization of rates for the volume of the experimental chamber (the
 232 measuring system) is distinguished from normalization for: (1) the volume or mass
 233 of the experimental sample; (2) the number of objects (cells, organisms); and (3)
 234 the concentration of mitochondrial markers in the chamber.
- 235 7. The consistent use of terms and symbols will facilitate transdisciplinary communication
 236 and support further developments of a database on bioenergetics and mitochondrial
 237 physiology. The present considerations are focused on studies with mitochondrial
 238 preparations. These will be extended in a series of reports on pathway control of
 239 mitochondrial respiration, the protonmotive force, respiratory states in intact cells,
 240 and harmonization of experimental procedures.

244 **Box 1: In brief – Mitochondria and Bioblasts**

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

249 **Mitochondria** are the oxygen-consuming electrochemical generators evolved from
 250 endosymbiotic bacteria (Margulis 1970; Lane 2005). They were described by Richard Altmann
 251 (1894) as ‘bioblasts’, which include not only the mitochondria as presently defined, but also
 252 symbiotic and free-living bacteria. The word ‘mitochondria’ (Greek mitos: thread; chondros:
 253 granule) was introduced by Carl Benda (1898).

254 Mitochondria form dynamic networks within eukaryotic cells and are morphologically
 255 enclosed by a double membrane. The mitochondrial inner membrane (mtIM) shows dynamic
 256 tubular to disk-shaped cristae that separate the mitochondrial matrix, *i.e.*, the negatively charged
 257 internal mitochondrial compartment, from the intermembrane space; the latter being enclosed
 258 by the mitochondrial outer membrane (mtOM) and positively charged with respect to the

259 matrix. The mtIM contains the non-bilayer phospholipid cardiolipin, which is not present in
260 any other eukaryotic cellular membrane. Cardiolipin stabilizes and promotes the formation of
261 respiratory supercomplexes (SC $I_nIII_nIV_n$), which are supramolecular assemblies based upon
262 specific, though dynamic interactions between individual respiratory complexes (Greggio *et al.*
263 2017; Lenaz *et al.* 2017). Membrane fluidity exerts an influence on functional properties of
264 proteins incorporated in the membranes (Waczulikova *et al.* 2007). In addition to mitochondrial
265 movement along microtubules, mitochondrial morphology can change in response to energy
266 requirements of the cell via processes known as fusion and fission, through which mitochondria
267 communicate within a network (Chan 2006). Intracellular stress factors may cause shrinking or
268 swelling of the mitochondrial matrix, that can ultimately result in permeability transition.

269 Mitochondria are the structural and functional elements of cell respiration. Mitochondrial
270 respiration is the reduction of molecular oxygen by electron transfer coupled to electrochemical
271 proton translocation across the mtIM. In the process of oxidative phosphorylation (OXPHOS),
272 the catabolic reaction of oxygen consumption is electrochemically coupled to the
273 transformation of energy in the form of adenosine triphosphate (ATP; Mitchell 1961, 2011).
274 Mitochondria are the powerhouses of the cell which contain the machinery of the OXPHOS-
275 pathways, including transmembrane respiratory complexes (proton pumps with FMN, Fe-S and
276 cytochrome *b*, *c*, *aa*₃ redox systems); alternative dehydrogenases and oxidases; the coenzyme
277 ubiquinone (Q); F-ATPase or ATP synthase; the enzymes of the tricarboxylic acid cycle, fatty
278 acid and amino acid oxidation; transporters of ions, metabolites and co-factors; iron/sulphur
279 cluster synthesis; and mitochondrial kinases related to energy transfer pathways. The
280 mitochondrial proteome comprises over 1,200 proteins (Calvo *et al.* 2015; 2017), mostly
281 encoded by nuclear DNA (nDNA), with a variety of functions, many of which are relatively
282 well known (*e.g.*, proteins regulating mitochondrial biogenesis or apoptosis), while others are
283 still under investigation, or need to be identified (*e.g.*, alanine transporter). Only lately it is
284 possible to use the mammalian mitochondrial proteome to discover and characterize the genetic
285 basis of mitochondrial diseases (Williams *et al.* 2016; Palmfeldt and Bross 2017).

286 There is a constant crosstalk between mitochondria and the other cellular components.
287 The crosstalk between mitochondria and endoplasmic reticulum is involved in the regulation of
288 calcium homeostasis, cell division, autophagy, differentiation, and anti-viral signaling (Murley
289 and Nunnari 2016). Mitochondria contribute to the formation of peroxisomes, which are hybrids
290 of mitochondrial and ER-derived precursors (Sugiura *et al.* 2017). Cellular mitochondrial
291 homeostasis (mitostasis) is maintained through regulation at both the transcriptional and post-
292 translational level. Cell signalling modules contribute to homeostatic regulation throughout the
293 cell cycle or even cell death by activating proteostatic modules (*e.g.*, the ubiquitin-proteasome
294 and autophagy-lysosome/vacuole pathways; specific proteases like LON) and genome stability
295 modules in response to varying energy demands and stress cues (Quiros *et al.* 2016).
296 Acetylation is a post-translational modification capable of influencing the bioenergetic
297 response, with clinically significant implications for health and disease (Carrico *et al.* 2018).
298 Mitochondria can traverse cell boundaries in a process known as horizontal mitochondrial
299 transfer (Torralba *et al.* 2016).

300 Mitochondria typically maintain several copies of their own circular genome known as
301 mitochondrial DNA (mtDNA; hundred to thousands per cell; Cummins 1998), which is
302 maternally inherited. Biparental mitochondrial inheritance is documented in mammals, birds,
303 fish, reptiles and invertebrate groups, and is even the norm in some bivalve taxonomic groups
304 (Breton *et al.* 2007; White *et al.* 2008). The mitochondrial genome of the angiosperm *Amborella*
305 contains a record of six mitochondrial genome equivalents acquired by horizontal transfer of
306 entire genomes, two from angiosperms, three from algae and one from mosses (Rice *et al.*
307 2016). However, some organisms such as *Cryptosporidium* species have morphologically and
308 functionally reduced mitochondria without DNA (Liu *et al.* 2016). In vertebrates but not all
309 invertebrates, mtDNA is compact (16.5 kB in humans) and encodes 13 protein subunits of the

310 transmembrane respiratory Complexes CI, CIII, CIV and F-ATPase, 22 tRNAs, and two RNAs.
311 Additional gene content has been suggested to include microRNAs, piRNA, smithRNAs, repeat
312 associated RNA, and even additional proteins (Duarte *et al.* 2014; Lee *et al.* 2015; Cobb *et al.*
313 2016). The mitochondrial genome requires nuclear-encoded mitochondrially targeted proteins
314 for its maintenance and expression (Rackham *et al.* 2012). Both genomes encode peptides of
315 the membrane spanning redox pumps (CI, CIII and CIV) and F-ATPase, leading to strong
316 constraints in the coevolution of both genomes (Blier *et al.* 2001).

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

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

326 327 **1. Introduction**

328
329 Mitochondria are the powerhouses of the cell with numerous physiological, molecular,
330 and genetic functions (**Box 1**). Every study of mitochondrial health and disease is faced with
331 Evolution, Age, Gender and sex, Lifestyle, and Environment (EAGLE) as essential background
332 conditions intrinsic to the individual person or cohort, species, tissue and to some extent even
333 cell line. As a large and coordinated group of laboratories and researchers, the mission of the
334 global MitoEAGLE Network is to generate the necessary scale, type, and quality of consistent
335 data sets and conditions to address this intrinsic complexity. Harmonization of experimental
336 protocols and implementation of a quality control and data management system are required to
337 interrelate results gathered across a spectrum of studies and to generate a rigorously monitored
338 database focused on mitochondrial respiratory function. In this way, researchers from a variety
339 of disciplines can compare their findings using clearly defined and accepted international
340 standards.

341 Reliability and comparability of quantitative results depend on the accuracy of
342 measurements under strictly-defined conditions. A conceptual framework is required to warrant
343 meaningful interpretation and comparability of experimental outcomes carried out by research
344 groups at different institutes. With an emphasis on quality of research, collected data can be
345 useful far beyond the specific question of a particular experiment. Standardization and
346 homogenization of terminology, methodology, and data sets could lead to the development of
347 open-access databases such as those that have been developed for National Institutes of Health
348 sponsored research in genetics, proteomics, and metabolomics. Enabling meta-analytic studies
349 is the most economic way of providing robust answers to biological questions (Cooper *et al.*
350 2009). Vague or ambiguous jargon can lead to confusion and may relegate valuable signals to
351 wasteful noise. For this reason, measured values must be expressed in standard units for each
352 parameter used to define mitochondrial respiratory function. Harmonization of nomenclature
353 and definition of technical terms are essential to improve the awareness of the intricate meaning
354 of current and past scientific vocabulary, for documentation and integration into databases in
355 general, and quantitative modelling in particular (Beard 2005). The focus on coupling states
356 and fluxes through metabolic pathways of aerobic energy transformation in mitochondrial
357 preparations is a first step in the attempt to generate a conceptually-oriented nomenclature in
358 bioenergetics and mitochondrial physiology. Coupling states of intact cells, the protonmotive
359 force, and respiratory control by fuel substrates and specific inhibitors of respiratory enzymes
360 will be reviewed in subsequent communications.

2. Oxidative phosphorylation and coupling states in mitochondrial preparations

‘Every professional group develops its own technical jargon for talking about matters of critical concern ... People who know a word can share that idea with other members of their group, and a shared vocabulary is part of the glue that holds people together and allows them to create a shared culture’ (Miller 1991).

Mitochondrial preparations are defined as either isolated mitochondria, or tissue and cellular preparations in which the barrier function of the plasma membrane is disrupted. Since this entails the loss of cell viability, mitochondrial preparations are not studied *in vivo*. In contrast to isolated mitochondria and tissue homogenate preparations, mitochondria in permeabilized tissues and cells are *in situ* relative to the plasma membrane. The plasma membrane separates the intracellular compartment including the cytosol, nucleus, and organelles from the extracellular environment. The plasma membrane consists of a lipid bilayer with embedded proteins and attached organic molecules that collectively control the selective permeability of ions, organic molecules, and particles across the cell boundary. The intact plasma membrane prevents the passage of many water-soluble mitochondrial substrates and inorganic ions—such as succinate, adenosine diphosphate (ADP) and inorganic phosphate (P_i), that must be controlled at kinetically-saturating concentrations for the analysis of respiratory capacities. Despite of the activity of solute carriers, *e.g.*, SLC13A3 and SLC20A2, that transport these metabolites across the plasma membrane of various cell types, this limits the scope of investigations into mitochondrial respiratory function in intact cells (**Figure 2A**).

The cholesterol content of the plasma membrane is high compared to mitochondrial membranes. Therefore, mild detergents—such as digitonin and saponin—can be applied to selectively permeabilize the plasma membrane by interaction with cholesterol and allow free exchange of organic molecules and inorganic ions between the cytosol and the immediate cell environment, while maintaining the integrity and localization of organelles, cytoskeleton, and the nucleus. Application of optimum concentrations of permeabilization agents (mild detergents or toxins) leads to washout of cytosolic marker enzymes—such as lactate dehydrogenase—and results in the complete loss of cell viability, tested by nuclear staining using membrane-impermeable dyes, while mitochondrial function remains intact, tested by cytochrome *c* addition, for example. Respiration of isolated mitochondria remains unaltered after the addition of low concentrations of digitonin or saponin, although care should be taken when isolating mitochondria from cancer cells since they have significantly higher contents of cholesterol in both membranes (Baggetto and Testa-Perussini, 1990). In addition to mechanical cell disruption during homogenization of tissue, permeabilization agents may be applied to ensure permeabilization of all cells in tissue homogenates. Suspensions of cells permeabilized in the respiration chamber and crude tissue homogenates contain all components of the cell at highly dilute concentrations. All mitochondria are retained in chemically-permeabilized mitochondrial preparations and crude tissue homogenates. In the preparation of isolated mitochondria, however, the mitochondria are separated from other cell fractions and purified by differential centrifugation, entailing the loss of a fraction of the total mitochondrial content. Typical mitochondrial recovery ranges from 30% to 80%. Using Percoll or sucrose density gradients to maximize the purity of isolated mitochondria may compromise the mitochondrial yield or structural and functional integrity. Therefore, protocols to isolate mitochondria need to be optimized according to each study. The term mitochondrial preparation does neither include further fractionation of mitochondrial components, nor submitochondrial particles.

2.1. Respiratory control and coupling

Respiratory coupling control states are established in studies of mitochondrial preparations to obtain reference values for various output variables (**Table 1**). Physiological

412 conditions *in vivo* deviate from these experimentally obtained states. Since kinetically-
 413 saturating concentrations, *e.g.*, of ADP or oxygen (O₂; dioxygen), may not apply to
 414 physiological intracellular conditions, relevant information is obtained in studies of kinetic
 415 responses to variations in [ADP] or [O₂] in the range between kinetically-saturating
 416 concentrations and anoxia (Gnaiger 2001).

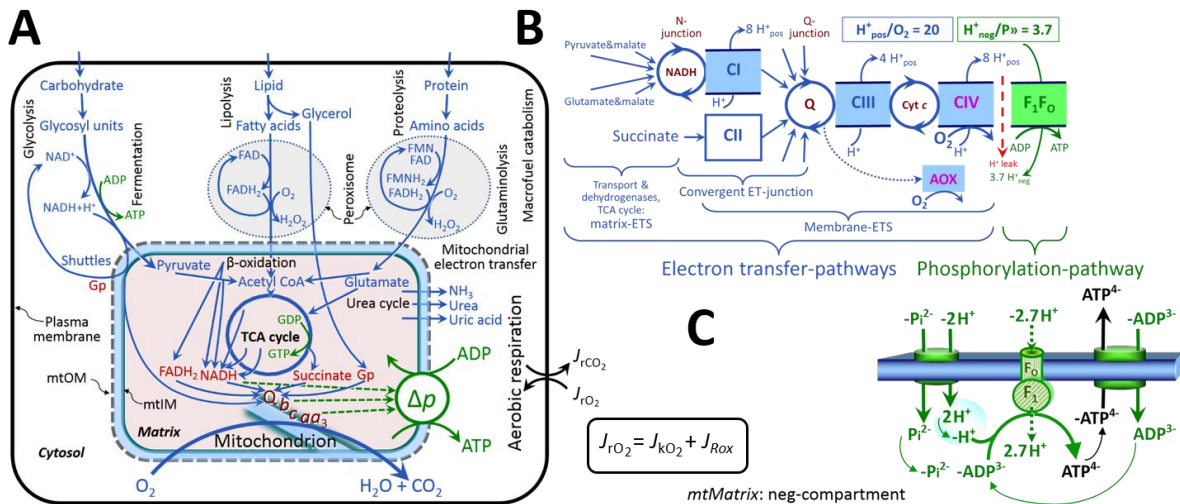
417 **The steady-state:** Mitochondria represent a thermodynamically open system in non-
 418 equilibrium states of biochemical energy transformation. State variables (protonmotive force;
 419 redox states) and metabolic *rates* (fluxes) are measured in defined mitochondrial respiratory
 420 *states*. Steady-states can be obtained only in open systems, in which changes by *internal*
 421 transformations, *e.g.*, O₂ consumption, are instantaneously compensated for by *external* fluxes,
 422 *e.g.*, O₂ supply, preventing a change of O₂ concentration in the system (Gnaiger 1993b).
 423 Mitochondrial respiratory states monitored in closed systems satisfy the criteria of pseudo-
 424 steady states for limited periods of time, when changes in the system (concentrations of O₂, fuel
 425 substrates, ADP, P_i, H⁺) do not exert significant effects on metabolic fluxes (respiration,
 426 phosphorylation). Such pseudo-steady states require respiratory media with sufficient buffering
 427 capacity and substrates maintained at kinetically-saturating concentrations, and thus depend on
 428 the kinetics of the processes under investigation.

429 **Specification of biochemical dose:** Substrates, uncouplers, inhibitors, and other
 430 chemical reagents are titrated to dissect mitochondrial function. Nominal concentrations of
 431 these substances are usually reported as initial amount of substance concentration [mol·L⁻¹] in
 432 the incubation medium. When aiming at the measurement of kinetically saturated processes—
 433 such as OXPHOS-capacities, the concentrations for substrates can be chosen according to the
 434 apparent equilibrium constant, K_m' . In the case of hyperbolic kinetics, only 80% of maximum
 435 respiratory capacity is obtained at a substrate concentration of four times the K_m' , whereas
 436 substrate concentrations of 5, 9, 19 and 49 times the K_m' are theoretically required for reaching
 437 83%, 90%, 95% or 98% of the maximal rate (Gnaiger 2001). Other reagents are chosen to
 438 inhibit or alter some processes. The amount of these chemicals in an experimental incubation
 439 is selected to maximize effect, avoiding unacceptable off-target consequences that would
 440 adversely affect the data being sought. Specifying the amount of substance in an incubation as
 441 nominal concentration in the aqueous incubation medium can be ambiguous (Doskey *et al.*
 442 2015), particularly for lipophilic substances (oligomycin, uncouplers, permeabilization agents)
 443 or cations (TPP⁺; fluorescent dyes such as safranin, TMRM; Chowdhury *et al.* 2015), which
 444 accumulate in biological membranes or in the mitochondrial matrix. For example, a dose of
 445 digitonin of 8 fmol·cell⁻¹ (10 pg·cell⁻¹; 10 μg·10⁻⁶ cells) is optimal for permeabilization of
 446 endothelial cells, and the concentration in the incubation medium has to be adjusted according
 447 to the cell density applied (Doerrier *et al.* 2018).

448 Generally, dose/exposure can be specified per unit of biological sample, *i.e.*, (nominal
 449 moles of xenobiotic)/(number of cells) [mol·cell⁻¹] or, as appropriate, per mass of biological
 450 sample [mol·kg⁻¹]. This approach to specification of dose/exposure provides a scalable
 451 parameter that can be used to design experiments, help interpret a wide variety of experimental
 452 results, and provide absolute information that allows researchers worldwide to make the most
 453 use of published data (Doskey *et al.* 2015).

454 **Phosphorylation, P_», and P_»/O₂ ratio:** *Phosphorylation* in the context of OXPHOS is
 455 defined as phosphorylation of ADP by P_i to form ATP. On the other hand, the term
 456 phosphorylation is used generally in many contexts, *e.g.*, protein phosphorylation. This justifies
 457 consideration of a symbol more discriminating and specific than P as used in the P/O ratio
 458 (phosphate to atomic oxygen ratio), where P indicates phosphorylation of ADP to ATP or GDP
 459 to GTP (**Figure 2**). We propose the symbol P_» for the endergonic (uphill) direction of
 460 phosphorylation ADP→ATP, and likewise the symbol P_« for the corresponding exergonic
 461 (downhill) hydrolysis ATP→ADP (**Figure 3**). P_» refers mainly to electrontransfer
 462 phosphorylation but may also involve substrate-level phosphorylation as part of the

463 tricarboxylic acid (TCA) cycle (succinyl-CoA ligase; phosphoglycerate kinase) and
 464 phosphorylation of ADP catalyzed by pyruvate kinase, and of GDP phosphorylated by
 465 phosphoenolpyruvate carboxykinase. Transphosphorylation is performed by adenylate kinase,
 466 creatine kinase (mtCK), hexokinase and nucleoside diphosphate kinase. In isolated mammalian
 467 mitochondria, ATP production catalyzed by adenylate kinase ($2 \text{ ADP} \leftrightarrow \text{ATP} + \text{AMP}$) proceeds
 468 without fuel substrates in the presence of ADP (Komlódi and Tretter 2017). Kinase cycles are
 469 involved in intracellular energy transfer and signal transduction for regulation of energy flux.
 470



471

472

Figure 2. Cell respiration and oxidative phosphorylation (OXPHOS)

473 Mitochondrial respiration is the oxidation of fuel substrates (electron donors) with electron
 474 transfer to O₂ as the electron acceptor. For explanation of symbols see also **Figure 1**.

475 **(A)** Respiration of intact cells: Extra-mitochondrial catabolism of macrofuels or uptake of small
 476 molecules by the cell provides the *mitochondrial* fuel substrates. Many fuel substrates are
 477 catabolized to acetyl-CoA or to glutamate, and further electron transfer reduces nicotinamide
 478 adenine dinucleotide to NADH or flavin adenine dinucleotide to FADH₂. In respiration,
 479 electron transfer is coupled to the phosphorylation of ADP to ATP, with energy transformation
 480 mediated by the protonmotive force, Δp . Anabolic reactions are linked to catabolism, both by
 481 ATP as the intermediary energy currency and by small organic precursor molecules as building
 482 blocks for biosynthesis (not shown). Glycolysis involves substrate-level phosphorylation of
 483 ADP to ATP in fermentation without utilization of O₂. In contrast, extra-mitochondrial
 484 oxidation of fatty acids and amino acids proceeds partially in peroxisomes without coupling to
 485 ATP production: acyl-CoA oxidase catalyzes the oxidation of FADH₂ with electron transfer to
 486 O₂; amino acid oxidases oxidize flavin mononucleotide FMNH₂ or FADH₂. Coenzyme Q, Q,
 487 and the cytochromes *b*, *c*, and *aa*₃ are redox systems of the mitochondrial inner membrane,
 488 mtIM. Dashed arrows indicate the connection between the redox proton pumps (respiratory
 489 Complexes CI, CIII and CIV) and the transmembrane Δp . Mitochondrial outer membrane,
 490 mtOM; glycerol-3-phosphate, Gp; tricarboxylic acid cycle, TCA cycle.

491 **(B)** Respiration in mitochondrial preparations: The mitochondrial electron transfer system
 492 (ETS) is (1) fuelled by diffusion and transport of substrates across the mitochondrial outer and
 493 inner membrane, and in addition consists of the (2) matrix-ETS, and (3) membrane-ETS.
 494 Upstream sections of ET-pathways converge at the N-junction. NADH mainly generated in the
 495 TCA cycle is oxidized by CI and electron entry into the Q-junction. Similarly, succinate is
 496 formed in the TCA cycle and oxidized by CII to fumarate. CII is part of both the TCA cycle
 497 and the ETS, and reduces FAD to FADH₂ with further reduction of ubiquinone to ubiquinol
 498 downstream of the TCA cycle in the Q-junction. Thus FADH₂ is not a substrate but is the
 499 product of CII, in contrast to erroneous metabolic maps shown in many textbooks and
 500 publications. Unspecified arrows converging at the Q-junction indicate additional ET-sections

501 with electron entry into Q through electron transferring flavoprotein, glycerophosphate
 502 dehydrogenase, dihydro-orotate dehydrogenase, proline dehydrogenase, choline
 503 dehydrogenase, and sulfide-ubiquinone oxidoreductase. The dotted arrow indicates the
 504 branched pathway of oxygen consumption by alternative quinol oxidase (AOX). ET-pathways
 505 are coupled to the phosphorylation-pathway. The H^+_{pos}/O_2 ratio is the outward proton flux from
 506 the matrix space to the positively (pos) charged vesicular compartment, divided by catabolic
 507 O_2 flux in the NADH-pathway. The H^+_{neg}/P_{\gg} ratio is the inward proton flux from the inter-
 508 membrane space to the negatively (neg) charged matrix space, divided by the flux of
 509 phosphorylation of ADP to ATP. These stoichiometries are not fixed due to ion leaks and proton
 510 slip.

511 (C) Chemiosmotic phosphorylation-pathway catalyzed by the proton pump F_1F_0 -ATPase (F-
 512 ATPase, ATP synthase), adenine nucleotide translocase, and inorganic phosphate transporter.
 513 The H^+_{neg}/P_{\gg} stoichiometry is the sum of the coupling stoichiometry in the F-ATPase reaction
 514 ($-2.7 H^+_{\text{pos}}$ from the positive intermembrane space, $2.7 H^+_{\text{neg}}$ to the matrix, *i.e.*, the negative
 515 compartment) and the proton balance in the translocation of ADP^{3-} , ATP^{4-} and P_i^{2-} . Modified
 516 from (B) Lemieux *et al.* (2017) and (C) Gnaiger (2014).

517
 518 The P_{\gg}/O_2 ratio ($P_{\gg}/4 e^-$) is two times the ‘P/O’ ratio ($P_{\gg}/2 e^-$) of classical bioenergetics.
 519 P_{\gg}/O_2 is a generalized symbol, not specific for determination of P_i consumption (P_i/O_2 flux
 520 ratio), ADP depletion (ADP/ O_2 flux ratio), or ATP production (ATP/ O_2 flux ratio). The
 521 mechanistic P_{\gg}/O_2 ratio—or P_{\gg}/O_2 stoichiometry—is calculated from the proton-to- O_2 and
 522 proton-to-phosphorylation coupling stoichiometries (**Figure 2B**):
 523

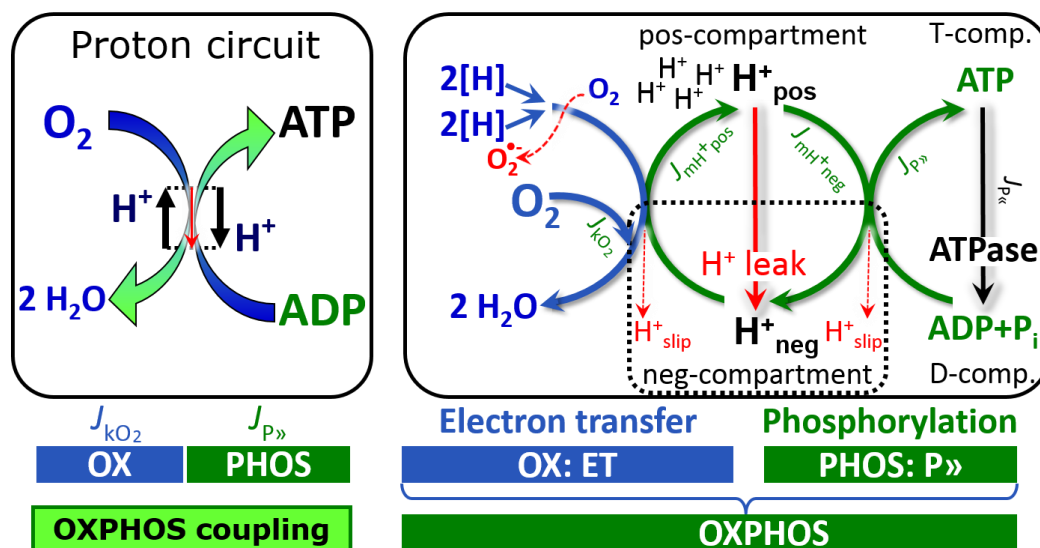
$$524 \quad P_{\gg}/O_2 = \frac{H^+_{\text{pos}}/O_2}{H^+_{\text{neg}}/P_{\gg}} \quad (1)$$

525
 526 The H^+_{pos}/O_2 coupling stoichiometry (referring to the full 4 electron reduction of O_2) depends
 527 on the relative involvement of the three coupling sites (respiratory Complexes I, III and IV; CI,
 528 CIII and CIV) in the catabolic ET-pathway from reduced fuel substrates (electron donors) to
 529 the reduction of O_2 (electron acceptor). This varies with: (1) a bypass of CI by single or multiple
 530 electron input into the Q-junction; and (2) a bypass of CIV by involvement of alternative
 531 oxidases, AOX, which are not expressed in mammalian mitochondria.

532 H^+_{pos}/O_2 is 12 in the ET-pathways involving CIII and CIV as proton pumps, increasing to
 533 20 for the NADH-pathway through CI (**Figure 2B**), but a general consensus on H^+_{pos}/O_2
 534 stoichiometries remains to be reached (Hinkle 2005; Wikström and Hummer 2012; Sazanov
 535 2015). The H^+_{neg}/P_{\gg} coupling stoichiometry (3.7; **Figure 2B**) is the sum of 2.7 H^+_{neg} required
 536 by the F-ATPase of vertebrate and most invertebrate species (Watt *et al.* 2010) and the proton
 537 balance in the translocation of ADP, ATP and P_i (**Figure 2C**). Taken together, the mechanistic
 538 P_{\gg}/O_2 ratio is calculated at 5.4 and 3.3 for NADH- and succinate-linked respiration, respectively
 539 (Eq. 1). The corresponding classical P \gg /O ratios (referring to the 2 electron reduction of 0.5 O_2)
 540 are 2.7 and 1.6 (Watt *et al.* 2010), in agreement with the measured P \gg /O ratio for succinate of
 541 1.58 ± 0.02 (Gnaiger *et al.* 2000).

542 The effective P_{\gg}/O_2 flux ratio ($Y_{P_{\gg}/O_2} = J_{P_{\gg}}/J_{kO_2}$; **Figure 3**) is diminished relative to the
 543 mechanistic P_{\gg}/O_2 ratio by intrinsic and extrinsic uncoupling and dyscoupling (**Figure 4**). Such
 544 generalized uncoupling is different from switching to mitochondrial pathways that involve
 545 fewer than three proton pumps (‘coupling sites’: Complexes CI, CIII and CIV), bypassing CI
 546 through multiple electron entries into the Q-junction, or CIII and CIV through AOX (**Figure**
 547 **2B**). Reprogramming of mitochondrial pathways leading to different types of substrates being
 548 oxidized may be considered as a switch of gears (changing the stoichiometry by altering the
 549 substrate that is oxidized) rather than uncoupling (loosening the tightness of coupling relative
 550 to a fixed stoichiometry). In addition, Y_{P_{\gg}/O_2} depends on several experimental conditions of flux
 551 control, increasing as a hyperbolic function of [ADP] to a maximum value (Gnaiger 2001).

552 **Control and regulation:** The terms metabolic *control* and *regulation* are frequently used
 553 synonymously, but are distinguished in metabolic control analysis: ‘We could understand the
 554 regulation as the mechanism that occurs when a system maintains some variable constant over
 555 time, in spite of fluctuations in external conditions (homeostasis of the internal state). On the
 556 other hand, metabolic control is the power to change the state of the metabolism in response to
 557 an external signal’ (Fell 1997). Respiratory control may be induced by experimental control
 558 signals that *exert* an influence on: (1) ATP demand and ADP phosphorylation-rate; (2) fuel
 559 substrate composition, pathway competition; (3) available amounts of substrates and O₂, *e.g.*,
 560 starvation and hypoxia; (4) the protonmotive force, redox states, flux–force relationships,
 561 coupling and efficiency; (5) Ca²⁺ and other ions including H⁺; (6) inhibitors, *e.g.*, nitric oxide
 562 or intermediary metabolites such as oxaloacetate; (7) signalling pathways and regulatory
 563 proteins, *e.g.*, insulin resistance, transcription factor hypoxia inducible factor 1. *Mechanisms* of
 564 respiratory control and regulation include adjustments of: (1) enzyme activities by allosteric
 565 mechanisms and phosphorylation; (2) enzyme content, concentrations of cofactors and
 566 conserved moieties—such as adenylates, nicotinamide adenine dinucleotide [NAD⁺/NADH],
 567 coenzyme Q, cytochrome *c*; (3) metabolic channeling by supercomplexes; and (4)
 568 mitochondrial density (enzyme concentrations and membrane area) and morphology (cristae
 569 folding, fission and fusion). Mitochondria are targeted directly by hormones, thereby affecting
 570 their energy metabolism (Lee *et al.* 2013; Gerö and Szabo 2016; Price and Dai 2016; Moreno
 571 *et al.* 2017). Evolutionary or acquired differences in the genetic and epigenetic basis of
 572 mitochondrial function (or dysfunction) between individuals; age; gender, biological sex, and
 573 hormone concentrations; life style including exercise and nutrition; and environmental issues
 574 including thermal, atmospheric, toxic and pharmacological factors, exert an influence on all
 575 control mechanisms listed above. For reviews, see Brown 1992; Gnaiger 1993a, 2009; 2014;
 576 Paradies *et al.* 2014; Morrow *et al.* 2017.
 577



578 **Figure 3. Coupling in oxidative phosphorylation (OXPHOS)**

579 2[H] indicates the reduced hydrogen equivalents of fuel substrates of the catabolic reaction *k*
 580 with oxygen. O₂ flux, J_{kO₂}, through the catabolic ET-pathway, is coupled to flux through the
 581 phosphorylation-pathway of ADP to ATP, J_{P»}. The redox proton pumps of the ET-pathway
 582 drive proton flux into the positive (pos) compartment, J_{mH⁺pos}, generating the output
 583 protonmotive force (motive, subscript *m*). F-ATPase is coupled to inward proton current into
 584 the negative (neg) compartment, J_{mH⁺neg}, to phosphorylate ADP to ATP. The system is defined
 585 by the boundaries (full black line) and is not a black box, but is analysed as a compartmental
 586 system. The negative compartment (neg–compartment, enclosed by the dotted line) is the
 587 matrix space, separated by the mtIM from the positive compartment (pos–compartment).

588 ADP+P_i and ATP are the substrate- and product-compartments (scalar ADP and ATP
 589 compartments, D-comp. and T-comp.), respectively. At steady-state proton turnover, $J_{\infty H^+}$, and
 590 ATP turnover, $J_{\infty P}$, maintain concentrations constant, when $J_{mH^+\infty} = J_{mH^+pos} = J_{mH^+neg}$, and $J_{P\infty}$
 591 = $J_{P\gg} = J_{P\ll}$. Modified from Gnaiger (2014).

592

593 **Respiratory control and response:** Lack of control by a metabolic pathway, *e.g.*,
 594 phosphorylation-pathway, means that there will be no response to a variable activating it, *e.g.*,
 595 [ADP]. The reverse, however, is not true as the absence of a response to [ADP] does not exclude
 596 the phosphorylation-pathway from having some degree of control. The degree of control of a
 597 component of the OXPHOS-pathway on an output variable—such as O₂ flux, will in general
 598 be different from the degree of control on other outputs—such as phosphorylation-flux or
 599 proton leak flux. Therefore, it is necessary to be specific as to which input and output are under
 600 consideration (Fell 1997).

601 **Respiratory coupling control and ET-pathway control:** Respiratory control refers to
 602 the ability of mitochondria to adjust O₂ flux in response to external control signals by engaging
 603 various mechanisms of control and regulation. Respiratory control is monitored in a
 604 mitochondrial preparation under conditions defined as respiratory states, preferentially under
 605 near-physiological conditions of pH, temperature and medium ionic composition, to generate
 606 data of higher biological relevance. When phosphorylation of ADP to ATP is stimulated or
 607 depressed, an increase or decrease is observed in electron transfer measured as O₂ flux in
 608 respiratory coupling states of intact mitochondria ('controlled states' in the classical
 609 terminology of bioenergetics). Alternatively, coupling of electron transfer with phosphorylation
 610 is disengaged by uncouplers. These protonophores are weak lipid-soluble acids which disrupt
 611 the barrier function of the mtIM and thus shortcircuit the protonmotive system, functioning like
 612 a clutch in a mechanical system. The corresponding coupling control state is characterized by
 613 a high O₂ flux without control by P_» (noncoupled or 'uncontrolled state').

614 ET-pathway control states are obtained in mitochondrial preparations by depletion of
 615 endogenous substrates and addition to the mitochondrial respiration medium of fuel substrates
 616 (**Figure 2**; 2[H] in **Figure 3**) and specific inhibitors, activating selected mitochondrial catabolic
 617 pathways, *k*, of electron transfer from the oxidation of fuel substrates to reduction of O₂ (**Figure**
 618 **2A**). Coupling control states and pathway control states are complementary, since
 619 mitochondrial preparations depend on an exogenous supply of pathway-specific fuel substrates
 620 and oxygen (Gnaiger 2014).

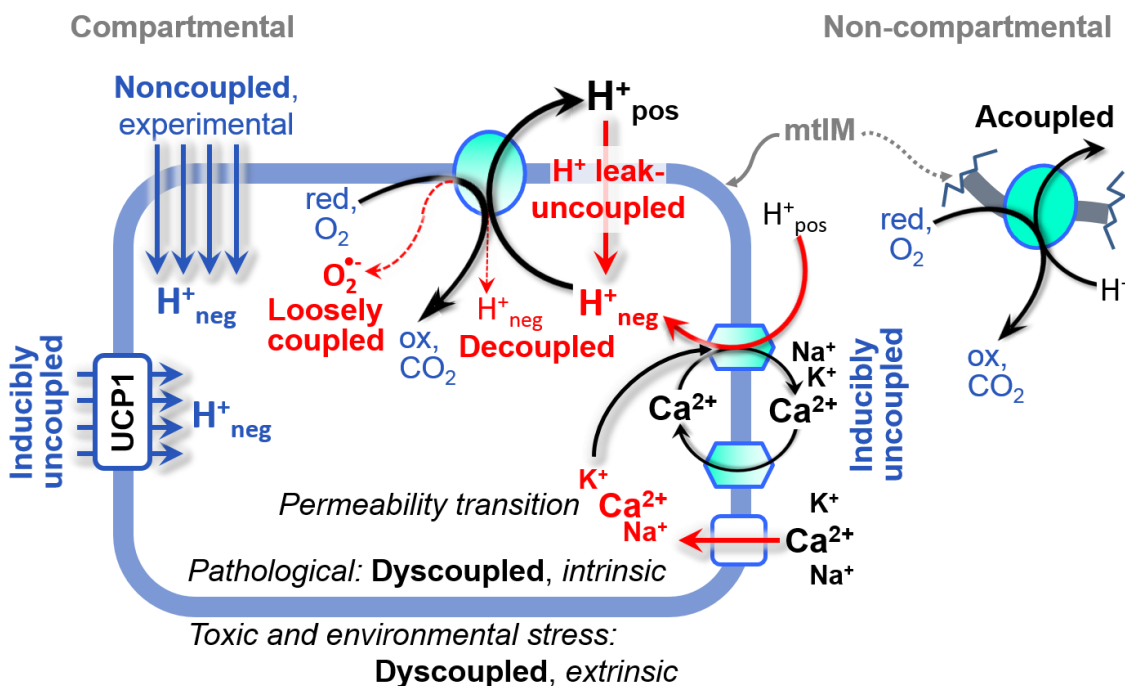
621 **Coupling:** In mitochondrial electron transfer, vectorial transmembrane proton flux is
 622 coupled through the redox proton pumps CI, CIII and CIV to the catabolic flux of scalar
 623 reactions, collectively measured as O₂ flux (**Figure 3**). Thus mitochondria are elements of
 624 energy transformation. Energy is a conserved quantity and cannot be lost or produced in any
 625 internal process (First Law of thermodynamics). Open and closed systems can gain or lose
 626 energy only by external fluxes—by exchange with the environment. Therefore, energy can
 627 neither be produced by mitochondria, nor is there any internal process without energy
 628 conservation. Exergy or Gibbs energy ('free energy') is the part of energy that can potentially
 629 be transformed into work under conditions of constant volume and pressure. *Coupling* is the
 630 interaction of an exergonic process (spontaneous, negative exergy change) with an endergonic
 631 process (positive exergy change) in energy transformations which conserve part of the exergy
 632 that would be irreversibly lost or dissipated in an uncoupled process.

633 **Uncoupling:** Uncoupling of mitochondrial respiration is a general term comprising
 634 diverse mechanisms:

- 635 1. Proton leak across the mtIM from the pos- to the neg-compartment (H⁺ leak-
 636 uncoupled; **Figure 4**).

- 637 2. Cycling of other cations, strongly stimulated by permeability transition; comparable
 638 to the use of protonophores, cation cycling is experimentally induced by valinomycin
 639 in the presence of K^+ ;
 640 3. Decoupling by proton slip in the redox proton pumps when protons are effectively not
 641 pumped (CI, CIII and CIV) or are not driving phosphorylation (F-ATPase);
 642 4. Loss of vesicular (compartmental) integrity when electron transfer is coupled;
 643 5. Electron leak in the loosely coupled univalent reduction of O_2 to superoxide ($O_2^{\cdot-}$;
 644 superoxide anion radical).

645 Differences of terms—uncoupled vs. noncoupled—are easily overlooked, although they relate
 646 to different meanings of uncoupling (**Figure 4** and **Table 2**).



649 **Figure 4. Mechanisms of respiratory uncoupling**
 650 An intact mitochondrial inner membrane, mtIM, is required for vectorial, compartmental
 651 coupling. ‘Acoupled’ respiration is the consequence of structural disruption with catalytic
 652 activity of non-compartmental mitochondrial fragments. Inducibly uncoupled (activation of
 653 UCP1) and experimentally noncoupled respiration (titration of protonophores) stimulate
 654 respiration to maximum O_2 flux. H^+ leak-uncoupled, decoupled, and loosely coupled respiration
 655 are components of intrinsic uncoupling. Pathological dysfunction may affect all types of
 656 uncoupling, including permeability transition, causing intrinsically dyscoupled respiration.
 657 Similarly, toxicological and environmental stress factors can cause extrinsically dyscoupled
 658 respiration.

662 2.2. Coupling states and respiratory rates

663
 664 **Respiratory capacities in coupling control states:** To extend the classical nomenclature
 665 on mitochondrial coupling states (Section 2.3) by a concept-driven terminology that explicitly
 666 incorporates information on the meaning of respiratory states, the terminology must be general
 667 and not restricted to any particular experimental protocol or mitochondrial preparation (Gnaiger
 668 2009). Concept-driven nomenclature aims at mapping the *meaning and concept behind the*
 669 words and acronyms onto the *forms* of words and acronyms (Miller 1991). The focus of

670 concept-driven nomenclature is primarily the conceptual ‘why’, along with clarification of the
 671 experimental ‘how’. Respiratory capacities delineate, comparable to channel capacity in
 672 information theory (Schneider 2006), the upper bound of the rate of respiration measured in
 673 defined coupling control states and electron transfer-pathway (ET-pathway) states (**Figure 5**).

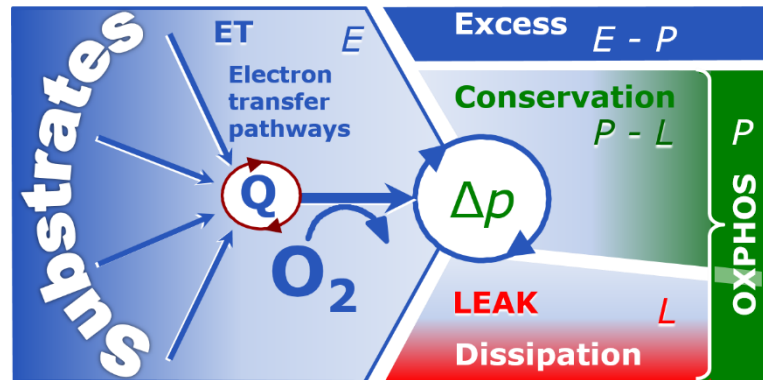
674
 675

676 **Figure 5. Four-compartment**
 677 **model of oxidative**
 678 **phosphorylation**

679 Respiratory states (ET,
 680 OXPHOS, LEAK; **Table 1**) and
 681 corresponding rates (E , P , L) are
 682 connected by the protonmotive
 683 force, Δp . ET-capacity, E (I), is
 684 partitioned into (2) dissipative
 685 LEAK-respiration, L , when the
 686 Gibbs energy change of catabolic

687 O_2 flux is irreversibly lost, (3) net OXPHOS-capacity, $P-L$, with partial conservation of the
 688 capacity to perform work, and (4) the excess capacity, $E-P$. Modified from Gnaiger (2014).

689
 690



691
 692

691 To provide a diagnostic reference for respiratory capacities of core energy metabolism,
 692 the capacity of *oxidative phosphorylation*, OXPHOS, is measured at kinetically-saturating
 693 concentrations of ADP and P_i . The *oxidative* ET-capacity reveals the limitation of OXPHOS-
 694 capacity mediated by the *phosphorylation*-pathway. The ET- and phosphorylation-pathways
 695 comprise coupled segments of the OXPHOS-system. ET-capacity is measured as noncoupled
 696 respiration by application of *external uncouplers*. The contribution of *intrinsically uncoupled*
 697 O_2 consumption is studied by preventing the stimulation of phosphorylation either in the
 698 absence of ADP or by inhibition of the phosphorylation-pathway. The corresponding states are
 699 collectively classified as LEAK-states, when O_2 consumption compensates mainly for ion
 700 leaks, including the proton leak. Defined coupling states are induced by: (1) adding cation
 701 chelators such as EGTA, binding free Ca^{2+} and thus limiting cation cycling; (2) adding ADP
 702 and P_i ; (3) inhibiting the phosphorylation-pathway; and (4) uncoupler titrations, while
 703 maintaining a defined ET-pathway state with constant fuel substrates and inhibitors of specific
 704 branches of the ET-pathway (**Figure 5**).

705 The three coupling states, ET, LEAK and OXPHOS, are shown schematically with the
 706 corresponding respiratory rates, abbreviated as E , L and P , respectively (**Figure 5**). We
 707 distinguish metabolic *pathways* from metabolic *states* and the corresponding metabolic *rates*;
 708 for example: ET-pathways, ET-states, and ET-capacities, E , respectively (**Table 1**). The
 709 protonmotive force is *high* in the OXPHOS-state when it drives phosphorylation, *maximum* in
 710 the LEAK-state of coupled mitochondria, driven by LEAK-respiration at a minimum back-flux
 711 of cations to the matrix side, and *very low* in the ET-state when uncouplers short-circuit the
 712 proton cycle (**Table 1**).

713

714 **LEAK-state (Figure 6A):**
 715 The LEAK-state is defined as a
 716 state of mitochondrial respiration
 717 when O_2 flux mainly
 718 compensates for ion leaks in the
 719 absence of ATP synthesis, at
 720 kinetically-saturating
 721 concentrations of O_2 , respiratory
 722 fuel substrates and P_i . LEAK-
 723 respiration is measured to obtain
 724 an estimate of *intrinsic*
 725 *uncoupling* without addition of an
 726 experimental uncoupler: (1) in the
 727 absence of adenylates, *i.e.*, AMP,
 728 ADP and ATP; (2) after depletion
 729 of ADP at a maximum ATP/ADP
 730 ratio; or (3) after inhibition of the
 731 phosphorylation-pathway by
 732 inhibitors of F-ATPase—such as
 733 oligomycin, or of adenine
 734 nucleotide translocase—such as
 735 carboxyatractyloside.
 736 Adjustment of the nominal
 737 concentration of these inhibitors
 738 to the density of biological
 739 sample applied can minimize or
 740 avoid inhibitory side-effects
 741 exerted on ET-capacity or even
 742 some dyscoupling.

743 **Proton leak and**
 744 **uncoupled respiration:** Proton
 745 leak is a leak current of protons.
 746 The intrinsic proton leak is the
 747 *uncoupled* process in which
 748 protons diffuse across the mtIM
 749 in the dissipative direction of the
 750 downhill protonmotive force
 751 without coupling to
 752 phosphorylation (Figure 6A).
 753 The proton leak flux depends
 754 non-linearly on the
 755 protonmotive force (Garlid *et al.*
 756 1989; Divakaruni and Brand
 757 2011), it is a temperature-
 758 dependent property of the mtIM
 759 and may be enhanced due to
 760 possible contaminations by free
 761 fatty acids. Inducible uncoupling
 762 mediated by uncoupling protein 1
 763 (UCP1) is physiologically
 controlled, *e.g.*, in brown adipose
 tissue. UCP1 is a member of the
 mitochondrial carrier family that
 is involved in the translocation of
 protons across the mtIM (Klingenberg
 2017).

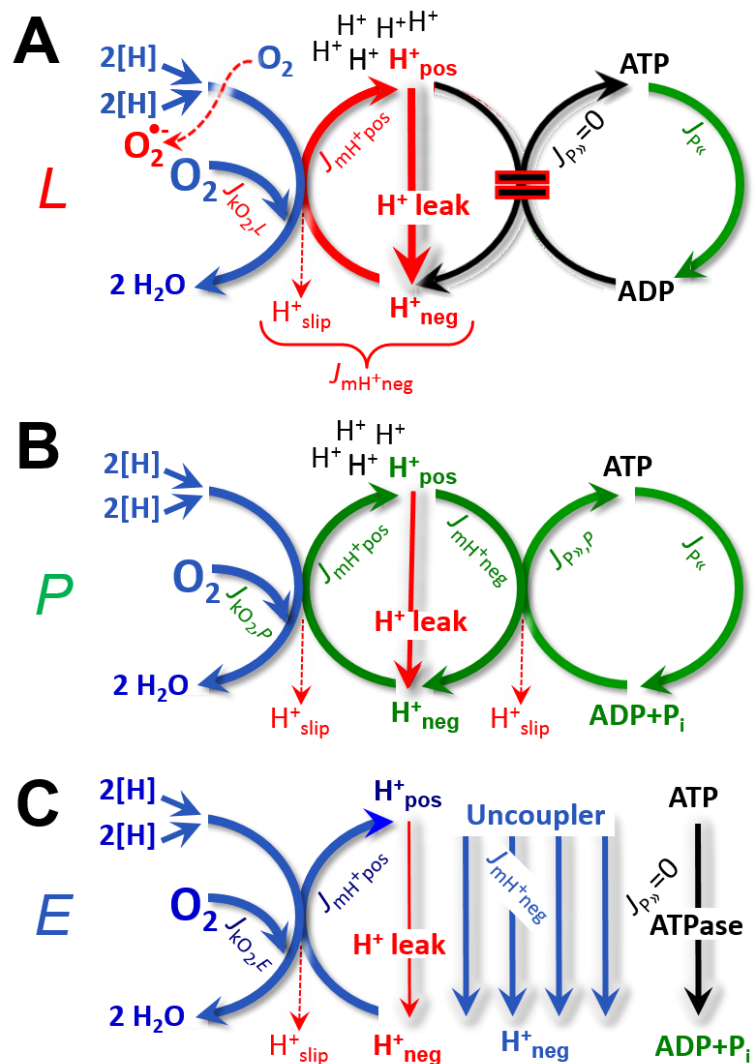


Figure 6. Respiratory coupling states

(A) **LEAK-state and rate, L:** Phosphorylation is arrested, $J_{P \gg} = 0$, and catabolic O_2 flux, $J_{kO_2,L}$, is controlled mainly by the proton leak, $J_{mH^+neg,L}$, at maximum protonmotive force (Figure 4). Extramitochondrial ATP may be hydrolyzed by extramitochondrial ATPases, $J_{P \ll}$.

(B) **OXPHOS-state and rate, P:** Phosphorylation, $J_{P \gg,P}$, is stimulated by kinetically-saturating [ADP] and [P_i], and is supported by a high protonmotive force. O_2 flux, $J_{kO_2,P}$, is well-coupled at a $P \gg / O_2$ ratio of $J_{P \gg,P} / J_{kO_2,P}$. Extramitochondrial ATPases may recycle ATP, $J_{P \ll}$.

(C) **ET-state and rate, E:** Noncoupled respiration, $J_{kO_2,E}$, is maximum at optimum exogenous uncoupler concentration and phosphorylation is zero, $J_{P \gg} = 0$. The F-ATPase may hydrolyze extramitochondrial ATP. See also Figure 3.

764 Consequently, the short-circuit diminishes the protonmotive force and stimulates electron
765 transfer to O₂ and heat dissipation without phosphorylation of ADP.

766 **Cation cycling:** There can be other cation contributors to leak current including calcium
767 and probably magnesium. Calcium influx is balanced by mitochondrial Na⁺/Ca²⁺ or H⁺/Ca²⁺
768 exchange, which is balanced by Na⁺/H⁺ or K⁺/H⁺ exchanges. This is another effective
769 uncoupling mechanism different from proton leak (**Table 2**).

770

771 **Table 1. Coupling states and residual oxygen consumption in mitochondrial**
772 **preparations in relation to respiration- and phosphorylation-flux, J_{kO_2} and $J_{P\gg}$,**
773 **and protonmotive force, Δp .** Coupling states are established at kinetically-
774 saturating concentrations of fuel substrates and O₂.

State	J_{kO_2}	$J_{P\gg}$	Δp	Inducing factors	Limiting factors
LEAK	L ; low, cation leak-dependent respiration	0	max.	back-flux of cations including proton leak, proton slip	$J_{P\gg} = 0$: (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]	$J_{P\gg}$ 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	inhibition of all ET-pathways; or absence of fuel substrates

775

776 **Proton slip and decoupled respiration:** Proton slip is the *decoupled* process in which
777 protons are only partially translocated by a redox proton pump of the ET-pathways and slip
778 back to the original vesicular compartment. The proton leak is the dominant contributor to the
779 overall leak current in mammalian mitochondria incubated under physiological conditions at
780 37 °C, whereas proton slip is increased at lower experimental temperature (Canton *et al.* 1995).
781 Proton slip can also happen in association with the F-ATPase, in which the proton slips downhill
782 across the pump to the matrix without contributing to ATP synthesis. In each case, proton slip
783 is a property of the proton pump and increases with the pump turnover rate.

784 **Electron leak and loosely coupled respiration:** Superoxide production by the ETS leads
785 to a bypass of redox proton pumps and correspondingly lower P_»/O₂ ratio. This depends on the
786 actual site of electron leak and the scavenging of hydrogen peroxide by cytochrome *c*, whereby
787 electrons may re-enter the ETS with proton translocation by CIV.

788 **Loss of compartmental integrity and acoupled respiration:** Electron transfer and catabolic
789 O₂ flux proceed without compartmental proton translocation in disrupted mitochondrial
790 fragments. Such fragments form during mitochondrial isolation, and may not fully fuse to re-
791 establish structurally intact mitochondria. Loss of mtIM integrity, therefore, is the cause of
792 acoupled respiration, which is a nonvectorial dissipative process without control by the
793 protonmotive force.

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Table 2. Terms on respiratory coupling and uncoupling.

Term	J_{kO_2}	P_{\gg}/O_2	Note	
acoupled		0	electron transfer in mitochondrial fragments without vectorial proton translocation (Figure 4)	
intrinsic, no protonophore added	uncoupled	L	0	non-phosphorylating LEAK-respiration (Figure 6A)
	proton leak-uncoupled		0	component of L , H^+ diffusion across the mtIM (Figure 4)
	decoupled		0	component of L , proton slip (Figure 4)
	loosely coupled		0	component of L , lower coupling due to superoxide formation and bypass of proton pumps (Figure 4)
	dyscoupled		0	pathologically, toxicologically, environmentally increased uncoupling, mitochondrial dysfunction
	inducibly uncoupled		0	by UCP1 or cation (<i>e.g.</i> , Ca^{2+}) cycling (Figure 4)
noncoupled	E	0	non-phosphorylating respiration stimulated to maximum flux at optimum exogenous uncoupler concentration (Figure 6C)	
well-coupled	P	high	phosphorylating respiration with an intrinsic LEAK component (Figure 6B)	
fully coupled	$P - L$	max.	OXPPOS-capacity corrected for LEAK-respiration (Figure 5)	

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Dyscoupled respiration: Mitochondrial injuries may lead to *dyscoupling* as a pathological or toxicological cause of *uncoupled* respiration. Dyscoupling may involve any type of uncoupling mechanism, *e.g.*, opening the permeability transition pore. Dyscoupled respiration is distinguished from the experimentally induced *noncoupled* respiration in the ET-state (**Table 2**).

OXPPOS-state (Figure 6B): The OXPPOS-state is defined as the respiratory state with kinetically-saturating concentrations of O_2 , respiratory and phosphorylation substrates, and absence of exogenous uncoupler, which provides an estimate of the maximal respiratory capacity in the OXPPOS-state for any given ET-pathway state. Respiratory capacities at kinetically-saturating substrate concentrations provide reference values or upper limits of performance, aiming at the generation of data sets for comparative purposes. Physiological activities and effects of substrate kinetics can be evaluated relative to the OXPPOS-capacity.

As discussed previously, 0.2 mM ADP does not fully saturate flux in isolated mitochondria (Gnaiger 2001; Puchowicz *et al.* 2004); greater ADP concentration is required, particularly in permeabilized muscle fibres and cardiomyocytes, to overcome limitations by intracellular diffusion and by the reduced conductance of the mtOM (Jepihhina *et al.* 2011, Illaste *et al.* 2012, Simson *et al.* 2016), either through interaction with tubulin (Rostovtseva *et al.* 2008) or other intracellular structures (Birkedal *et al.* 2014). In addition, saturating ADP concentrations need to be evaluated under different experimental conditions such as temperature (Lemieux *et al.* 2017) and with different animal models (Blier and Guderley, 1993). In permeabilized muscle fibre bundles of high respiratory capacity, the apparent K_m for ADP increases up to 0.5 mM (Saks *et al.* 1998), consistent with experimental evidence that >90% saturation is reached only at >5 mM ADP (Pesta and Gnaiger 2012). Similar ADP concentrations are also required for accurate determination of OXPPOS-capacity in human

821 clinical cancer samples and permeabilized cells (Klepinin *et al.* 2016; Koit *et al.* 2017).
 822 Whereas 2.5 to 5 mM ADP is sufficient to obtain the actual OXPHOS-capacity in many types
 823 of permeabilized tissue and cell preparations, experimental validation is required in each
 824 specific case.

825 **Electron transfer-state (Figure 6C):** O₂ flux determined in the ET-state yields an
 826 estimate of ET-capacity. The ET-state is defined as the *noncoupled* state with kinetically-
 827 saturating concentrations of O₂, respiratory substrate and optimum *exogenous* uncoupler
 828 concentration for maximum O₂ flux. As a consequence of the nearly collapsed protonmotive
 829 force, the driving force is insufficient for phosphorylation, and $J_{P\gg} = 0$. The most frequently
 830 used uncouplers are carbonyl cyanide m-chloro phenyl hydrazone, carbonyl cyanide *p*-
 831 trifluoromethoxyphenylhydrazone or dinitrophenol (CCCP, FCCP, DNP). Stepwise titration
 832 of uncouplers stimulates respiration up to or above the level of O₂ consumption rates in the
 833 OXPHOS-state, but inhibition of respiration is observed above optimum uncoupler
 834 concentrations (Mitchell 2011). Data obtained with a single dose of uncoupler must be
 835 evaluated with caution, particularly when a fixed uncoupler concentration is used in studies
 836 exploring a treatment or disease that may alter the mitochondrial content or mitochondrial
 837 sensitivity to inhibition by uncouplers. The effect on ET-capacity of the reversed function of F-
 838 ATPase ($J_{P\ll}$; Figure 6C) can be evaluated in the presence and absence of extramitochondrial
 839 ATP.

840 **ROX state and *Rox*:** Besides the three fundamental coupling states of mitochondrial
 841 preparations, the state of residual O₂ consumption, ROX, is relevant to assess respiratory
 842 function (Figure 1). ROX is not a coupling state. The rate of residual oxygen consumption,
 843 *Rox*, is defined as O₂ consumption due to oxidative reactions measured after inhibition of ET—
 844 with rotenone, malonic acid and antimycin A. Cyanide and azide inhibit not only CIV but
 845 catalase and several peroxidases involved in *Rox*. However, high concentrations of antimycin
 846 A, but not rotenone or cyanide, inhibit peroxisomal acyl-CoA oxidase and D-amino acid
 847 oxidase (Vamecq *et al.* 1987). ROX represents a baseline that is used to correct respiration
 848 measured in defined coupling states. *Rox*-corrected *L*, *P* and *E* not only lower the values of total
 849 fluxes, but also changes the flux control ratios *L/P* and *L/E*. *Rox* is not necessarily equivalent
 850 to non-mitochondrial reduction of O₂, considering O₂-consuming reactions in mitochondria that
 851 are not related to ET—such as O₂ consumption in reactions catalyzed by monoamine oxidases
 852 (type A and B), monooxygenases (cytochrome P450 monooxygenases), dioxygenase (sulfur
 853 dioxygenase and trimethyllysine dioxygenase), and several hydroxylases. Even isolated
 854 mitochondrial fractions, especially those obtained from liver, may be contaminated by
 855 peroxisomes. This fact makes the exact determination of mitochondrial O₂ consumption and
 856 mitochondria-associated generation of reactive oxygen species complicated (Schönfeld *et al.*
 857 2009; Speijer 2016; Figure 2). The dependence of ROX-linked O₂ consumption needs to be
 858 studied in detail together with non-ET enzyme activities, availability of specific substrates, O₂
 859 concentration, and electron leakage leading to the formation of reactive oxygen species.

860 **Quantitative relations:** *E* may exceed or be equal to *P*. $E > P$ is observed in many types
 861 of mitochondria, varying between species, tissues and cell types (Gnaiger 2009). $E - P$ is the
 862 excess ET-capacity pushing the phosphorylation-flux (Figure 2C) to the limit of its *capacity of*
 863 *utilizing* the protonmotive force. In addition, the magnitude of $E - P$ depends on the tightness of
 864 respiratory coupling or degree of uncoupling, since an increase of *L* causes *P* to increase
 865 towards the limit of *E*. The *excess E-P* capacity, $E - P$, therefore, provides a sensitive diagnostic
 866 indicator of specific injuries of the phosphorylation-pathway, under conditions when *E* remains
 867 constant but *P* declines relative to controls (Figure 5). Substrate cocktails supporting
 868 simultaneous convergent electron transfer to the Q-junction for reconstitution of TCA cycle
 869 function establish pathway control states with high ET-capacity, and consequently increase the
 870 sensitivity of the $E - P$ assay.

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

878 The net OXPHOS-capacity is calculated by subtracting L from P (**Figure 5**). The net
 879 $P \gg O_2$ equals $P \gg / (P - L)$, wherein the dissipative LEAK component in the OXPHOS-state may
 880 be overestimated. This can be avoided by measuring LEAK-respiration in a state when the
 881 protonmotive force is adjusted to its slightly lower value in the OXPHOS-state—by titration of
 882 an ET inhibitor (Divakaruni and Brand 2011). Any turnover-dependent components of proton
 883 leak and slip, however, are underestimated under these conditions (Garlid *et al.* 1993). In
 884 general, it is inappropriate to use the term *ATP production* or *ATP turnover* for the difference
 885 of O₂ flux measured in the OXPHOS and LEAK states. $P - L$ is the upper limit of OXPHOS-
 886 capacity that is freely available for ATP production (corrected for LEAK-respiration) and is
 887 fully coupled to phosphorylation with a maximum mechanistic stoichiometry (**Figure 5**).

888 The rates of LEAK respiration and OXPHOS capacity depend on (1) the tightness of
 889 coupling under the influence of the respiratory uncoupling mechanisms (**Figure 4**), and (2) the
 890 coupling stoichiometry, which varies as a function of the substrate type undergoing oxidation
 891 in ET-pathways with either two or three coupling sites (**Figure 2B**). When cocktails with
 892 NADH-linked substrates and succinate are used, the relative contribution of ET-pathways with
 893 three or two coupling sites cannot be controlled experimentally, is difficult to determine, and
 894 may shift in transitions between LEAK-, OXPHOS- and ET-states (Gnaiger 2014). Under these
 895 experimental conditions, we cannot separate the tightness of coupling *versus* coupling
 896 stoichiometry as the mechanisms of respiratory control in the shift of L/P ratios. The tightness
 897 of coupling and fully coupled O₂ flux, $P - L$ (**Table 2**), therefore, are obtained from
 898 measurements of coupling control of LEAK respiration, OXPHOS- and ET-capacities in well
 899 defined pathway states, using either pyruvate and malate as substrates or the classical succinate
 900 and rotenone substrate-inhibitor combination (**Figure 2B**).

901 2.3. Classical terminology for isolated mitochondria

902 ‘When a code is familiar enough, it ceases appearing like a code; one forgets that there
 903 is a decoding mechanism. The message is identical with its meaning’ (Hofstadter 1979).

904 Chance and Williams (1955; 1956) introduced five classical states of mitochondrial
 905 respiration and cytochrome redox states. **Table 3** shows a protocol with isolated mitochondria
 906 in a closed respirometric chamber, defining a sequence of respiratory states. States and rates
 907 are not specifically distinguished in this nomenclature.

911 **Table 3. Metabolic states of mitochondria (Chance and Williams, 1956; Table V).**

State	[O ₂]	ADP level	Substrate level	Respiration rate	Rate-limiting substance
1	>0	low	low	slow	ADP
2	>0	high	~0	slow	substrate
3	>0	high	high	fast	respiratory chain
4	>0	low	high	slow	ADP
5	0	high	high	0	oxygen

915 **State 1** is obtained after addition of isolated mitochondria to air-saturated
 916 isoosmotic/isotonic respiration medium containing P_i , but no fuel substrates and no adenylates,
 917 *i.e.*, AMP, ADP, ATP.

918 **State 2** is induced by addition of a 'high' concentration of ADP (typically 100 to 300
 919 μM), which stimulates respiration transiently on the basis of endogenous fuel substrates and
 920 phosphorylates only a small portion of the added ADP. State 2 is then obtained at a low
 921 respiratory activity limited by exhausted endogenous fuel substrate availability (**Table 3**). If
 922 addition of specific inhibitors of respiratory complexes—such as rotenone—does not cause a
 923 further decline of O_2 flux, State 2 is equivalent to the ROX state (See below.). If inhibition is
 924 observed, undefined endogenous fuel substrates are a confounding factor of pathway control,
 925 contributing to the effect of subsequently externally added substrates and inhibitors. In contrast
 926 to the original protocol, an alternative sequence of titration steps is frequently applied, in which
 927 the alternative 'State 2' has an entirely different meaning, when this second state is induced by
 928 addition of fuel substrate without ADP or ATP (LEAK-state; in contrast to State 2 defined in
 929 **Table 1** as a ROX state). Some researchers have called this condition as "pseudostate 4"
 930 because it has no significant concentrations of adenine nucleotides and hence it is not a near-
 931 physiological condition, although it should be used for calculating the net OXPHOS-capacity,
 932 *P-L*.

933 **State 3** is the state stimulated by addition of fuel substrates while the ADP concentration
 934 is still high (**Table 3**) and supports coupled energy transformation through oxidative
 935 phosphorylation. 'High ADP' is a concentration of ADP specifically selected to allow the
 936 measurement of State 3 to State 4 transitions of isolated mitochondria in a closed respirometric
 937 chamber. Repeated ADP titration re-establishes State 3 at 'high ADP'. Starting at O_2
 938 concentrations near air-saturation (193 or 238 μM O_2 at 37 °C or 25 °C and sea level at 1 atm
 939 or 101.32 kPa, and an oxygen solubility of respiration medium at 0.92 times that of pure water;
 940 Forstner and Gnaiger 1983), the total ADP concentration added must be low enough (typically
 941 100 to 300 μM) to allow phosphorylation to ATP at a coupled O_2 flux that does not lead to O_2
 942 depletion during the transition to State 4. In contrast, kinetically-saturating ADP concentrations
 943 usually are 10-fold higher than 'high ADP', *e.g.*, 2.5 mM in isolated mitochondria. The
 944 abbreviation State 3u is occasionally used in bioenergetics, to indicate the state of respiration
 945 after titration of an uncoupler, without sufficient emphasis on the fundamental difference
 946 between OXPHOS-capacity (*well-coupled* with an *endogenous* uncoupled component) and ET-
 947 capacity (*noncoupled*).

948 **State 4** is a LEAK-state that is obtained only if the mitochondrial preparation is intact
 949 and well-coupled. Depletion of ADP by phosphorylation to ATP causes a decline of O_2 flux in
 950 the transition from State 3 to State 4. Under the conditions of State 4, a maximum protonmotive
 951 force and high ATP/ADP ratio are maintained. The gradual decline of $Y_{P\gg\text{O}_2}$ towards
 952 diminishing [ADP] at State 4 must be taken into account for calculation of $P\gg\text{O}_2$ ratios (Gnaiger
 953 2001). State 4 respiration, L_T (**Table 1**), reflects intrinsic proton leak and ATP hydrolysis
 954 activity. O_2 flux in State 4 is an overestimation of LEAK-respiration if the contaminating ATP
 955 hydrolysis activity recycles some ATP to ADP, $J_{P\ll}$, which stimulates respiration coupled to
 956 phosphorylation, $J_{P\gg} > 0$. Some degree of mechanical disruption and loss of mitochondrial
 957 integrity allows the exposed mitochondrial F-ATPases to hydrolyze the ATP synthesized by
 958 the fraction of coupled mitochondria. This can be tested by inhibition of the phosphorylation-
 959 pathway using oligomycin, ensuring that $J_{P\gg} = 0$ (State 4o). On the other hand, the state 4
 960 respiration reached after exhaustion of added ADP is a more physiological condition (*i.e.*,
 961 presence of ATP, ADP and even AMP). Sequential ADP titrations re-establish State 3, followed
 962 by State 3 to State 4 transitions while sufficient O_2 is available. Anoxia may be reached,
 963 however, before exhaustion of ADP (State 5).

964 **State 5** is the state after exhaustion of O_2 in a closed respirometric chamber. Diffusion of
 965 O_2 from the surroundings into the aqueous solution may be a confounding factor preventing

966 complete anoxia (Gnaiger 2001). Chance and Williams (1955) provide an alternative definition
 967 of State 5, which gives it the different meaning of ROX versus anoxia: ‘State 5 may be obtained
 968 by antimycin A treatment or by anaerobiosis’.

969 In **Table 3**, only States 3 and 4 are coupling control states, with the restriction that O₂
 970 flux in State 3 may be limited kinetically by non-saturating ADP concentrations.

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973 3. Normalization: flows and fluxes

974

975 3.1. Normalization: system or sample

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977 The term *rate* is not sufficiently defined to be useful for reporting data (**Figure 7**). The
 978 inconsistency of the meanings of rate becomes apparent when considering Galileo Galilei’s
 979 famous principle, that ‘bodies of different weight all fall at the same rate (have a constant
 980 acceleration)’ (Coopersmith 2010).

981 **Flow per system, I :** In a generalization of electrical terms, flow as an extensive quantity
 982 (I ; per system) is distinguished from flux as a size-specific quantity (J ; per system size) (**Figure**
 983 **7A**). Electric current is flow, I_{el} [$A \equiv C \cdot s^{-1}$] per system (extensive quantity). When dividing this
 984 extensive quantity by system size (cross-sectional area of a ‘wire’), a size-specific quantity is
 985 obtained, which is flux (current density), J_{el} [$A \cdot m^{-2} = C \cdot s^{-1} \cdot m^{-2}$] (**Box 2**).

986

987 **Box 2: Metabolic flows and fluxes: vectorial and scalar**

988 *Flows, I_{tr} ,* are defined for all transformations as extensive quantities. Electric charge per
 989 unit time is electric flow or current, $I_{el} = dQ_{el} \cdot dt^{-1}$ [A]. When expressed per unit cross-sectional
 990 area, A [m^2], a vector flux is obtained, which is current density (surface-density of flow)
 991 perpendicular to the direction of flux, $J_{el} = I_{el} \cdot A^{-1}$ [$A \cdot m^{-2}$] (Cohen et al. 2008). Fluxes with
 992 *spatial* geometric direction and magnitude are *vectors*. Vector and scalar *fluxes* are related to
 993 flows as $J_{tr} = I_{tr} \cdot A^{-1}$ [$mol \cdot s^{-1} \cdot m^{-2}$] and $J_{tr} = I_{tr} \cdot V^{-1}$ [$mol \cdot s^{-1} \cdot m^{-3}$], expressing flux as an area-specific
 994 vector or volume-specific vectorial or scalar quantity, respectively (Gnaiger 1993b). We use
 995 the metre–kilogram–second–ampere (MKSA) international system of units (*SI*) for general
 996 cases ([m], [kg], [s] and [A]), with decimal *SI* prefixes for specific applications (**Table 4**).

997 We suggest to define: (1) *vectorial* fluxes, which are translocations as functions of
 998 *gradients* with direction in geometric space in continuous systems; (2) *vectorial* fluxes, which
 999 describe translocations in discontinuous systems and are restricted to information on
 1000 *compartmental differences* (**Figure 3**, transmembrane proton flux); and (3) *scalar* fluxes, which
 1001 are transformations in a *homogenous* system (**Figure 3**, catabolic O₂ flux, J_{kO_2}).

1002 Vectorial transmembrane proton fluxes, J_{mH^+pos} and J_{mH^+neg} , are analyzed in a
 1003 heterogenous compartmental system as a quantity with *directional* but not *spatial* information.
 1004 Translocation of protons across the mtIM has a defined direction, either from the negative
 1005 compartment (matrix space; negative, neg–compartment) to the positive compartment (inter-
 1006 membrane space; positive, pos–compartment) or *vice versa* (**Figure 3**). The arrows defining
 1007 the direction of the translocation between the two vesicular compartments may point upwards
 1008 or downwards, right or left, without any implication that these are actual directions in space.
 1009 The pos–compartment is neither above nor below the neg–compartment in a spatial sense, but
 1010 can be visualized arbitrarily in a figure in the upper position (**Figure 3**). In general, the
 1011 *compartmental direction* of vectorial translocation from the neg–compartment to the pos–
 1012 compartment is defined by assigning the initial and final state as *ergodynamic compartments*,
 1013 $H^+_{neg} \rightarrow H^+_{pos}$ or $0 = -1 H^+_{neg} + 1 H^+_{pos}$, related to work (erg = work) that must be performed to
 1014 lift the proton from a lower to a higher electrochemical potential or from the lower to the higher
 1015 ergodynamic compartment (Gnaiger 1993b).
 1016

1017 In analogy to *vectorial* translocation, the direction of a *scalar* chemical reaction, $A \rightarrow B$
 1018 or $0 = -1 A + 1 B$, is defined by assigning substrates and products, A and B, as ergodynamic
 1019 compartments. O_2 is defined as a substrate in respiratory O_2 consumption (electron acceptor),
 1020 which together with the fuel substrates (electron donors) comprises the substrate compartment
 1021 of the catabolic reaction. Volume-specific scalar O_2 flux is coupled to vectorial translocation,
 1022 yielding the H^+_{pos}/O_2 ratio (**Figure 2B**).

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Figure 7. Flow and flux, and normalization in structure-function analysis

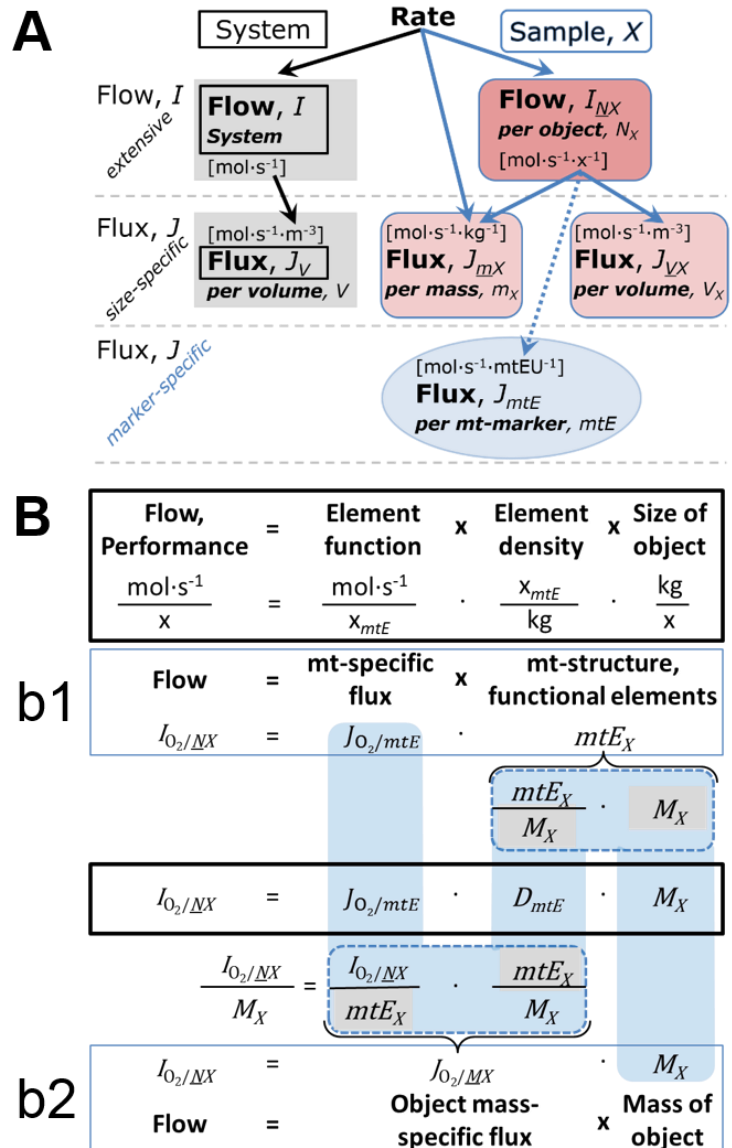
1025 **(A)** Different meanings of rate may lead to confusion, if the normalization is not sufficiently
 1026 specified. Results are frequently expressed as mass-specific *flux*,
 1027 J_{mX} , per mg protein, dry or wet weight (mass). Cell volume, V_{ce} ,
 1028 may be used for normalization (volume-specific flux, J_{Vce}),
 1029 which must be clearly distinguished from flow per cell,
 1030 I_{Nce} , or flux, J_V , expressed for methodological reasons per
 1031 volume of the measurement system.

1032 **(B)** O_2 flow, $I_{O_2/NX}$, is the product of performance per functional
 1033 element (element function, mitochondria-specific flux),
 1034 element density (mitochondrial density, D_{mtE}), and size of entity X
 1035 (mass, M_X). **(b1)** Structured analysis: performance is the
 1036 product of mitochondrial *function* (mt-specific flux) and *structure*
 1037 (functional elements; D_{mtE} times mass of X). **(b2)** Unstructured
 1038 analysis: performance is the product of *entity mass-specific*
 1039 *flux*, $J_{O_2/MX} = I_{O_2/NX}/M_X$ [$\text{mol}\cdot\text{s}^{-1}\cdot\text{kg}^{-1}$] and *size of entity*,
 1040 expressed as mass of X ; $M_X = m_X \cdot N_X^{-1}$ [$\text{kg}\cdot\text{x}^{-1}$]. Modified from Gnaiger (2014). For further details see **Table 4**.

1059

1060 **Extensive quantities:** An extensive quantity increases proportionally with system size.
 1061 The magnitude of an extensive quantity is completely additive for non-interacting
 1062 subsystems—such as mass or flow expressed per defined system. The magnitude of these
 1063 quantities depends on the extent or size of the system (Cohen *et al.* 2008).

1064 **Size-specific quantities:** ‘The adjective *specific* before the name of an extensive quantity
 1065 is often used to mean *divided by mass*’ (Cohen *et al.* 2008). In this system-paradigm, mass-
 1066 specific flux is flow divided by mass of the *system* (the total mass of everything within the
 1067 measuring chamber or reactor). A mass-specific quantity is independent of the extent of non-



1068 interacting homogenous subsystems. Tissue-specific quantities (related to the *sample* in
 1069 contrast to the *system*) are of fundamental interest in the field of comparative mitochondrial
 1070 physiology, where *specific* refers to the *type of the sample* rather than *mass of the system*. The
 1071 term *specific*, therefore, must be clarified; *sample-specific*, e.g., muscle mass-specific
 1072 normalization, is distinguished from *system-specific* quantities (mass or volume; **Figure 7**).

1073

1074 3.2. Normalization for system-size: flux per chamber volume

1075

1076 **System-specific flux, J_{V,O_2} :** The experimental system (experimental chamber) is part of
 1077 the measurement apparatus, separated from the environment as an isolated, closed, open,
 1078 isothermal or non-isothermal system (**Table 4**). On another level, we distinguish between (1)
 1079 the *system* with volume V and mass m defined by the system boundaries, and (2) the *sample* or
 1080 *objects* with volume V_X and mass m_X that are enclosed in the experimental chamber (**Figure 7**).
 1081 Metabolic O_2 flow per object, $I_{O_2/NX}$, is the total O_2 flow in the system divided by the number of
 1082 objects, N_X , in the system. $I_{O_2/NX}$ increases as the mass of the object is increased. Sample mass-
 1083 specific O_2 flux, $J_{O_2/mX}$ should be independent of the mass of the sample studied in the
 1084 instrument chamber, but system volume-specific O_2 flux, J_{V,O_2} (per volume of the instrument
 1085 chamber), should increase in direct proportion to the mass of the sample in the chamber.
 1086 Whereas J_{V,O_2} depends on mass-concentration of the sample in the chamber, it should be
 1087 independent of the chamber (system) volume at constant sample mass. There are practical
 1088 limitations to increase the mass-concentration of the sample in the chamber, when one is
 1089 concerned about crowding effects and instrumental time resolution.

1090 N_X and m_X indicate the number format and mass format, respectively, for expressing the
 1091 quantity of a sample X . When different formats are indicated in symbols of derived quantities,
 1092 the format (\underline{N} , \underline{m}) is shown as a subscript (*underlined italic*), as in $I_{O_2/\underline{NX}}$ and $J_{O_2/\underline{mX}}$. Oxygen flow
 1093 and flux are expressed in the molar format, n_{O_2} [mol], but in the volume format, V_{O_2} [m³] in
 1094 ergometry. For mass-specific flux these formats can be distinguished as $J_{\underline{n}O_2/\underline{mX}}$ and $J_{\underline{V}O_2/\underline{mX}}$,
 1095 respectively. Further examples are given in **Figure 7** and **Table 4**.

1096 When the reactor volume does not change during the reaction, which is typical for liquid
 1097 phase reactions, the volume-specific flux of a chemical reaction r is the time derivative of the
 1098 advancement of the reaction per unit volume, $J_{V,rB} = d_r\zeta_B/dt \cdot V^{-1}$ [(mol·s⁻¹)·L⁻¹]. The *rate of*
 1099 *concentration change* is dc_B/dt [(mol·L⁻¹)·s⁻¹], where concentration is $c_B = n_B/V$. There is a
 1100 difference between (1) J_{V,rO_2} [mol·s⁻¹·L⁻¹] and (2) rate of concentration change [mol·L⁻¹·s⁻¹].
 1101 These merge to a single expression only in closed systems. In open systems, external fluxes
 1102 (such as O_2 supply) are distinguished from internal transformations (catabolic flux, O_2
 1103 consumption). In a closed system, external flows of all substances are zero and O_2 consumption
 1104 (internal flow of catabolic reactions k), I_{kO_2} [pmol·s⁻¹], causes a decline of the amount of O_2 in
 1105 the system, n_{O_2} [nmol]. Normalization of these quantities for the volume of the system, V [L \equiv
 1106 dm³], yields volume-specific O_2 flux, $J_{V,kO_2} = I_{kO_2}/V$ [nmol·s⁻¹·L⁻¹], and O_2 concentration, $[O_2]$
 1107 or $c_{O_2} = n_{O_2}/V$ [μ mol·L⁻¹ = μ M = nmol·mL⁻¹]. Instrumental background O_2 flux is due to external
 1108 flux into a non-ideal closed respirometer; then total volume-specific flux has to be corrected for
 1109 instrumental background O_2 flux— O_2 diffusion into or out of the instrumental chamber. J_{V,kO_2}
 1110 is relevant mainly for methodological reasons and should be compared with the accuracy of
 1111 instrumental resolution of background-corrected flux, e.g., ± 1 nmol·s⁻¹·L⁻¹ (Gnaiger 2001).
 1112 ‘Metabolic’ or catabolic indicates O_2 flux, J_{kO_2} , corrected for: (1) instrumental background O_2
 1113 flux; (2) chemical background O_2 flux due to autoxidation of chemical components added to
 1114 the incubation medium; and (3) R_{ox} for O_2 -consuming side reactions unrelated to the catabolic
 1115 pathway k .

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1119 3.3. Normalization: per sample

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1121 The challenges of measuring mitochondrial respiratory flux are matched by those of
 1122 normalization. Application of common and defined units is required for direct transfer of
 1123 reported results into a database. The second [s] is the *SI* unit for the base quantity *time*. It is also
 1124 the standard time-unit used in solution chemical kinetics. A rate may be considered as the
 1125 numerator and normalization as the complementary denominator, which are tightly linked in
 1126 reporting the measurements in a format commensurate with the requirements of a database.
 1127 Normalization (**Table 4**) is guided by physicochemical principles, methodological
 1128 considerations, and conceptual strategies (**Figure 7**).

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Table 4. Sample concentrations and normalization of flux.

Expression	Symbol	Definition	Unit	Notes
Sample				
identity of sample	X	object: cell, tissue, animal, patient		
number of sample entities X	N_X	number of objects	x	1
mass of sample X	m_X		kg	2
mass of object X	M_X	$M_X = m_X \cdot N_X^{-1}$	$\text{kg} \cdot \text{x}^{-1}$	2
Mitochondria				
Mitochondria	mt	$X = \text{mt}$		
amount of mt-elements	mtE	quantity of mt-marker	mtEU	
Concentrations				
object number concentration	C_{NX}	$C_{NX} = N_X \cdot V^{-1}$	$\text{x} \cdot \text{m}^{-3}$	3
sample mass concentration	C_{mX}	$C_{mX} = m_X \cdot V^{-1}$	$\text{kg} \cdot \text{m}^{-3}$	
mitochondrial concentration	C_{mtE}	$C_{mtE} = mtE \cdot V^{-1}$	$\text{mtEU} \cdot \text{m}^{-3}$	4
specific mitochondrial density	D_{mtE}	$D_{mtE} = mtE \cdot m_X^{-1}$	$\text{mtEU} \cdot \text{kg}^{-1}$	5
mitochondrial content, mtE per object X	mtE_{NX}	$mtE_{NX} = mtE \cdot N_X^{-1}$	$\text{mtEU} \cdot \text{x}^{-1}$	6
O₂ flow and flux				
flow, system	I_{O_2}	internal flow	$\text{mol} \cdot \text{s}^{-1}$	7
volume-specific flux	J_{V,O_2}	$J_{V,O_2} = I_{O_2} \cdot V^{-1}$	$\text{mol} \cdot \text{s}^{-1} \cdot \text{m}^{-3}$	8
flow per object X	$I_{O_2/NX}$	$I_{O_2/NX} = J_{V,O_2} \cdot C_{NX}^{-1}$	$\text{mol} \cdot \text{s}^{-1} \cdot \text{x}^{-1}$	9
mass-specific flux	$J_{O_2/mX}$	$J_{O_2/mX} = J_{V,O_2} \cdot C_{mX}^{-1}$	$\text{mol} \cdot \text{s}^{-1} \cdot \text{kg}^{-1}$	10
mitochondria-specific flux	$J_{O_2/mtE}$	$J_{O_2/mtE} = J_{V,O_2} \cdot C_{mtE}^{-1}$	$\text{mol} \cdot \text{s}^{-1} \cdot \text{mtEU}^{-1}$	11

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- 1 The unit x for a number is not used by IUPAC. To avoid confusion, the units $[\text{kg} \cdot \text{x}^{-1}]$ and $[\text{kg}]$ distinguish the mass per object from the mass of a sample that may contain any number of objects. Similarly, the units for flow per system *versus* flow per object are $[\text{mol} \cdot \text{s}^{-1}]$ (Note 8) and $[\text{mol} \cdot \text{s}^{-1} \cdot \text{x}^{-1}]$ (Note 10).
- 2 Units are given in the MKSA system (**Box 2**). The *SI* prefix k is used for the *SI* base unit of mass (kg = 1,000 g). In praxis, various *SI* prefixes are used for convenience, to make numbers easily readable, e.g., 1 mg tissue, cell or mitochondrial mass instead of 0.000001 kg.
- 3 In case of cells (sample $X = \text{cells}$), the object number concentration is $C_{Nce} = N_{ce} \cdot V^{-1}$, and volume may be expressed in $[\text{dm}^3 \equiv \text{L}]$ or $[\text{cm}^3 = \text{mL}]$. See **Table 5** for different object types.
- 4 mt-concentration is an experimental variable, dependent on sample concentration: (1) $C_{mtE} = mtE \cdot V^{-1}$; (2) $C_{mtE} = mtE_X \cdot C_{NX}$; (3) $C_{mtE} = C_{mX} \cdot D_{mtE}$.
- 5 If the amount of mitochondria, mtE , is expressed as mitochondrial mass, then D_{mtE} is the mass fraction of mitochondria in the sample. If mtE is expressed as mitochondrial volume, V_{mt} , and the

- 1145 mass of sample, m_X , is replaced by volume of sample, V_X , then D_{mtE} is the volume fraction of
 1146 mitochondria in the sample.
- 1147 6 $mtE_{NX} = mtE \cdot N_X^{-1} = C_{mtE} \cdot C_{NX}^{-1}$.
- 1148 7 O_2 can be replaced by other chemicals B to study different reactions, e.g., ATP, H_2O_2 , or vesicular
 1149 compartmental translocations, e.g., Ca^{2+} .
- 1150 8 I_{O_2} and V are defined per instrument chamber as a system of constant volume (and constant
 1151 temperature), which may be closed or open. I_{O_2} is abbreviated for I_{rO_2} , i.e., the metabolic or internal
 1152 O_2 flow of the chemical reaction r in which O_2 is consumed, hence the negative stoichiometric
 1153 number, $\nu_{O_2} = -1$. $I_{rO_2} = d_r n_{O_2} / dt \cdot \nu_{O_2}^{-1}$. If r includes all chemical reactions in which O_2 participates, then
 1154 $d_r n_{O_2} = dn_{O_2} - d_e n_{O_2}$, where dn_{O_2} is the change in the amount of O_2 in the instrument chamber and $d_e n_{O_2}$
 1155 is the amount of O_2 added externally to the system. At steady state, by definition $dn_{O_2} = 0$, hence $d_r n_{O_2}$
 1156 $= -d_e n_{O_2}$.
- 1157 9 J_{V,O_2} is an experimental variable, expressed per volume of the instrument chamber.
- 1158 10 $I_{O_2/NX}$ is a physiological variable, depending on the size of entity X .
- 1159 11 There are many ways to normalize for a mitochondrial marker, that are used in different experimental
 1160 approaches: (1) $J_{O_2/mtE} = J_{V,O_2} \cdot C_{mtE}^{-1}$; (2) $J_{O_2/mtE} = J_{V,O_2} \cdot C_{mX}^{-1} \cdot D_{mtE}^{-1} = J_{O_2/mX} \cdot D_{mtE}^{-1}$; (3) $J_{O_2/mtE} =$
 1161 $J_{V,O_2} \cdot C_{NX}^{-1} \cdot mtE_{NX}^{-1} = I_{O_2/NX} \cdot mtE_{NX}^{-1}$; (4) $J_{O_2/mtE} = I_{O_2} \cdot mtE^{-1}$. The mt-elemental unit [mtEU] varies depending
 1162 on the mt-marker.

1164 **Sample concentration, C_{mX} :** Normalization for sample concentration is required to
 1165 report respiratory data. Considering a tissue or cells as the sample, X , the sample mass is m_X
 1166 [mg], which is frequently measured as wet or dry weight, W_w or W_d [mg], respectively, or as
 1167 amount of tissue or cell protein, m_{protein} . In the case of permeabilized tissues, cells, and
 1168 homogenates, the sample concentration, $C_{mX} = m_X/V$ [$g \cdot L^{-1} = mg \cdot mL^{-1}$], is the mass of the
 1169 subsample of tissue that is transferred into the instrument chamber.

1170 **Mass-specific flux, $J_{O_2/mX}$:** Mass-specific flux is obtained by expressing respiration per
 1171 mass of sample, m_X [mg]. X is the type of sample—isolated mitochondria, tissue homogenate,
 1172 permeabilized fibres or cells. Volume-specific flux is divided by mass concentration of X , $J_{O_2/mX}$
 1173 $= J_{V,O_2}/C_{mX}$; or flow per cell is divided by mass per cell, $J_{O_2/mce} = I_{O_2/cc}/M_{ce}$. If mass-specific O_2
 1174 flux is constant and independent of sample size (expressed as mass), then there is no interaction
 1175 between the subsystems. A 1.5 mg and a 3.0 mg muscle sample respire at identical mass-
 1176 specific flux. Mass-specific O_2 flux, however, may change with the mass of a tissue sample,
 1177 cells or isolated mitochondria in the measuring chamber, in which the nature of the interaction
 1178 becomes an issue. Therefore, cell density must be optimized, particularly in experiments carried
 1179 out in wells, considering the confluency of the cell monolayer or clumps of cells (Salabei *et al.*
 1180 2014).

1181 **Number concentration, C_{NX} :** C_{NX} is the experimental *number concentration* of sample
 1182 X . In the case of cells or animals, e.g., nematodes, $C_{NX} = N_X/V$ [$X \cdot L^{-1}$], where N_X is the number
 1183 of cells or organisms in the chamber (**Table 4**).

1184 **Flow per object, $I_{O_2/NX}$:** A special case of normalization is encountered in respiratory
 1185 studies with permeabilized (or intact) cells. If respiration is expressed per cell, the O_2 flow per
 1186 measurement system is replaced by the O_2 flow per cell, $I_{O_2/Nce}$ (**Table 4**). O_2 flow can be
 1187 calculated from volume-specific O_2 flux, J_{V,O_2} [$nmol \cdot s^{-1} \cdot L^{-1}$] (per V of the measurement chamber
 1188 [L]), divided by the number concentration of cells, $C_{Nce} = N_{ce}/V$ [$X \cdot L^{-1}$], where N_{ce} is the number
 1189 of cells in the chamber. The total cell count is the sum of viable and dead cells, $N_{ce} = N_{vce} + N_{dce}$
 1190 (**Table 5**). The cell viability index, $CVI = N_{vce}/N_{ce}$, is the ratio of viable cells (N_{vce} ; before
 1191 experimental permeabilization) per total cell count. After experimental permeabilization, all
 1192 cells are permeabilized, $N_{pce} = N_{ce}$. The cell viability index can be used to normalize respiration
 1193 for the number of cells that have been viable before experimental permeabilization, $I_{O_2/Nvce} =$
 1194 $I_{O_2/Nce}/CVI$, considering that mitochondrial respiratory dysfunction in dead cells should be
 1195 eliminated as a confounding factor.

1196 Cellular O_2 flow can be compared between cells of identical size. To take into account
 1197 changes and differences in cell size, normalization is required to obtain cell size-specific or
 1198 mitochondrial marker-specific O_2 flux (Renner *et al.* 2003).

1199 The complexity changes when the sample is a whole organism studied as an experimental
 1200 model. The scaling law in respiratory physiology reveals a strong interaction of O₂ flow and
 1201 individual body mass of an organism, since *basal* metabolic rate (flow) does not increase
 1202 linearly with body mass, whereas *maximum* mass-specific O₂ flux, $\dot{V}_{O_{2max}}$ or $\dot{V}_{O_{2peak}}$, is
 1203 approximately constant across a large range of individual body mass (Weibel and Hoppeler
 1204 2005), with individuals, breeds, and species deviating substantially from this relationship. For
 1205 comparison of units, $\dot{V}_{O_{2peak}}$ of human endurance athletes is 60 to 80 mL O₂·min⁻¹·kg⁻¹ body
 1206 mass, converted to $J_{O_{2peak}/M_{org}}$ of 45 to 60 nmol·s⁻¹·g⁻¹ (Gnaiger 2014; **Table 6**).
 1207
 1208

Table 5. Sample types, X, abbreviations, and quantification.

Identity of sample	X	N _X	Mass ^a	Volume	mt-Marker
mitochondrial preparation		[x]	[kg]	[m ³]	[mtEU]
isolated mitochondria	imt		m_{mt}	V_{mt}	mtE
tissue homogenate	thom		m_{thom}		mtE_{thom}
permeabilized tissue	pti		m_{pti}		mtE_{pti}
permeabilized fibre	pfi		m_{pfi}		mtE_{pfi}
permeabilized cell	pce	N_{pce}	M_{pce}	V_{pce}	mtE_{pce}
cells ^b	ce	N_{ce}	M_{ce}	V_{ce}	mtE_{ce}
intact cell, viable cell	vce	N_{vce}	M_{vce}	V_{vce}	
dead cell	dce	N_{dce}	M_{dce}	V_{dce}	
organism	org	N_{org}	M_{org}	V_{org}	

1209 ^a Instead of mass, the wet weight or dry weight is frequently stated, W_w or W_d .
 1210 m_X is mass of the sample [kg], M_X is mass of the object [kg·x⁻¹] (**Table 4**).

1211 ^b Total cell count, $N_{ce} = N_{vce} + N_{dce}$
 1212

1213 3.4. Normalization for mitochondrial content

1214
 1215 Tissues can contain multiple cell populations that may have distinct mitochondrial
 1216 subtypes. Mitochondria undergo dynamic fission and fusion cycles, and can exist in multiple
 1217 stages and sizes that may be altered by a range of factors. The isolation of mitochondria (often
 1218 achieved through differential centrifugation) can therefore yield a subsample of the
 1219 mitochondrial types present in a tissue, depending on the isolation protocols utilized (*e.g.*,
 1220 centrifugation speed). This possible bias should be taken into account when planning
 1221 experiments using isolated mitochondria. Different sizes of mitochondria are enriched at
 1222 specific centrifugation speeds, which can be used strategically for isolation of mitochondrial
 1223 subpopulations.

1224 Part of the mitochondrial content of a tissue is lost during preparation of isolated
 1225 mitochondria. The fraction of isolated mitochondria obtained from a tissue sample is expressed
 1226 as mitochondrial recovery. At a high mitochondrial recovery the fraction of isolated
 1227 mitochondria is more representative of the total mitochondrial population than in preparations
 1228 characterized by low recovery. Determination of the mitochondrial recovery and yield is based
 1229 on measurement of the concentration of a mitochondrial marker in the stock of isolated
 1230 mitochondria, $C_{mtE,stock}$, and crude tissue homogenate, $C_{mtE,thom}$, which simultaneously provides
 1231 information on the specific mitochondrial density in the sample, D_{mtE} (**Table 4**).

1232 Normalization is a problematic subject; it is essential to consider the question of the study.
 1233 If the study aims at comparing tissue performance—such as the effects of a treatment on a
 1234 specific tissue, then normalization for tissue mass or protein content is appropriate. However,
 1235 if the aim is to find differences on mitochondrial function independent of mitochondrial density
 1236 (**Table 4**), then normalization to a mitochondrial marker is imperative (**Figure 7**). One cannot

1237 assume that quantitative changes in various markers—such as mitochondrial proteins—
 1238 necessarily occur in parallel with one another. It should be established that the marker chosen
 1239 is not selectively altered by the performed treatment. In conclusion, the normalization must
 1240 reflect the question under investigation to reach a satisfying answer. On the other hand, the goal
 1241 of comparing results across projects and institutions requires standardization on normalization
 1242 for entry into a databank.

1243 **Mitochondrial concentration, C_{mtE} , and mitochondrial markers:** Mitochondrial
 1244 organelles comprise a dynamic cellular reticulum in various states of fusion and fission. Hence,
 1245 the definition of an "amount" of mitochondria is often misconceived: mitochondria cannot be
 1246 counted reliably as a number of occurring elements. Therefore, quantification of the "amount"
 1247 of mitochondria depends on the measurement of chosen mitochondrial markers. 'Mitochondria
 1248 are the structural and functional elemental units of cell respiration' (Gnaiger 2014). The
 1249 quantity of a mitochondrial marker can reflect the amount of *mitochondrial elements, mtE*,
 1250 expressed in various mitochondrial elemental units [mtEU] specific for each measured mt-
 1251 marker (**Table 4**). However, since mitochondrial quality may change in response to stimuli—
 1252 particularly in mitochondrial dysfunction (Campos *et al.* 2017) and after exercise training (Pesta
 1253 *et al.* 2011) and during aging (Daum *et al.* 2013)—some markers can vary while others are
 1254 unchanged: (1) Mitochondrial volume and membrane area are structural markers, whereas
 1255 mitochondrial protein mass is frequently used as a marker for isolated mitochondria. (2)
 1256 Molecular and enzymatic mitochondrial markers (amounts or activities) can be selected as
 1257 matrix markers, *e.g.*, citrate synthase activity, mtDNA; mtIM-markers, *e.g.*, cytochrome *c*
 1258 oxidase activity, *aa₃* content, cardiolipin, or mtOM-markers, *e.g.*, the voltage-dependent anion
 1259 channel (VDAC), TOM20. (3) Extending the measurement of mitochondrial marker enzyme
 1260 activity to mitochondrial pathway capacity, ET- or OXPHOS-capacity can be considered as an
 1261 integrative functional mitochondrial marker.

1262 Depending on the type of mitochondrial marker, the mitochondrial elements, *mtE*, are
 1263 expressed in marker-specific units. Mitochondrial concentration in the measurement chamber
 1264 and the tissue of origin are quantified as (1) a quantity for normalization in functional analyses,
 1265 C_{mtE} , and (2) a physiological output that is the result of mitochondrial biogenesis and
 1266 degradation, D_{mtE} , respectively (**Table 4**). It is recommended, therefore, to distinguish
 1267 *experimental mitochondrial concentration*, $C_{mtE} = mtE/V$ and *physiological mitochondrial*
 1268 *density*, $D_{mtE} = mtE/m_X$. Then mitochondrial density is the amount of mitochondrial elements
 1269 per mass of tissue, which is a biological variable (**Figure 7**). The experimental variable is
 1270 mitochondrial density multiplied by sample mass concentration in the measuring chamber, C_{mtE}
 1271 $= D_{mtE} \cdot C_{mX}$, or mitochondrial content multiplied by sample number concentration, $C_{mtE} =$
 1272 $mtE_X \cdot C_{NX}$ (**Table 4**).

1273 **Mitochondria-specific flux, $J_{O_2/mtE}$:** Volume-specific metabolic O_2 flux depends on: (1)
 1274 the sample concentration in the volume of the instrument chamber, C_{mX} , or C_{NX} ; (2) the
 1275 mitochondrial density in the sample, $D_{mtE} = mtE/m_X$ or $mtE_X = mtE/N_X$; and (3) the specific
 1276 mitochondrial activity or performance per elemental mitochondrial unit, $J_{O_2/mtE} = J_{V,O_2}/C_{mtE}$
 1277 $[mol \cdot s^{-1} \cdot mtEU^{-1}]$ (**Table 4**). Obviously, the numerical results for $J_{O_2/mtE}$ vary with the type of
 1278 mitochondrial marker chosen for measurement of *mtE* and $C_{mtE} = mtE/V [mtEU \cdot m^{-3}]$.

1279

1280 3.5. Evaluation of mitochondrial markers

1281

1282 Different methods are implicated in the quantification of mitochondrial markers and have
 1283 different strengths. Some problems are common for all mitochondrial markers, *mtE*: (1)
 1284 Accuracy of measurement is crucial, since even a highly accurate and reproducible
 1285 measurement of O_2 flux results in an inaccurate and noisy expression if normalized by a biased
 1286 and noisy measurement of a mitochondrial marker. This problem is acute in mitochondrial
 1287 respiration because the denominators used (the mitochondrial markers) are often small moieties

1288 of which accurate and precise determination is difficult. This problem can be avoided when O₂
 1289 fluxes measured in substrate-uncoupler-inhibitor titration protocols are normalized for flux in
 1290 a defined respiratory reference state, which is used as an *internal* marker and yields flux control
 1291 ratios, *FCRs*. *FCRs* are independent of *externally* measured markers and, therefore, are
 1292 statistically robust, considering the limitations of ratios in general (Jasienski and Bazzaz 1999).
 1293 *FCRs* indicate qualitative changes of mitochondrial respiratory control, with highest
 1294 quantitative resolution, separating the effect of mitochondrial density or concentration on $J_{O_2/mX}$
 1295 and $I_{O_2/NX}$ from that of function per elemental mitochondrial marker, $J_{O_2/mtE}$ (Pesta *et al.* 2011;
 1296 Gnaiger 2014). (2) If mitochondrial quality does not change and only the amount of
 1297 mitochondria varies as a determinant of mass-specific flux, any marker is equally qualified in
 1298 principle; then in practice selection of the optimum marker depends only on the accuracy and
 1299 precision of measurement of the mitochondrial marker. (3) If mitochondrial flux control ratios
 1300 change, then there may not be any best mitochondrial marker. In general, measurement of
 1301 multiple mitochondrial markers enables a comparison and evaluation of normalization for a
 1302 variety of mitochondrial markers. Particularly during postnatal development, the activity of
 1303 marker enzymes—such as cytochrome *c* oxidase and citrate synthase—follows different time
 1304 courses (Drahota *et al.* 2004). Evaluation of mitochondrial markers in healthy controls is
 1305 insufficient for providing guidelines for application in the diagnosis of pathological states and
 1306 specific treatments.

1307 In line with the concept of the respiratory control ratio (Chance and Williams 1955a), the
 1308 most readily used normalization is that of flux control ratios and flux control factors (Gnaiger
 1309 2014). Selection of the state of maximum flux in a protocol as the reference state has the
 1310 advantages of: (1) internal normalization; (2) statistically validated linearization of the response
 1311 in the range of 0 to 1; and (3) consideration of maximum flux for integrating a large number of
 1312 elemental steps in the OXPHOS- or ET-pathways. This reduces the risk of selecting a functional
 1313 marker that is specifically altered by the treatment or pathology, yet increases the chance that
 1314 the highly integrative pathway is disproportionately affected, *e.g.*, the OXPHOS- rather than
 1315 ET-pathway in case of an enzymatic defect in the phosphorylation-pathway. In this case,
 1316 additional information can be obtained by reporting flux control ratios based on a reference
 1317 state which indicates stable tissue-mass specific flux.

1318 Stereological determination of mitochondrial content via two-dimensional transmission
 1319 electron microscopy can have limitations due to the dynamics of mitochondrial size (Meinild
 1320 Lundby *et al.* 2017). Accurate determination of three-dimensional volume by two-dimensional
 1321 microscopy can be both time consuming and statistically challenging (Larsen *et al.* 2012).

1322 The validity of using mitochondrial marker enzymes (citrate synthase activity, Complex
 1323 I–IV amount or activity) for normalization of flux is limited in part by the same factors that
 1324 apply to flux control ratios. Strong correlations between various mitochondrial markers and
 1325 citrate synthase activity (Reichmann *et al.* 1985; Boushel *et al.* 2007; Mogensen *et al.* 2007)
 1326 are expected in a specific tissue of healthy persons and in disease states not specifically
 1327 targeting citrate synthase. Citrate synthase activity is acutely modifiable by exercise
 1328 (Tonkonogi *et al.* 1997; Leek *et al.* 2001). Evaluation of mitochondrial markers related to a
 1329 selected age and sex cohort cannot be extrapolated to provide recommendations for
 1330 normalization in respirometric diagnosis of disease, in different states of development and
 1331 ageing, different cell types, tissues, and species. mtDNA normalized to nDNA via qPCR is
 1332 correlated to functional mitochondrial markers including OXPHOS- and ET-capacity in some
 1333 cases (Puntschart *et al.* 1995; Wang *et al.* 1999; Menshikova *et al.* 2006; Boushel *et al.* 2007;
 1334 Ehinger *et al.* 2015), but lack of such correlations have been reported (Menshikova *et al.* 2005;
 1335 Schultz and Wiesner 2000; Pesta *et al.* 2011). Several studies indicate a strong correlation
 1336 between cardiolipin content and increase in mitochondrial function with exercise (Menshikova
 1337 *et al.* 2005; Menshikova *et al.* 2007; Larsen *et al.* 2012; Faber *et al.* 2014), but it has not been
 1338 evaluated as a general mitochondrial biomarker in disease. With no single best mitochondrial

1339 marker, a good strategy is to quantify several different biomarkers to minimize the decorrelating
 1340 effects caused by diseases, treatments, or other factors. Determination of multiple markers,
 1341 particularly a matrix marker and a marker from the mtIM, allows tracking changes in
 1342 mitochondrial quality defined by their ratio.

1343

1344 3.6. Conversion: units

1345

1346 Many different units have been used to report the O₂ consumption rate, OCR (**Table 6**).
 1347 *SI* base units provide the common reference to introduce the theoretical principles (**Figure 7**),
 1348 and are used with appropriately chosen *SI* prefixes to express numerical data in the most
 1349 practical format, with an effort towards unification within specific areas of application (**Table**
 1350 **7**). Reporting data in *SI* units—including the mole [mol], coulomb [C], joule [J], and second
 1351 [s]—should be encouraged, particularly by journals which propose the use of *SI* units.

1352

1353 **Table 6. Conversion of various formats and units used in respirometry and**
 1354 **ergometry.** e^- is the number of electrons or reducing equivalents. z_B is the charge
 1355 number of entity B.

1356

Format	1 Unit		Multiplication factor	<i>SI</i> -unit	Note
\underline{n}	ng.atom O·s ⁻¹	(2 e ⁻)	0.5	nmol O ₂ ·s ⁻¹	
\underline{n}	ng.atom O·min ⁻¹	(2 e ⁻)	8.33	pmol O ₂ ·s ⁻¹	
\underline{n}	natom O·min ⁻¹	(2 e ⁻)	8.33	pmol O ₂ ·s ⁻¹	
\underline{n}	nmol O ₂ ·min ⁻¹	(4 e ⁻)	16.67	pmol O ₂ ·s ⁻¹	
\underline{n}	nmol O ₂ ·h ⁻¹	(4 e ⁻)	0.2778	pmol O ₂ ·s ⁻¹	
\underline{V} to \underline{n}	mL O ₂ ·min ⁻¹ at STPD ^a		0.744	μmol O ₂ ·s ⁻¹	1
\underline{e} to \underline{n}	W = J/s at -470 kJ/mol O ₂		-2.128	μmol O ₂ ·s ⁻¹	
\underline{e} to \underline{n}	mA = mC·s ⁻¹	($z_{H^+} = 1$)	10.36	nmol H ⁺ ·s ⁻¹	2
\underline{e} to \underline{n}	mA = mC·s ⁻¹	($z_{O_2} = 4$)	2.59	nmol O ₂ ·s ⁻¹	2
\underline{n} to \underline{e}	nmol H ⁺ ·s ⁻¹	($z_{H^+} = 1$)	0.09649	mA	3
\underline{n} to \underline{e}	nmol O ₂ ·s ⁻¹	($z_{O_2} = 4$)	0.38594	mA	3

1357 1 At standard temperature and pressure dry (STPD: 0 °C = 273.15 K and 1 atm = 101.325
 1358 kPa = 760 mmHg), the molar volume of an ideal gas, V_m , and V_{m,O_2} is 22.414 and 22.392
 1359 L·mol⁻¹, respectively. Rounded to three decimal places, both values yield the conversion
 1360 factor of 0.744. For comparison at normal temperature and pressure dry (NTPD: 20 °C),
 1361 V_{m,O_2} is 24.038 L·mol⁻¹. Note that the *SI* standard pressure is 100 kPa.

1362 2 The multiplication factor is $10^{6/(z_B \cdot F)}$.

1363 3 The multiplication factor is $z_B \cdot F / 10^6$.

1364

1365 Although volume is expressed as m³ using the *SI* base unit, the litre [dm³] is a
 1366 conventional unit of volume for concentration and is used for most solution chemical kinetics.
 1367 If one multiplies $I_{O_2/N_{cc}}$ by $C_{N_{cc}}$, then the result will not only be the amount of O₂ [mol] consumed
 1368 per time [s⁻¹] in one litre [L⁻¹], but also the change in O₂ concentration per second (for any
 1369 volume of an ideally closed system). This is ideal for kinetic modeling as it blends with
 1370 chemical rate equations where concentrations are typically expressed in mol·L⁻¹ (Wagner *et al.*
 1371 2011). In studies of multinuclear cells—such as differentiated skeletal muscle cells—it is easy
 1372 to determine the number of nuclei but not the total number of cells. A generalized concept,
 1373 therefore, is obtained by substituting cells by nuclei as the sample entity. This does not hold,
 1374 however, for enucleated platelets.

1375 For studies of cells, we recommend that respiration be expressed, as far as possible, as:
 1376 (1) O₂ flux normalized for a mitochondrial marker, for separation of the effects of mitochondrial
 1377 quality and content on cell respiration (this includes *FCRs* as a normalization for a functional
 1378 mitochondrial marker); (2) O₂ flux in units of cell volume or mass, for comparison of respiration
 1379 of cells with different cell size (Renner *et al.* 2003) and with studies on tissue preparations, and
 1380 (3) O₂ flow in units of attomole (10⁻¹⁸ mol) of O₂ consumed in a second by each cell
 1381 [amol·s⁻¹·cell⁻¹], numerically equivalent to [pmol·s⁻¹·10⁻⁶ cells]. This convention allows
 1382 information to be easily used when designing experiments in which O₂ flow must be considered.
 1383 For example, to estimate the volume-specific O₂ flux in an instrument chamber that would be
 1384 expected at a particular cell number concentration, one simply needs to multiply the flow per
 1385 cell by the number of cells per volume of interest. This provides the amount of O₂ [mol]
 1386 consumed per time [s⁻¹] per unit volume [L⁻¹]. At an O₂ flow of 100 amol·s⁻¹·cell⁻¹ and a cell
 1387 density of 10⁹ cells·L⁻¹ (10⁶ cells·mL⁻¹), the volume-specific O₂ flux is 100 nmol·s⁻¹·L⁻¹ (100
 1388 pmol·s⁻¹·mL⁻¹).
 1389
 1390

Table 7. Conversion of units with preservation of numerical values.

Name	Frequently used unit	Equivalent unit	Note
volume-specific flux, J_{V,O_2}	pmol·s ⁻¹ ·mL ⁻¹ mmol·s ⁻¹ ·L ⁻¹	nmol·s ⁻¹ ·L ⁻¹ mol·s ⁻¹ ·m ⁻³	1
cell-specific flow, $I_{O_2/cell}$	pmol·s ⁻¹ ·10 ⁻⁶ cells pmol·s ⁻¹ ·10 ⁻⁹ cells	amol·s ⁻¹ ·cell ⁻¹ zmol·s ⁻¹ ·cell ⁻¹	2 3
cell number concentration, C_{Nce}	10 ⁶ cells·mL ⁻¹	10 ⁹ cells·L ⁻¹	
mitochondrial protein concentration, C_{mtE}	0.1 mg·mL ⁻¹	0.1 g·L ⁻¹	
mass-specific flux, $J_{O_2/m}$	pmol·s ⁻¹ ·mg ⁻¹	nmol·s ⁻¹ ·g ⁻¹	4
catabolic power, P_k	μW·10 ⁻⁶ cells	pW·cell ⁻¹	1
Volume	1,000 L L mL μL fL	m ³ (1,000 kg) dm ³ (kg) cm ³ (g) mm ³ (mg) μm ³ (pg)	5
amount of substance concentration	M = mol·L ⁻¹	mol·dm ⁻³	

1391
 1392 1 pmol: picomole = 10⁻¹² mol 4 nmol: nanomole = 10⁻⁹ mol
 1393 2 amol: attomole = 10⁻¹⁸ mol 5 fL: femtolitre = 10⁻¹⁵ L
 1394 3 zmol: zeptomole = 10⁻²¹ mol
 1395

1396 ET-capacity in human cell types including HEK 293, primary HUVEC and fibroblasts
 1397 ranges from 50 to 180 amol·s⁻¹·cell⁻¹, measured in intact cells in the noncoupled state (see
 1398 Gnaiger 2014). At 100 amol·s⁻¹·cell⁻¹ corrected for *Rox*, the current across the mt-membranes,
 1399 I_{H^+e} , approximates 193 pA·cell⁻¹ or 0.2 nA per cell. See Rich (2003) for an extension of
 1400 quantitative bioenergetics from the molecular to the human scale, with a transmembrane proton
 1401 flux equivalent to 520 A in an adult at a catabolic power of -110 W. Modelling approaches
 1402 illustrate the link between protonmotive force and currents (Willis *et al.* 2016).

1403 We consider isolated mitochondria as powerhouses and proton pumps as molecular
 1404 machines to relate experimental results to energy metabolism of the intact cell. The cellular
 1405 P_»/O₂ based on oxidation of glycogen is increased by the glycolytic (fermentative) substrate-
 1406 level phosphorylation of 3 P_»/Glyc or 0.5 mol P_» for each mol O₂ consumed in the complete
 1407 oxidation of a mol glycosyl unit (Glyc). Adding 0.5 to the mitochondrial P_»/O₂ ratio of 5.4
 1408 yields a bioenergetic cell physiological P_»/O₂ ratio close to 6. Two NADH equivalents are

1409 formed during glycolysis and transported from the cytosol into the mitochondrial matrix, either
 1410 by the malate-aspartate shuttle or by the glycerophosphate shuttle (**Figure 2A**) resulting in
 1411 different theoretical yields of ATP generated by mitochondria, the energetic cost of which
 1412 potentially must be taken into account. Considering also substrate-level phosphorylation in the
 1413 TCA cycle, this high P_{H}/O_2 ratio not only reflects proton translocation and OXPHOS studied
 1414 in isolation, but integrates mitochondrial physiology with energy transformation in the living
 1415 cell (Gnaiger 1993a).

1416

1417 **4. Conclusions**

1418

1419 Catabolic cell respiration is the process of exergonic and exothermic energy
 1420 transformation in which scalar redox reactions are coupled to vectorial ion translocation across
 1421 a semipermeable membrane, which separates the small volume of a bacterial cell or
 1422 mitochondrion from the larger volume of its surroundings. The electrochemical exergy can be
 1423 partially conserved in the phosphorylation of ADP to ATP or in ion pumping, or dissipated in
 1424 an electrochemical short-circuit. Respiration is thus clearly distinguished from fermentation as
 1425 the counterpart of cellular core energy metabolism. An O_2 flux balance scheme illustrates the
 1426 relationships and general definitions (**Figures 1 and 2**).

1427 Experimentally, respiration is separated in mitochondrial preparations from the
 1428 interactions with the fermentative pathways of the intact cell. OXPHOS analysis (**Figure 3**) is
 1429 based on the study of mitochondrial preparations complementary to bioenergetic investigations
 1430 of intact cells and organisms—from model organisms to the human species including healthy
 1431 and diseased persons (patients). Different mechanisms of respiratory uncoupling have to be
 1432 distinguished (**Figure 4**). Metabolic fluxes measured in defined coupling and pathway control
 1433 states (**Figures 5 and 6**) provide insights into the meaning of cellular and organismic
 1434 respiration.

1435 The optimal choice for expressing mitochondrial and cell respiration as O_2 flow per
 1436 biological sample, and normalization for specific tissue-markers (volume, mass, protein) and
 1437 mitochondrial markers (volume, protein, content, mtDNA, activity of marker enzymes,
 1438 respiratory reference state) is guided by the scientific question under study. Interpretation of
 1439 the data depends critically on appropriate normalization (**Figure 7**).

1440 MitoEAGLE can serve as a gateway to better diagnose mitochondrial respiratory
 1441 adaptations and defects linked to genetic variation, age-related health risks, sex-specific
 1442 mitochondrial performance, lifestyle with its effects on degenerative diseases, and thermal and
 1443 chemical environment. The present recommendations on coupling control states and rates,
 1444 linked to the concept of the protonmotive force, are focused on studies with mitochondrial
 1445 preparations (**Box 3**). These will be extended in a series of reports on pathway control of
 1446 mitochondrial respiration, respiratory states in intact cells, and harmonization of experimental
 1447 procedures.

1448

1449

1450 **Box 3: Recommendations for studies with mitochondrial preparations**

1451

- 1452 • Normalization of respiratory rates should be provided as far as possible:
 - 1453 1. *Biophysical normalization*: on a per cell basis as O_2 flow; this may not be possible
 1454 when dealing with coenocytic organisms or tissues without cross-walls
 1455 separating individual cells (e.g., filamentous fungi, muscle fibers)
 - 1456 2. *Cellular normalization*: per g protein; per cell- or tissue-mass as mass-specific
 1457 O_2 flux; per cell volume as cell volume-specific flux
 - 1458 3. *Mitochondrial normalization*: per mitochondrial marker as mt-specific flux.

- 1459 With information on cell size and the use of multiple normalizations, maximum potential
 1460 information is available (Renner *et al.* 2003; Wagner *et al.* 2011; Gnaiger 2014). Reporting
 1461 flow in a respiratory chamber [$\text{nmol}\cdot\text{s}^{-1}$] is discouraged, since it restricts the analysis to intra-
 1462 experimental comparison of relative (qualitative) differences.
- 1463 ● Catabolic mitochondrial respiration is distinguished from residual O_2 consumption. Fluxes
 1464 in mitochondrial coupling states should be, as far as possible, corrected for residual O_2
 1465 consumption.
 - 1466 ● Different mechanisms of uncoupling should be distinguished by defined terms. The tightness
 1467 of coupling relates to these uncoupling mechanisms, whereas the coupling stoichiometry
 1468 varies as a function the substrate type involved in ET-pathways with either three or two
 1469 redox proton pumps operating in series. Separation of tightness of coupling from the
 1470 pathway-dependent coupling stoichiometry is possible only when the substrate type
 1471 undergoing oxidation remains the same for respiration in LEAK-, OXPHOS-, and ET-states.
 1472 In studies of the tightness of coupling, therefore, simple substrate-inhibitor combinations
 1473 should be applied to exclude a shift in substrate competition which may occur when
 1474 providing physiological substrate cocktails.
 - 1475 ● In studies of isolated mitochondria, the mitochondrial recovery and yield should be reported.
 1476 Experimental criteria for evaluation of purity versus integrity should be considered.
 1477 Mitochondrial markers—such as citrate synthase activity as an enzymatic matrix marker—
 1478 provide a link to the tissue of origin on the basis of calculating the mitochondrial recovery,
 1479 *i.e.*, the fraction of mitochondrial marker obtained from a unit mass of tissue. Total
 1480 mitochondrial protein is frequently applied as a mitochondrial marker, which is restricted to
 1481 isolated mitochondria.
 - 1482 ● In studies of permeabilized cells, the viability of the cell culture or cell suspension of origin
 1483 should be reported. Normalization should be evaluated for total cell count or viable cell
 1484 count.
 - 1485 ● Terms and symbols are summarized in **Table 8**. Their use will facilitate transdisciplinary
 1486 communication and support further developments towards a consistent theory of
 1487 bioenergetics and mitochondrial physiology. Technical terms related to and defined with
 1488 normal words can be used as index terms in databases, support the creation of ontologies
 1489 towards semantic information processing (MitoPedia), and help in communicating analytical
 1490 findings as impactful data-driven stories. *‘Making data available without making it*
 1491 *understandable may be worse than not making it available at all’* (National Academies of
 1492 Sciences, Engineering, and Medicine 2018). Success will depend on taking next steps: (1)
 1493 exhaustive text-mining considering Omics data and functional data; (2) network analysis of
 1494 Omics data with bioinformatics tools; (3) cross-validation with distinct bioinformatics
 1495 approaches; (4) correlation with functional data; (5) guidelines for biological validation of
 1496 network data. This is a call to carefully contribute to FAIR principles (Findable, Accessible,
 1497 Interoperable, Reusable) for the sharing of scientific data.

1499
 1500
 1501 **Table 8. Terms, symbols, and units.**

Term	Symbol	Unit	Links and comments
1502 alternative quinol oxidase	AOX		Figure 2B
1503 amount of substance B	n_B	[mol]	
1504 ATP yield per O_2	$Y_{P\gg/O_2}$		$P\gg/O_2$ ratio measured in any respiratory state
1505 catabolic reaction	k		Figure 1 and 3

1513	catabolic respiration	J_{kO_2}	<i>varies</i>	Figure 1 and 3
1514	cell number	N_{ce}	[x]	Table 5; $N_{ce} = N_{vce} + N_{dce}$
1515	cell respiration	J_{rO_2}	<i>varies</i>	Figure 1
1516	cell viability index	CVI		$CVI = N_{vce}/N_{ce} = 1 - N_{dce}/N_{ce}$
1517	Complexes I to IV	CI to CIV		respiratory ET Complexes; Figure 2B
1518	concentration of substance B	$c_B = n_B \cdot V^{-1}$; [B]	[mol·m ⁻³]	Box 2
1519	dead cell number	N_{dce}	[x]	Table 5; non-viable cells, loss of plasma membrane barrier function
1520				
1521	electric format	e	[C]	Table 6
1522	electron transfer system	ETS		Figure 2B, Figure 5; state
1523	flow, for substance B	I_B	[mol·s ⁻¹]	system-related extensive quantity; Figure 7
1524				
1525	flux, for substance B	J_B	<i>varies</i>	size-specific quantity; Figure 7
1526	inorganic phosphate	P_i		Figure 3
1527	intact cell number, viable cell number	N_{vce}	[x]	Table 5; viable cells, intact of plasma membrane barrier function
1528				
1529	LEAK	LEAK		Table 1, Figure 5; state
1530	mass format	m	[kg]	Table 4, Figure 7
1531	mass of sample X	m_X	[kg]	Table 4
1532	mass of entity X	M_X	[kg]	mass of object X; Table 4
1533	MITOCARTA			https://www.broadinstitute.org/scientific-community/science/programs/metabolic-disease-program/publications/mitocarta/mitocarta-in-0
1534				
1535				
1536				
1537				
1538	MitoPedia			http://www.bioblast.at/index.php/MitoPedia
1539	mitochondria or mitochondrial	mt		Box 1
1540	mitochondrial DNA	mtDNA		Box 1
1541	mitochondrial concentration	$C_{mtE} = mtE \cdot V^{-1}$	[mtEU·m ⁻³]	Table 4
1542	mitochondrial content	$mtE_X = mtE \cdot N_X^{-1}$	[mtEU·x ⁻¹]	Table 4
1543	mitochondrial element	mtE	[mtEU]	Table 4, quantity of mt-marker
1544	mitochondrial elemental unit	mtEU	<i>varies</i>	Table 4, specific units for mt-marker
1545	mitochondrial inner membrane	mtIM		Figure 2; MIM is widely used; the first M is replaced by mt; Box 1
1546				
1547	mitochondrial outer membrane	mtOM		Figure 2; MOM is widely used; the first M is replaced by mt; Box 1
1548				
1549	mitochondrial recovery	Y_{mtE}		fraction of <i>mtE</i> recovered in sample from the tissue of origin
1550	mitochondrial yield	$Y_{mtE/m}$		mt-yield per tissues mass; $Y_{mtE/m} = Y_{mtE} \cdot D_{mtE}$
1551				
1552				
1553	molar format	n	[mol]	Table 6
1554	negative	neg		Figure 3
1555	number concentration of X	C_{NX}	[x·m ⁻³]	Table 4
1556	number format	N	[x]	Table 4, Figure 7
1557	number of entities X	N_X	[x]	Table 4, Figure 7
1558	number of entity B	N_B	[x]	Table 4
1559	oxidative phosphorylation	OXPHOS		Table 1, Figure 5; state
1560	oxygen concentration	$c_{O_2} = n_{O_2} \cdot V^{-1}$; [O ₂]	[mol·m ⁻³]	Section 3.2
1561	oxygen flux, in reaction r	J_{rO_2}	<i>varies</i>	Figure 1
1562	permeabilized cell number	N_{pce}	[x]	Table 5; experimental permeabilization of plasma membrane; $N_{pce} = N_{ce}$
1563				
1564	phosphorylation of ADP to ATP	P»		Section 2.2
1565	positive	pos		Figure 3
1566	proton in the negative compartment	H^{+neg}		Figure 3
1567	proton in the positive compartment	H^{+pos}		Figure 3
1568	rate of electron transfer in ET state	E		ET-capacity; Table 1
1569	rate of LEAK respiration	L		Table 1
1570	rate of oxidative phosphorylation	P		OXPHOS capacity; Table 1
1571	rate of residual oxygen consumption	Rox		Table 1, Figure 1
1572	residual oxygen consumption	ROX		Table 1; state

1573	respiratory supercomplex	$SC I_n III_n IV_n$	Box 1; supramolecular assemblies composed of variable copy numbers (n) of CI, CIII and CIV
1574			
1575			
1576	specific mitochondrial density	$D_{mtE} = mtE \cdot m_X^{-1}$	[mtEU·kg ⁻¹] Table 4
1577	volume	V	[m ³] Table 7
1578	volume format	\underline{V}	[m ³] Table 6
1579	weight, dry weight	W_d	[kg] used as mass of sample X ; Figure 7
1580	weight, wet weight	W_w	[kg] used as mass of sample X ; Figure 7
1581			

1582

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