1 MitoEAGLE preprint 2018-02-18(26)

2

3 **Mitochondrial respiratory states and rates:**

4 **Building blocks of mitochondrial physiology**

5 **Part 1.**

6

7 <http://www.mitoeagle.org/index.php/MitoEAGLE_preprint_2018-02-08>

8 Preprint version 26 (2018-02-18)

9

10 **MitoEAGLE Network**

11 Corresponding author: Gnaiger E

12 Contributing co-authors

13 Acuna-Castroviejo D, Ahn B, Alves MG, Amati F, Aral C, Arandarčikaitė O, Åsander

14 Frostner E, Bailey DM, Bastos Sant'Anna Silva AC, Battino M, Beard DA, Ben-Shachar D,

15 Bishop D, Borutaitė V, Breton S, Brown GC, Brown RA, Buettner GR, Burtscher J, Calabria

16 E, Cardoso LHD, Carvalho E, Casado Pinna M, Cervinkova Z, Chang SC, Chen Q, Chicco

17 AJ, Chinopoulos C, Coen PM, Collins JL, Crisóstomo L, Davis MS, Dias T, Distefano G,

18 Doerrier C, Drahota Z, Duchen MR, Ehinger J, Elmer E, Endlicher R, Fell DA, Ferko M,

19 Ferreira JCB, Filipovska A, Fisar Z, Fisher J, Garcia-Roves PM, Garcia-Souza LF, Genova

20 ML, Gonzalo H, Goodpaster BH, Gorr TA, Grefte S, Han J, Harrison DK, Hellgren KT,

21 Hernansanz P, Holland O, Hoppel CL, Houstek J, Hunger M, Iglesias-Gonzalez J, Irving BA,

22 Iyer S, Jackson CB, Jadiya P, Jansen-Dürr P, Jespersen NR, Jha RK, Kaambre T, Kane DA,

23 Kappler L, Karabatsiakis A, Keijer J, Keppner G, Komlodi T, Kopitar-Jerala N, Krako

24 Jakovljevic N, Kuang J, Kucera O, Labieniec-Watala M, Lai N, Laner V, Larsen TS, Lee HK,

25 Lemieux H, Lerfall J, Lucchinetti E, MacMillan-Crow LA, Makrecka-Kuka M, Meszaros AT,

26 Michalak S, Moisoi N, Molina AJA, Montaigne D, Moore AL, Moreira BP, Mracek T,

27 Muntane J, Muntean DM, Murray AJ, Nedergaard J, Nemec M, Newsom S, Nozickova K,

28 O'Gorman D, Oliveira PF, Oliveira PJ, Orynbayeva Z, Pak YK, Palmeira CM, Patel HH,

29 Pecina P, Pereira da Silva Grilo da Silva F, Pesta D, Petit PX, Pichaud N, Pirkmajer S, Porter

30 RK, Pranger F, Prochownik EV, Puurand M, Radenkovic F, Reboredo P, Renner-Sattler K,

31 Robinson MM, Rohlena J, Røsland GV, Rossiter HB, Rybacka-Mossakowska J, Saada A,

32 Salvadego D, Scatena R, Schartner M, Scheibye-Knudsen M, Schilling JM, Schlattner U,

33 Schoenfeld P, Schwarzer C, Scott GR, Shabalina IG, Sharma P, Shevchuk I, Siewiera K,

34 Singer D, Sobotka O, Sokolova I, Spinazzi M, Stankova P, Stier A, Stocker R, Sumbalova Z,

35 Suravajhala P, Tanaka M, Tandler B, Tepp K, Tomar D, Towheed A, Tretter L, Trivigno C,

36 Tronstad KJ, Trougakos IP, Tyrrell DJ, Urban T, Valentine JM, Velika B, Vendelin M,

37 Vercesi AE, Victor VM, Villena JA, Wagner BA, Ward ML, Watala C, Wei YH, Wieckowski

38 MR, Wohlwend M, Wolff J, Wuest RCI, Zaugg K, Zaugg M, Zorzano A

39

40 Supporting co-authors:

41 Bakker BM, Bernardi P, Boetker HE, Borsheim E, Bouitbir J, Calbet JA, Calzia E, Chaurasia

42 B, Clementi E, Coker RH, Collin A, Das AM, De Palma C, Dubouchaud H, Durham WJ,

43 Dyrstad SE, Engin AB, Fischer M, Fornaro M, Gan Z, Garlid KD, Garten A, Gourlay CW,

44 Granata C, Haas CB, Haavik J, Haendeler J, Hand SC, Hepple RT, Hickey AJ, Hoel F, Jang

45 DH, Kainulainen H, Khamoui AV, Klingenspor M, Koopman WJH, Kowaltowski AJ,

46 Krajcova A, Lane N, Lenaz G, Liu J, Liu SS, Malik A, Markova M, Mazat JP, Menze MA,

47 Methner A, Neuzil J, Oliveira MT, Pallotta ML, Parajuli N, Pettersen IKN, Porter C,

48 Pulinilkunnil T, Ropelle ER, Salin K, Sandi C, Sazanov LA, Silber AM, Skolik R, Smenes

49 BT, Soares FAA, Sonkar VK, Swerdlow RH, Szabo I, Trifunovic A, Thyfault JP, Vieyra A,

50 Votion DM, Williams C, Zischka H

51

52

53

54

55

56

57

58

59

60

61

62

63

64

65

66

67

68

69

70

71

72

73

74

75

76

77

78

79

80

81

82

83

84

85

86

87

88

89

90

91

92

93

94

95

96

97

98

99

100

**Updates and discussion:**

<http://www.mitoeagle.org/index.php/MitoEAGLE_preprint_2018-02-08>

Correspondence: Gnaiger E

*Chair COST Action CA15203 MitoEAGLE* – [http://www.mitoeagle.org](http://www.mitoeagle.org/) *Department of Visceral, Transplant and Thoracic Surgery, D. Swarovski Research Laboratory, Medical University of Innsbruck, Innrain 66/4, A-6020 Innsbruck, Austria Email:* [erich.gnaiger@i-med.ac.at](mailto:erich.gnaiger@i-med.ac.at)

*Tel: +43 512 566796, Fax: +43 512 566796 20*

**Contents**

**Abstract**

**Executive summary**

**1. Introduction** – Box 1: In brief: Mitochondria and Bioblasts

**2. Oxidative phosphorylation and coupling states in mitochondrial preparations**

Mitochondrial preparations

*2.1. Respiratory control and coupling*

The steady-state

Specification of biochemical dose Phosphorylation, P», and P»/O2 ratio Control and regulation

Respiratory control and response

Respiratory coupling control and ET-pathway control

Coupling

Uncoupling

*2.2. Coupling states and respiratory rates* Respiratory capacities in coupling control states LEAK, OXPHOS, ET, ROX

*2.3. Classical terminology for isolated mitochondria*

States 1–5

**3. Normalization: fluxes and flows**

*3.1. Normalization: system or sample*

Flow per system, *I*

Extensive quantities

Size-specific quantities – Box 2: Metabolic fluxes and flows: vectorial and scalar

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

System-specific flux, *JV*,O2

*3.3. Normalization: per sample*

Sample concentration, *CmX* Mass-specific flux, *J*O2/*mX* Number concentration, *CNX* Flow per object, *I*O2/*X*

*3.4. Normalization for mitochondrial content*

Mitochondrial concentration, *CmtE*, and mitochondrial markers

Mitochondria-specific flux, *J*O2/*mtE*

*3.5. Evaluation of mitochondrial markers*

*3.6. Conversion: units*

**4. Conclusions** – Box 3: Mitochondrial and cell respiration

**5. References**

102

103

104

105

106

107

108

109

110

111

112

113

114

115

116

117

118

119

120

121

122

123

124

125

126

127

128

129

130

131

132

133

134

135

136

137

138

139

140

141

142

143

144

145

146

147

148

149

150

151

152

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

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

**Executive summary**

1. In view of broad implications on health care, mitochondrial researchers face an increasing responsibility to disseminate their fundamental knowledge and novel discoveries to a wide range of stakeholders and scientists beyond the group of specialists. This requires implementation of a commonly accepted terminology within the discipline and standardization in the translational context. Authors, reviewers, journal editors, and lecturers are challenged to collaborate with the aim to harmonize the nomenclature in the growing field of mitochondrial physiology and bioenergetics.

2. Aerobic energy metabolism in mammalian mitochondria depends on the coupling of ADP → ATP phosphorylation to oxygen consumption in catabolic reactions. In this process of oxidative phosphorylation, coupling is mediated by translocation of protons through respiratory proton pumps operating across the inner mitochondrial membrane and generating or utilizing the protonmotive force measured between the mitochondrial matrix and intermembrane compartment. Compartmental coupling thus distinguishes vectorial oxidative phosphorylation from fermentation as the counterpart of cellular core energy metabolism.

3. To exclude fermentation and other cytosolic interactions from exerting an effect on mitochondrial metabolism, the barrier function of the plasma membrane must be disrupted. Selective removal or permeabilization of the plasma membrane yields mitochondrial preparations—including isolated mitochondria, tissue and cellular preparations—with structural and functional integrity. Then extra-mitochondrial concentrations of fuel substrates transported into the mitochondrial matrix, ADP, ATP, inorganic phosphate, and cations including H+ can be controlled to determine mitochondrial function under a set of conditions defined as coupling control states.

153

154

155

156

157

158

159

160

161

162

163

164

165

166

167

168

169

170

171

172

173

174

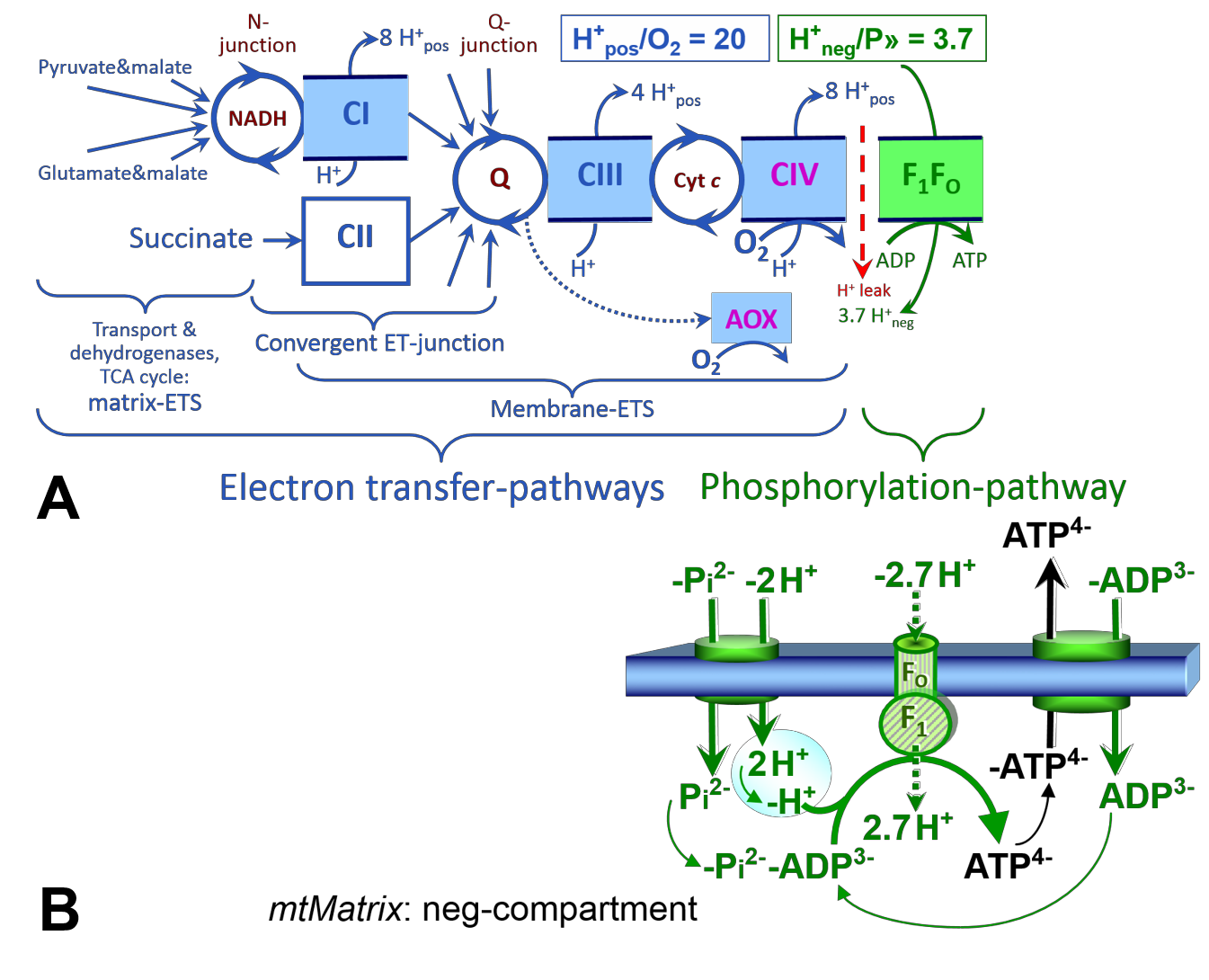
175

176

177

178

A concept-driven terminology of bioenergetics incorporates in its terms and symbols explicitly information on the nature of respiratory states, that makes the technical terms readily recognized and easy to understand.



**Fig. 1. The oxidative phosphorylation (OXPHOS) system. (A)** The mitochondrial electron transfer system (ETS) is fuelled by diffusion and transport of substrates across the mtOM and mtIM and consists of the matrix-ETS and membrane-ETS. ET-pathways are coupled to the phosphorylation-pathway. ET-pathways converge at the N-junction and Q-junction. Additional arrows indicate electron entry into the Q-junction through electron transferring flavoprotein, glycerophosphate dehydrogenase, dihydro-orotate dehydrogenase, choline dehydrogenase, and sulfide-ubiquinone oxidoreductase. The dotted arrow indicates the branched pathway of oxygen consumption by alternative quinol oxidase (AOX). The H+pos/O2 ratio is the outward proton flux from the matrix space to the positively (pos) charged compartment, divided by catabolic O2 flux in the NADH-pathway. The H+neg/P» ratio is the inward proton flux from the inter-membrane space to the negatively (neg) charged matrix space, divided by the flux of phosphorylation of ADP to ATP (Eq. 1). These are not fixed stoichiometries due to ion leaks and proton slip. (**B**) Phosphorylation-pathway catalyzed by the proton pump F1FO•ATPase (F-ATPase), adenine nucleotide translocase, and inorganic phosphate transporter. The H+neg/P» stoichiometry is the sum of the coupling stoichiometry in the F-ATPase reaction (•2.7 H+pos from the positive intermembrane space, 2.7 H+neg to the matrix, *i.e.*, the negative compartment) and the proton balance in the translocation of ADP2•, ATP3- and Pi2-. Modified from (A) Lemieux *et al*. (2017) and (B) Gnaiger (2014).

180

181

182

183

184

185

186

187

188

189

190

191

192

193

194

195

196

197

198

199

200

201

202

203

204

205

206

207

208

209

210

211

212

213

214

215

216

217

218

219

220

221

222

223

224

225

226

227

228

229

230

4. Mitochondrial coupling states are defined according to the control of respiratory oxygen consumption by the protonmotive force. Capacities of oxidative phosphorylation and electron transfer capacities are measured at kinetically saturating concentrations of fuel substrates, ADP and inorganic phosphate, or at optimal uncoupler concentrations, respectively. Respiratory capacities are a measure of the upper bound (limit?) of the rates of respiration, providing reference values for the diagnosis of health and disease, and for evaluation of the effects of **E**volutionary background, **A**ge, **G**ender and sex, **L**ifestyle and **E**nvironment (EAGLE).

5. Some degree of uncoupling is a characteristic of energy-transformations across membranes. Uncoupling is caused by a variety of physiological, pathological, toxicological, pharmacological and environmental conditions that exert an influence not only on the proton leak and cation cycling, but also on proton slip within the proton pumps and the structural integrity of the mitochondria. A more loosely coupled state is induced by stimulation of mitochondrial superoxide formation and the bypass of proton pumps. In addition, uncoupling by application of protonophores represents an experimental intervention for the transition from a well-coupled to the noncoupled state of mitochondrial respiration.

6. Respiratory oxygen consumption rates have to be carefully normalized to enable meta- analytic studies beyond the specific question of a particular experiment. Therefore, all raw data should be published in a supplemental table or open access data repository. Normalization of rates for the volume of the experimental chamber (the measuring system) is distinguished from normalization for (*1*) the volume or mass of the experimental sample, (*2*) the number of objects (cells, organisms), and (*3*) the concentration of mitochondrial markers in the chamber.

7. The consistent use of terms and symbols discussed in this MitoEAGLE position statement will facilitate transdisciplinary communication and support further developments of a database on bioenergetics and mitochondrial physiology. The present considerations are focused on studies with mitochondrial preparations. These will be extended in a series of reports on pathway control of mitochondrial respiration, the protonmotive force, respiratory states in intact cells, and harmonization of experimental procedures.

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

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

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

We now recognize mitochondria as dynamic organelles with a double membrane that are contained within eukaryotic cells. The mitochondrial inner membrane (mtIM) shows dynamic tubular to disk-shaped cristae that separate the mitochondrial matrix, *i.e*., the negatively charged

231

232

233

234

235

236

237

238

239

240

241

242

243

244

245

246

247

248

249

250

251

252

253

254

255

256

257

258

259

260

261

262

263

264

265

266

267

268

269

270

271

272

273

274

275

276

277

278

279

280

281

internal mitochondrial compartment, and the intermembrane space; the latter being positively charged and enclosed by the mitochondrial outer membrane (mtOM). The mtIM contains the non-bilayer phospholipid cardiolipin, which is not present in any other eukaryotic cellular membrane. Cardiolipin promotes the formation of respiratory supercomplexes, which are supramolecular assemblies based upon specific, though dynamic, interactions between individual respiratory complexes (Greggio *et al.* 2017; Lenaz *et al.* 2017). Membrane fluidity exerts an influence on functional properties of proteins incorporated in the membranes (Waczulikova *et al.* 2007).

Mitochondria are the structural and functional elements of cell respiration. Cell respiration is the consumption of oxygen by electron transfer coupled to electrochemical proton translocation across the mtIM. In the process of oxidative phosphorylation (OXPHOS), the reduction of O2 is electrochemically coupled to the transformation of energy in form of adenosine triphosphate (ATP; Mitchell 1961, 2011). Mitochondria are the powerhouses of the cell which contain among others the machinery of the OXPHOS-pathways, including transmembrane respiratory complexes—proton pumps with FMN, Fe-S and cytochrome *b*, *c*, *aa*3 redox systems); alternative dehydrogenases and oxidases; the coenzyme ubiquinone (Q); F-ATPase or ATP synthase; the enzymes of the tricarboxylic acid cycle and fatty acid oxidation; transporters of ions, metabolites and co-factors; and mitochondrial kinases related to energy transfer pathways. The mitochondrial proteome comprises over 1,200 proteins (Calvo *et al.* 2015; 2017), mostly encoded by nuclear DNA (nDNA), with a variety of functions, many of which are relatively well known (*e.g.,* apoptosis-regulating proteins), while others are still under investigation, or need to be identified (*e.g.,* alanine transporter).

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

Mitochondria typically maintain several copies of their own genome known as mitochondrial DNA (mtDNA; hundreds to thousands per cell; Cummins 1998), which is maternally inherited. One exception to strictly maternal inheritance in animals is found in bivalves (Breton *et al.* 2007; White *et al.* 2008). mtDNA is 16.5 kB in length, contains 13 protein-coding genes for subunits of the transmembrane respiratory Complexes CI, CIII, CIV and F-ATPase, and also encodes 22 tRNAs and the mitochondrial 16S and 12S rRNA. Additional gene content is encoded in the mitochondrial genome, *e.g.,* microRNAs, piRNA, smithRNAs, repeat associated RNA, and even additional proteins (Duarte *et al.* 2014; Lee *et*

*al.* 2015; Cobb *et al.* 2016). The mitochondrial genome is regulated and supplemented by nuclear-encoded mitochondrial targeted proteins.

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

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

282

283

284

285

286

287

288

289

290

291

292

293

294

295

296

297

298

299

300

301

302

303

304

305

306

307

308

309

310

311

312

313

314

315

316

317

318

319

320

321

322

323

324

325

326

327

328

329

330

331

332

**1. Introduction**

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

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

**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 environment of the cell. The plasma membrane consists of a lipid bilayer, 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 (Pi),

333

334

335

336

337

338

339

340

341

342

343

344

345

346

347

348

349

350

351

352

353

354

355

356

357

358

359

360

361

362

363

364

365

366

367

368

369

370

371

372

373

374

375

376

377

378

379

380

381

382

383

that must be controlled at kinetically-saturating concentrations for the analysis of respiratory capacities; this limits the scope of investigations into mitochondrial respiratory function in intact cells.

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 the complete loss of cell viability, tested by nuclear staining and washout of cytosolic marker enzymes—such as lactate dehydrogenase, while mitochondrial function remains intact. The respiration rate of isolated mitochondria remains unaltered after the addition of low concertations of digitonin or saponin. In addition to mechanical permeabilization during homogenization of tissue, peremeabilization agents may be applied to ensure permeabilization of all cells. 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, the cells or tissues are homogenized, and the mitochondria are separated from other cell fractions and purified by differential centrifugation and eventually density gradient centrifugation, entailing the loss of a fraction of mitochondria. Typical mitochondrial recovery ranges from 30% to 80%. Maximization of the purity of isolated mitochondria may compromise not only the mitochondrial yield but also the structural and functional integrity. Therefore, protocols to isolate mitochondria need to be optimized according to each study. The term mitochondrial preparation does not include further fractionation of mitochondrial components, neither 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. Physiological conditions *in vivo* deviate from these experimentally obtained states. Since kinetically-saturating concentrations, *e.g.,* of ADP or oxygen, may not apply to physiological intracellular conditions, relevant information is obtained in studies of kinetic responses to variations in [ADP] or [O2] in the range between kinetically-saturating concentrations and anoxia (Gnaiger 2001).

**The steady-state:** Mitochondria represent a thermodynamically open system in non- equilibrium states of biochemical energy transformation. State variables (protonmotive force; redox states) and metabolic *rates* (fluxes) are measured in defined mitochondrial respiratory *states*. Steady-states can be obtained only in open systems, in which changes by *internal*

transformations, *e.g.*, O2 consumption, are instantaneously compensated for by *external* fluxes,

*e.g.*, O2 supply, preventing a change of oxygen concentration in the system (Gnaiger 1993b). Mitochondrial respiratory states monitored in closed systems satisfy the criteria of pseudo-

steady states for limited periods of time, when changes in the system (concentrations of O2, fuel substrates, ADP, Pi, H+) do not exert significant effects on metabolic fluxes (respiration, phosphorylation). Such pseudo-steady states require respiratory media with sufficient buffering capacity and substrates maintained at kinetically-saturating concentrations, and thus depend on the kinetics of the processes under investigation.

**Specification of biochemical dose:** Substrates, uncouplers, inhibitors, and other biochemical reagents are titrated to dissect mitochondrial function. Nominal concentrations of these substances are usually reported as initial amount of substance concentration [mol∙L-1] in the incubation medium. When aiming at the measurement of kinetically saturated processes— such as OXPHOS-capacities, the concentrations for substrates can be chosen according to the

384

385

386

387

388

389

390

391

392

393

394

395

396

397

398

399

400

401

402

403

404

405

406

407

408

409

410

411

412

413

414

415

416

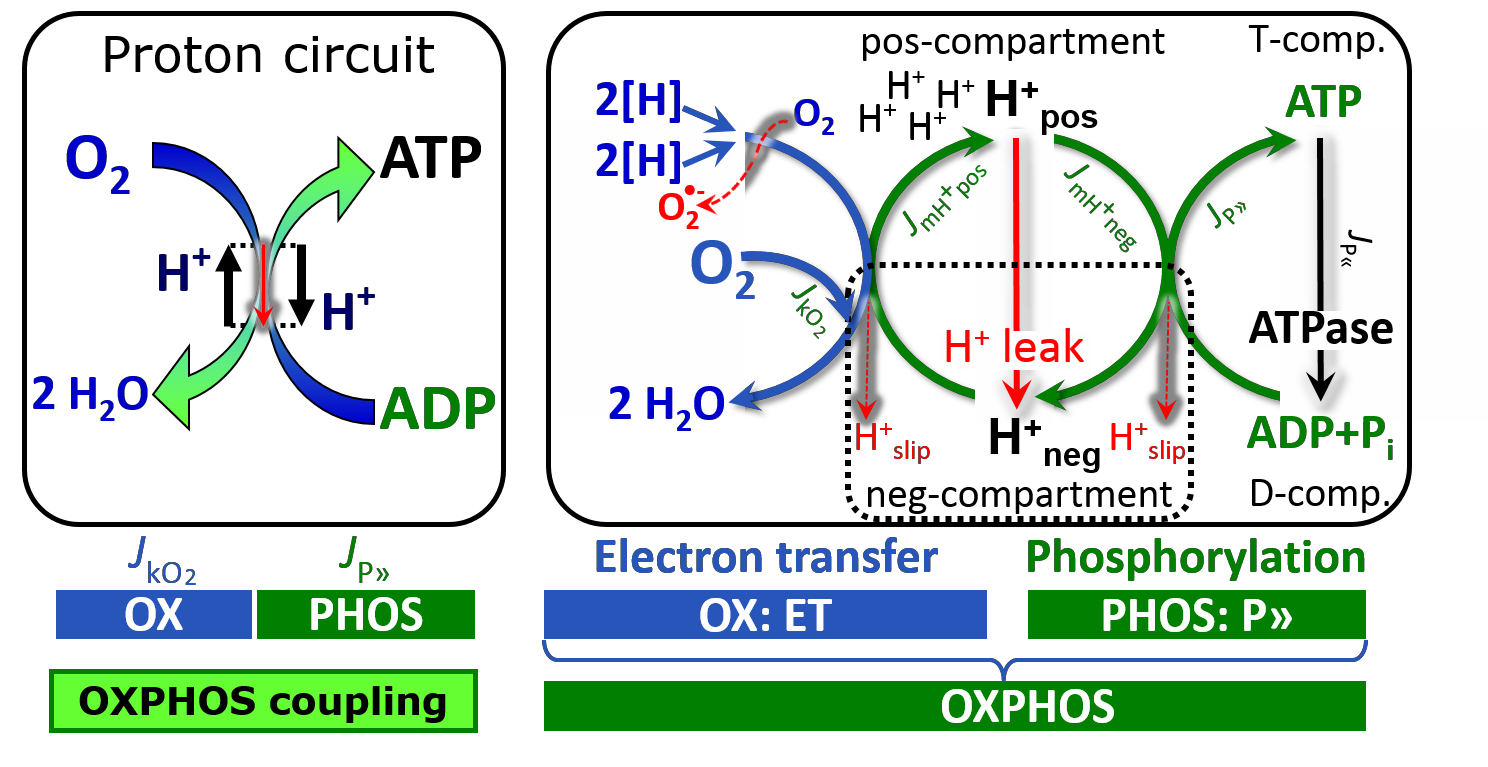
417

418

apparent equilibrium constant, *K*m’. In the case of hyperbolic kinetics, only 80% of maximum respiratory capacity is obtained at a substrate concentration of four times the *K*m’, whereas substrate concentrations of 5, 9, 19 and 49 times the *K*m’ are theoretically required for reaching

83%, 90%, 95% or 98% of the maximal rate (Gnaiger 2001). Other reagents are chosen to inhibit or alter some process. The amount of these chemicals in an experimental incubation is selected to maximize effect, yet not lead to unacceptable off-target consequences that would adversely affect the data being sought. Specifying the amount of substance in an incubation as nominal concentration in the aqueous incubation medium can be ambiguous (Doskey *et al*.

2015), particularly when lipophilic substances (oligomycin; uncouplers, permeabilization agents) or cations (TPP+; fluorescent dyes such as safranin, TMRM) are applied which accumulate in biological membranes or the mitochondrial matrix. For example, a dose of digitonin of 8 fmol∙cell•1 (10 pg∙cell•1; 10 µg∙10•6 cells) is optimal for permeabilization of endothelial cells, and the concentration in the incubation medium has to be adjusted according to the cell density applied (Doerrier *et al.* 2018). Generally, dose/exposure can be specified per unit of biological sample, *i.e.*, (nominal moles of xenobiotic)/(number of cells) [mol∙cell-1] or, as appropriate, per mass of biological sample [mol∙kg-1]. This approach to specification of dose/exposure provides a scalable parameter that can be used to design experiments, help interpret a wide variety of experimental results, and provide absolute information that allows researchers worldwide to make the most use of published data (Doskey *et al.* 2015).



**Fig. 2. The proton circuit and coupling in oxidative phosphorylation (OXPHOS).** 2[H] indicates the reduced hydrogen equivalents of fuel substrates of the catabolic reaction k with oxygen. Oxygen flux, *J*kO2, through the catabolic ET-pathway, is coupled to flux through the phosphorylation-pathway of ADP to ATP, *J*P». The proton pumps of the ET-pathway drive proton flux into the positive (pos) compartment, *J*mH+pos, generating the output protonmotive force (motive, subscript m). F-ATPase is coupled to inward proton current into the negative (neg) compartment, *J*mH+neg, to phosphorylate ADP+Pi to ATP. The system defined by the boundaries (full black line) is not a black box, but is analysed as a compartmental system. The negative compartment (neg–compartment, enclosed by the dotted line) is the matrix space, separated by the mtIM from the positive compartment (pos–compartment). ADP+Pi and ATP are the substrate- and product–compartments (scalar ADP and ATP compartments, D–comp. and T–comp.), respectively. At steady-state proton turnover, *J*∞H+, and ATP turnover, *J*∞P, maintain concentrations constant, when *J*mH+∞ = *J*mH+pos = *J*mH+neg, and *J*P∞ = *J*P» = *J*P«. Modified from Gnaiger (2014).

419

420

421

422

423

424

425

426

427

428

429

430

431

432

433

434

435

436

437

438

439

**Phosphorylation, P», and P»/O2 ratio:** *Phosphorylation* in the context of OXPHOS is defined as phosphorylation of ADP by Pi to ATP. On the other hand, the term phosphorylation is used generally in many contexts, *e.g.,* protein phosphorylation. This justifies consideration of a symbol more discriminating and specific than P as used in the P/O ratio (phosphate to atomic oxygen ratio), where P indicates phosphorylation of ADP to ATP or GDP to GTP. We propose the symbol P» for the endergonic (uphill) direction of phosphorylation ADP→ATP, and likewise the symbol P« for the corresponding exergonic (downhill) hydrolysis ATP→ADP (**Fig. 2**). P» refers mainly to electrontransfer phosphorylation but may also involve substrate- level phosphorylation as part of the tricarboxylic acid (TCA) cycle (succinyl-CoA ligase) and phosphorylation of ADP catalyzed by phosphoenolpyruvate carboxykinase. Transphosphorylation is performed by adenylate kinase, creatine kinase, hexokinase and nucleoside diphosphate kinase. In isolated mammalian mitochondria, ATP production catalyzed by adenylate kinase (2 ADP ↔ ATP + AMP) proceeds without fuel substrates in the presence of ADP (Komlódi and Tretter 2017). Kinase cycles are involved in intracellular energy transfer and signal transduction for regulation of energy flux.

The P»/O2 ratio (P»/4 e-) is two times the ‘P/O’ ratio (P»/2 e-) of classical bioenergetics. P»/O2 is a generalized symbol, independent phosphorylation assessment by determination of Pi consumption (Pi/O2 flux ratio), ADP depletion (ADP/O2 flux ratio), or ATP production (ATP/O2 flux ratio). The mechanistic P»/O2 ratio—or P»/O2 stoichiometry—is calculated from

the proton–to–oxygen and proton–to–phosphorylation coupling stoichiometries (**Fig. 1A**),

+ /O

Hpos 2

neg

440

441

P»/O2 =

H+ /P» (1)

442

443

444

445

446

447

448

449

450

451

452

453

454

455

456

457

458

459

460

461

462

463

464

465

466

467

468

The H+pos/O2 *coupling stoichiometry* (referring to the full 4 electron reduction of O2) depends on the ET-pathway control state which defines the relative involvement of the three coupling sites (CI, CIII and CIV) in the catabolic pathway of electrons to O2. This varies with: (*1*) a bypass of CI by single or multiple electron input into the Q-junction; and (*2*) a bypass of CIV by involvement of AOX. H+pos/O2 is 12 in the ET-pathways involving CIII and CIV as proton pumps, increasing to 20 for the NADH-pathway (**Fig. 1A**), but a general consensus on H+pos/O2 stoichiometries remains to be reached (Hinkle 2005; Wikström and Hummer 2012; Sazanov

2015). The H+neg/P» coupling stoichiometry (3.7; **Fig. 1A**) is the sum of 2.7 H+neg required by the F-ATPase of vertebrate and most invertebrate species (Watt *et al.* 2010) and the proton

balance in the translocation of ADP, ATP and Pi (**Fig. 1B**). Taken together, the mechanistic P»/O2 ratio is calculated at 5.4 and 3.3 for NADH- and succinate-linked respiration, respectively (Eq. 1). The corresponding classical P»/O ratios (referring to the 2 electron reduction of 0.5 O2) are 2.7 and 1.6 (Watt *et al.* 2010), in agreement with the measured P»/O ratio for succinate of

1.58 ± 0.02 (Gnaiger *et al.* 2000).

The effective P»/O2 flux ratio (*Y*P»/O2 = *J*P»/*J*kO2) is diminished relative to the mechanistic P»/O2 ratio by intrinsic and extrinsic uncoupling and dyscoupling (**Fig. 3**). Such generalized uncoupling is different from switching to mitochondrial pathways that involve fewer than three proton pumps (‘coupling sites’: Complexes CI, CIII and CIV), bypassing CI through multiple electron entries into the Q-junction, or CIII and CIV through AOX (**Fig. 1**). Reprogramming of mitochondrial pathways may be considered as a switch of gears (changing the stoichiometry) rather than uncoupling (loosening the stoichiometry). In addition, *Y*P»/O2 depends on several experimental conditions of flux control, increasing as a hyperbolic function of [ADP] to a maximum value (Gnaiger 2001).

**Control and regulation:** The terms metabolic *control* and *regulation* are frequently used synonymously, but are distinguished in metabolic control analysis: ‘We could understand the regulation as the mechanism that occurs when a system maintains some variable constant over time, in spite of fluctuations in external conditions (homeostasis of the internal state). On the

469

470

471

472

473

474

475

476

477

478

479

480

481

482

483

484

485

486

487

488

489

490

491

492

493

494

495

496

497

498

499

500

501

502

503

504

505

506

507

508

509

510

511

512

513

514

515

516

517

518

519

other hand, metabolic control is the power to change the state of the metabolism in response to an external signal’ (Fell 1997). Respiratory control may be induced by experimental control signals that *exert* an influence on: (*1*) ATP demand and ADP phosphorylation-rate; (*2*) fuel substrate composition, pathway competition; (*3*) available amounts of substrates and oxygen, *e.g.*, starvation and hypoxia; (*4*) the protonmotive force, redox states, flux–force relationships, coupling and efficiency; (*5*) Ca2+ and other ions including H+; (*6*) inhibitors, *e.g.*, nitric oxide or intermediary metabolites such as oxaloacetate; (*7*) signalling pathways and regulatory proteins, *e.g.,* insulin resistance, transcription factor hypoxia inducible factor 1. *Mechanisms* of respiratory control and regulation include adjustments of: (*1*) enzyme activities by allosteric mechanisms and phosphorylation; (*2*) enzyme content, concentrations of cofactors and conserved moieties—such as adenylates, nicotinamide adenine dinucleotide [NAD+/NADH], coenzyme Q, cytochrome *c*); (*3*) metabolic channeling by supercomplexes; and *(4)* mitochondrial density (enzyme concentrations and membrane area) and morphology (cristae folding, fission and fusion). Mitochondria are targeted directly by hormones, thereby affecting their energy metabolism (Lee *et al.* 2013; Gerö and Szabo 2016; Price and Dai 2016; Moreno *et al.* 2017). Evolutionary or acquired differences in the genetic and epigenetic basis of mitochondrial function (or dysfunction) between subjects and gene therapy; age; gender, biological sex, and hormone concentrations; life style including exercise and nutrition; and environmental issues including thermal, atmospheric, toxicological and pharmacological factors, exert an influence on all control mechanisms listed above. For reviews, see Brown

1992; Gnaiger 1993a, 2009; 2014; Paradies *et al.* 2014; Morrow *et al*. 2017.

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

**Respiratory coupling control and ET-pathway control:** Respiratory control refers to the ability of mitochondria to adjust oxygen consumption in response to external control signals by engaging various mechanisms of control and regulation. Respiratory control is monitored in a mitochondrial preparation under conditions defined as respiratory states. When phosphorylation of ADP to ATP is stimulated or depressed, an increase or decrease is observed in electron flux linked to oxygen consumption in respiratory coupling states of intact mitochondria (‘controlled states’ in the classical terminology of bioenergetics). Alternatively, coupling of electron transfer with phosphorylation is disengaged by disruption of the integrity of the mtIM or by uncouplers, functioning like a clutch in a mechanical system. The corresponding coupling control state is characterized by high levels of oxygen consumption without control by P» (‘uncontrolled state’).

ET-pathway control states are obtained in mitochondrial preparations by depletion of endogenous substrates and addition to the mitochondrial respiration medium of fuel substrates (CHNO; 2[H] in **Fig. 2**) and specific inhibitors, activating selected mitochondrial catabolic pathways, k (**Fig. 1**). Coupling control states and pathway control states are complementary, since mitochondrial preparations depend on an exogenous supply of pathway-specific fuel substrates and oxygen (Gnaiger 2014).

**Coupling:** In mitochondrial electron transfer (**Fig. 1**), vectorial transmembrane proton flux is coupled through the proton pumps CI, CIII and CIV to the catabolic flux of scalar reactions, collectively measured as oxygen flux (**Fig. 2**). Thus mitochondria are elements of energy transformation. Energy cannot be lost or produced in any internal process (First Law of thermodynamics). Open and closed systems can gain or loose energy only by external fluxes—

520

521

522

523

524

525

526

527

528

529

530

531

532

533

534

535

536

537

538

539

540

541

542

543

544

545

546

547

548

549

550

551

552

by exchange with the environment. Energy is a conserved quantity. Therefore, energy can neither be produced by mitochondria, nor is there any internal process without energy conservation. Exergy is defined as the ‘free energy’ with the potential to perform work. *Coupling* is the mechanistic linkage of an exergonic process (spontaneous, negative exergy change) with an endergonic process (positive exergy change) in energy transformations which conserve part of the exergy that would be irreversible lost or dissipated in an uncoupled process.

**Uncoupling:** Uncoupling of mitochondrial respiration is a general term comprising diverse mechanisms. Differences of terms—uncoupled *vs.* noncoupled—are easily overlooked, although they relate to different mechanisms of uncoupling (**Fig. 3**).

1. Proton leak across the mtIM from the pos– to the neg–compartment (**Fig. 2**);

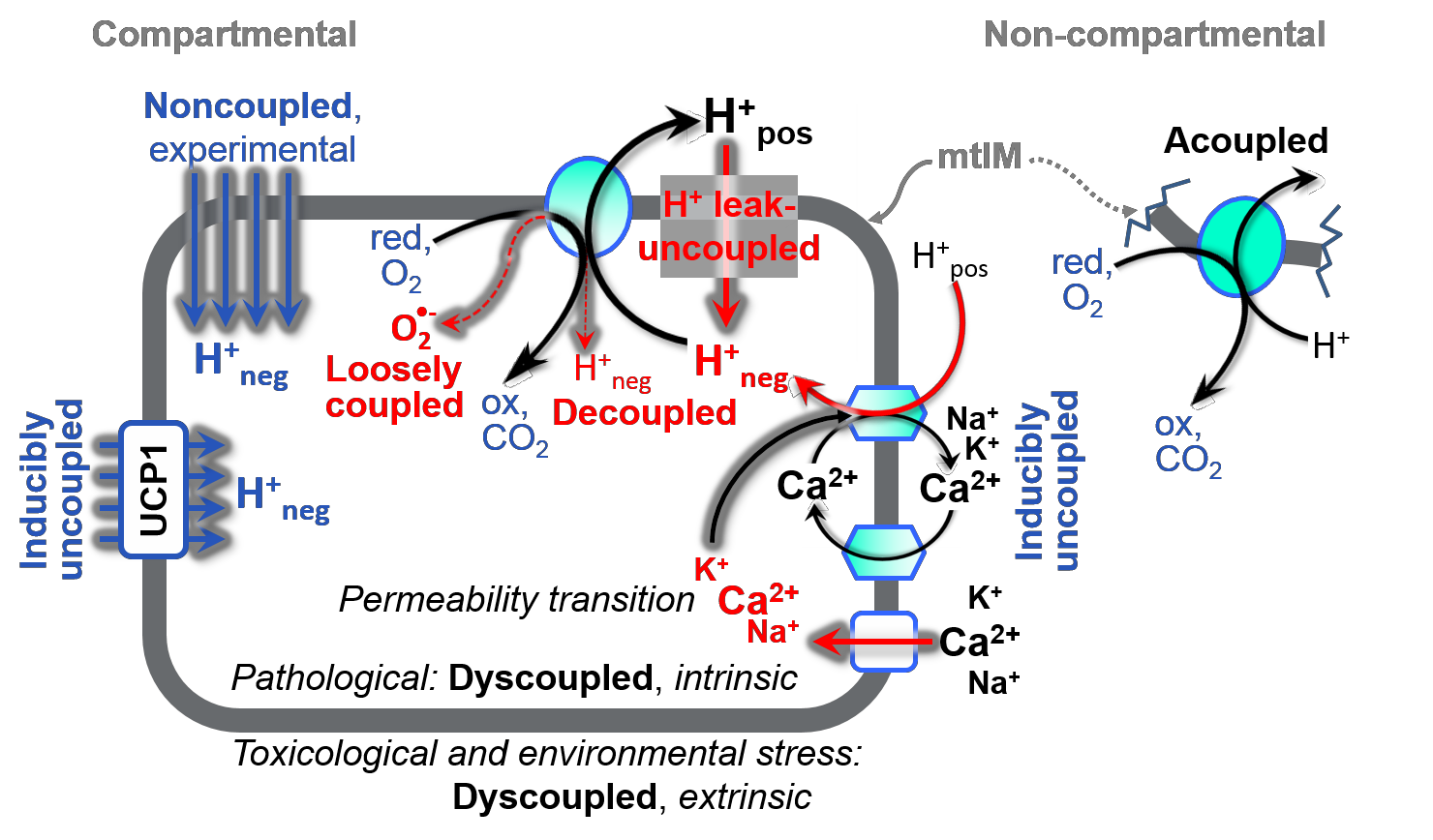
2. Cycling of other cations, strongly stimulated by permeability transition;

3. Proton slip in the proton pumps when protons are effectively not pumped (CI, CIII and

CIV) or are not driving phosphorylation (F-ATPase);

4. Loss of compartmental integrity when electron transfer is acoupled;

5. Electron leak in the loosely coupled univalent reduction of oxygen (O2; dioxygen) to superoxide (O2•–; superoxide anion radical).



**Fig 3. Mechanisms of respiratory uncoupling.** An intact mitochondrial inner membrane, mtIM, is required for vectorial, compartmental coupling. ‘Acoupled’ respiration is the consequence of structural disruption with catalytic activity of non-compartmental mitochondrial fragments. Inducibly uncoupled (activation of UCP1) and experimentally noncoupled respiration (titration of protonophores) stimulate respiration to maximum oxygen flux. H+ leak-uncoupled, decoupled, and loosely coupled respiration are components of intrinsic uncoupling. Pathological dysfunction may affect all types of uncoupling, including permeability transition, causing intrinsically dyscoupled respiration. Similarly, toxicological and environmental stress factors can cause extrinsically dyscoupled respiration.

*2.2. Coupling states and respiratory rates*

**Respiratory capacities in coupling control states:** To extend the classical nomenclature on mitochondrial coupling states (Section 2.3) by a concept-driven terminology that incorporates explicitly information on the nature of respiratory states, the terminology must be

553

554

555

556

557

558

559

560

561

562

563

564

565

566

567

568

569

570

571

572

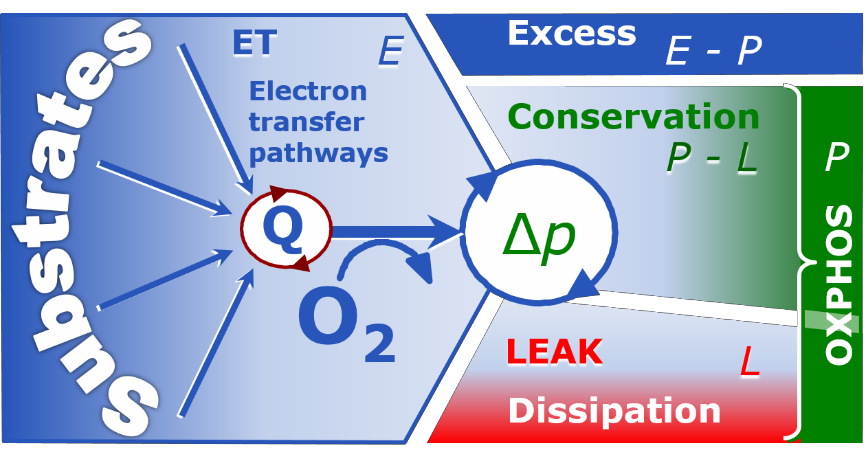
573

574

575

576

general and not restricted to any particular experimental protocol or mitochondrial preparation (Gnaiger 2009). We focus primarily on the conceptual ‘why’, along with clarification of the experimental ‘how’. Respiratory capacities delineate, comparable to channel capacity in information theory (Schneider 2006), the upper bound of the rate of respiration measured in defined coupling control states and electron transfer-pathway (ET-pathway) states (**Fig. 4**).

**Fig. 4. Four-compartment model of oxidative phosphorylation.** Respiratory states (ET, OXPHOS, LEAK; **Table 1**) and corresponding rates (*E, P, L*) are connected by the protonmotive force, ∆*p*. ET- capacity, *E*, is partitioned into (*1*) dissipative LEAK-respiration, *L*, when the Gibbs energy change of catabolic O2 consumption is

irreversibly lost, (*2*) net OXPHOS-capacity, *P-L*, with partial conservation of the capacity to perform work, and (*3*) the excess capacity, *E-P*. Modified from Gnaiger (2014).

Table 1. Coupling states and residual oxygen consumption in mitochondrial preparations in relation to respiration- and phosphorylation-rate, JkO2 and JP», and protonmotive force, ∆p. Coupling states are established at kinetically-saturating concentrations of fuel substrates and O2.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **State** | ***J*kO2** | ***J*P»** | ∆***p*** | **Inducing factors** | **Limiting factors** |
| LEAK | *L*; low, cation leak-dependent respiration | 0 | max. | proton leak, slip, and cation cycling | *J*P» = 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 [Pi] | *J*P» by phosphorylation- pathway; or *J*kO2 by ET- capacity |
| ET | *E*; max., noncoupled respiration | 0 | low | optimal external uncoupler concentration for max. *J*O2,*E* | *J*kO2 by ET-capacity |
| ROX | *Rox*; min., residual O2 consumption | 0 | 0 | *J*O2,*Rox* in non-ET- pathway oxidation  reactions | full inhibition of ET- pathway; or absence of fuel substrates |

577

578

579

580

581

582

583

To provide a diagnostic reference for respiratory capacities of core energy metabolism, the capacity of *oxidative phosphorylation*, OXPHOS, is measured at kinetically-saturating concentrations of ADP and Pi. The *oxidative* ET-capacity reveals the limitation of OXPHOS- capacity mediated by the *phosphorylation*-pathway. The ET- and phosphorylation-pathways comprise coupled segments of the OXPHOS-system. ET-capacity is measured as noncoupled respiration by application of *external uncouplers*. The contribution of *intrinsically uncoupled*

584

585

586

587

588

589

590

591

592

593

594

595

596

597

598

599

600

601

602

603

604

605

606

607

608

609

610

611

612

613

614

615

616

617

618

619

620

621

622

623

624

625

626

627

628

629

630

631

oxygen consumption is studied in the absence of ADP—by not stimulating phosphorylation, or by inhibition of the phosphorylation-pathway. The corresponding states are collectively classified as LEAK-states, when oxygen consumption compensates mainly for ion leaks, including the proton leak. Defined coupling states are induced by: (*1*) adding cation chelators such as EGTA, binding free Ca2+ and thus limiting cation cycling; (*2*) adding ADP and Pi; (*3*) inhibiting the phosphorylation-pathway; and (*4*) uncoupler titrations, while maintaining a defined ET-pathway state with constant fuel substrates and inhibitors of specific branches of the ET-pathway (**Fig. 1**).

The three coupling states, ET, LEAK and OXPHOS, are shown schematically with the corresponding respiratory rates, abbreviated as *E*, *L* and *P*, respectively (**Fig. 4**). We distinguish metabolic *pathways* from metabolic *states* and the corresponding metabolic *rates*; for example: ET-pathways (**Fig. 4**), ET-state (**Fig. 5C**), and ET-capacity, *E*, respectively (**Table 1**). The protonmotive force is *high* in the OXPHOS-state when it drives phosphorylation, *maximum* in the LEAK-state of coupled mitochondria, driven by LEAK-respiration at a minimum back flux of cations to the matrix side, and *very low* in the ET-state when uncouplers short-circuit the proton cycle (**Table 1**).

*E* may exceed or be equal to *P*. *E* > *P* is observed in many types of mitochondria, varying between species, tissues and cell types (Gnaiger 2009). *E*-*P* is the excess ET-capacity pushing the phosphorylation-flux (**Fig. 1B**) to the limit of its *capacity of utilizing* the protonmotive force. In addition, the magnitude of *E*-*P* depends on the tightness of respiratory coupling or degree of uncoupling, since an increase of *L* causes *P* to increase towards the limit of *E*. The *excess E-P* capacity, *E*-*P*, therefore, provides a sensitive diagnostic indicator of specific injuries of the phosphorylation-pathway, under conditions when *E* remains constant but *P* declines relative to controls (**Fig. 4**). Substrate cocktails supporting simultaneous convergent electron transfer to the Q-junction for reconstitution of TCA cycle function establish pathway control states with high ET-capacity, and consequently increase the sensitivity of the *E-P* assay.

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

The net OXPHOS-capacity is calculated by subtracting *L* from *P* (**Fig. 4**). Then the net P»/O2 equals P»/(*P-L*), wherein the dissipative LEAK component in the OXPHOS-state may be overestimated. This can be avoided by measuring LEAK-respiration in a state when the

protonmotive force is adjusted to its slightly lower value in the OXPHOS-state—by titration of an ET inhibitor (Divakaruni and Brand 2011). Any turnover-dependent components of proton leak and slip, however, are underestimated under these conditions (Garlid *et al.* 1993). In general, it is inappropriate to use the term *ATP production* or *ATP turnover* for the difference of oxygen consumption measured in states *P* and *L*. The difference *P-L* is the upper limit of the part of OXPHOS-capacity that is freely available for ATP production (corrected for LEAK- respiration) and is fully coupled to phosphorylation with a maximum mechanistic stoichiometry (**Fig. 4**).

**LEAK-state** (**Fig. 5A**): The LEAK-state is defined as a state of mitochondrial respiration when O2 flux mainly compensates for ion leaks in the absence of ATP synthesis, at kinetically- saturating concentrations of O2 and respiratory fuel substrates. LEAK-respiration is measured to obtain an estimate of *intrinsic uncoupling* without addition of an experimental uncoupler: (*1*)

632

633

634

635

636

637

638

639

640

641

642

643

644

645

646

647

648

649

650

651

652

653

654

655

656

657

658

659

660

661

662

663

664

665

666

667

668

669

670

671

672

673

674

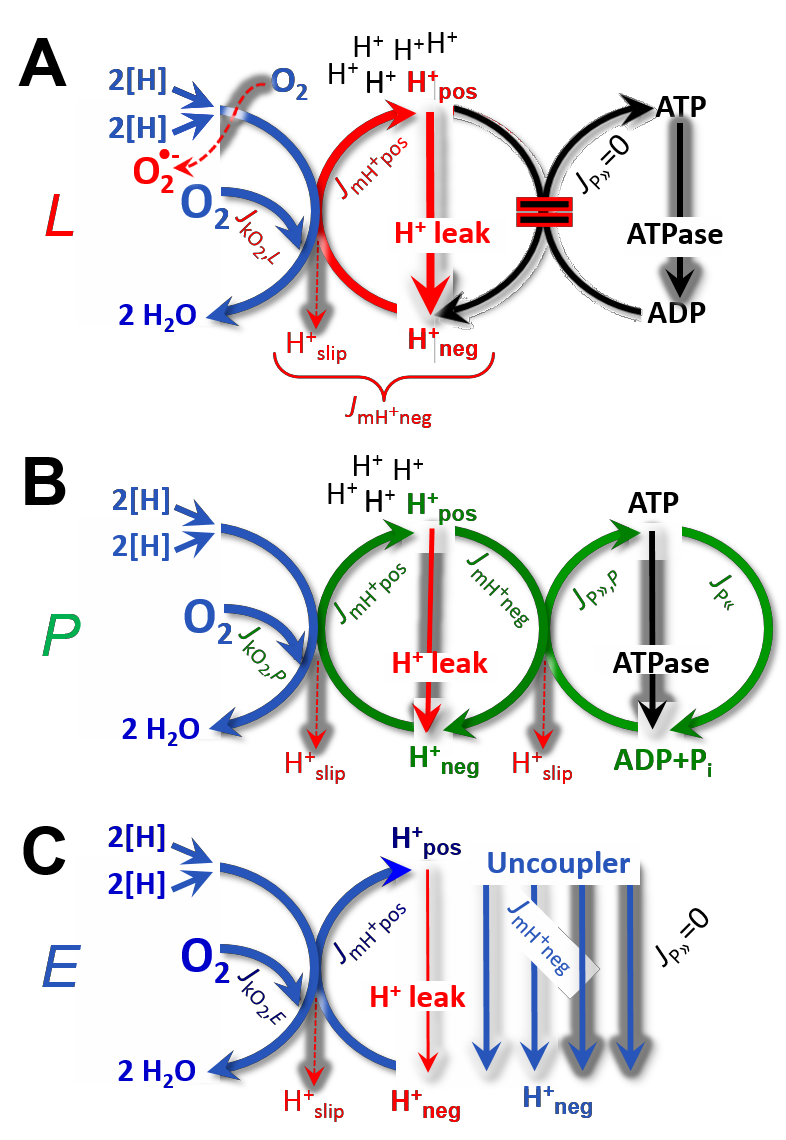
in the absence of adenylates; (*2*) after depletion of ADP at a maximum ATP/ADP ratio; or (*3*) after inhibition of the phosphorylation-pathway by inhibitors of F-ATPase—such as oligomycin, or of adenine nucleotide translocase—such as carboxyatractyloside.

Adjustment of the nominal concentration of these inhibitors to the density of biological sample applied can minimize or avoid inhibitory side-effects exerted on ET-capacity or even some dyscoupling.

**Proton leak and uncoupled respiration:** Proton leak is a leak current of protons. The intrinsic proton leak is the *uncoupled* process in which protons diffuse across the mtIM in the dissipative direction of the downhill protonmotive force without coupling to phosphorylation (**Fig. 5A**). The proton leak flux depends non- linearly on the protonmotive force (Garlid *et al.* 1989; Divakaruni and Brand 2011), it is a property of the mtIM and may be enhanced due to possible contaminations by free fatty acids. Inducible uncoupling mediated by uncoupling protein

1 (UCP1) is physiologically controlled, *e.g.*, in brown adipose tissue. UCP1 is a member of the mitochondrial carrier family which is involved in the translocation of protons across the mtIM (Klingenberg

2017). Consequently, the short-



**Fig. 5. Respiratory coupling states. A: LEAK-state and rate,** *L***:** Phosphorylation is arrested, *J*P» = 0, and catabolic oxygen flux, *J*kO2,*L*, is controlled mainly by the proton leak, *J*mH+neg,*L*, at maximum protonmotive force (**Fig. 3**). **B: OXPHOS-state and rate,** *P***:** Phosphorylation, *J*P», is stimulated by kinetically- saturating [ADP] and [Pi], and is supported by a high protonmotive force. O2 flux, *J*kO2,*P*, is well-coupled at a P»/O2 ratio of *J*P»,*P*/*J*O2,*P*. **C: ET-state and rate,** *E***:** Noncoupled respiration, *J*kO2,*E*, is maximum at optimum exogenous uncoupler concentration and phosphorylation is zero, *J*P» = 0. See also **Fig. 2**.

675

676

677

678

679

680

681

682

circuit diminishes the protonmotive force and stimulates electron transfer to O2 and heat dissipation without phosphorylation of ADP.

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

683

Table 2. Terms on respiratory coupling and uncoupling.

|  |  |  |  |
| --- | --- | --- | --- |
| **Term** | ***J*kO2** | **P»/O2** | **Note** |
| acoupled |  | 0 | electron transfer in mitochondrial fragments without vectorial proton translocation (**Fig. 3**) |

uncoupled *L* 0 non-phosphorylating LEAK-respiration (**Fig. 5A**)



intrinsic, no protonophore added

proton leak- uncoupled

0 component of *L*, H+ diffusion across the mtIM (**Fig. 3**)

decoupled 0 component of *L*, proton slip (**Fig. 3**)

loosely coupled

0 component of *L*, lower coupling due to superoxide formation and bypass of proton pumps (**Fig. 3**)

dyscoupled 0 pathologically, toxicologically, environmentally increased uncoupling, mitochondrial dysfunction

inducibly uncoupled

0 by UCP1 or cation (*e.g.,* Ca2+) cycling (**Fig. 3**)

684

685

686

687

688

689

690

691

692

693

694

695

696

697

698

699

700

701

702

703

704

705

706

707

708

709

noncoupled *E* 0 non-phosphorylating respiration stimulated to maximum flux at optimum exogenous uncoupler concentration (**Fig. 5C**)

well-coupled *P* high phosphorylating respiration with an intrinsic

LEAK component (**Fig. 5B**)

fully coupled *P* – *L* max. OXPHOS-capacity corrected for LEAK-

respiration (**Fig. 4**)

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

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

**Loss of compartmental integrity and acoupled respiration:** Electron transfer and O2 consumption proceed without compartmental proton translocation in disrupted mitochondrial fragments. Such fragments form during mitochondrial isolation, and may not fully fuse to re-

establish structurally intact mitochondria. Loss of mtIM integrity, therefore, is the cause of acoupled respiration, which is a nonvectorial dissipative process without control by the protonmotive force.

**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 (**Fig. 3**).

**OXPHOS-state** (**Fig. 5B**): The OXPHOS-state is defined as the respiratory state with kinetically-saturating concentrations of O2, respiratory and phosphorylation substrates, and

710

711

712

713

714

715

716

717

718

719

720

721

722

723

724

725

726

727

728

729

730

731

732

733

734

735

736

737

738

739

740

741

742

743

744

745

746

747

748

749

750

751

752

753

754

755

756

757

758

759

760

absence of exogenous uncoupler, which provides an estimate of the maximal respiratory capacity in the OXPHOS-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 OXPHOS-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 a reduced conductance of the mtOM (Jepihhina *et al.* 2011, Illaste *et al.* 2012, Simson *et al*. 2016), e.g. via regulation of VDAC permeability by tubulin (Rostovtseva *et al.* 2008) or other intracellular structures (Birkedal *et al*. 2014). 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 OXPHOS-capacity in human clinical cancer samples and permeabilized cells (Klepinin *et al.* 2016; Koit *et al.* 2017). Whereas 2.5 to 5 mM ADP is sufficient to obtain the actual OXPHOS-capacity in many types of permeabilized tissue and cell preparations, experimental validation is required in each specific case, e.g. by an adequate titration series of ADP. The latter can also report any changes in the accessibility of ADP to OXPHOS. In contrast, low ADP concentration is sufficient if ADP is regenerated in the mtIM, e.g. by mitochondrial isoforms of creatine kinase (mtCK) or nucleoside disphosphate kinase (NDPK-D/NME4), when adding Cr or any NDP (other than ADP) in the experimental system.

**Electron transfer*-*state** (**Fig. 5C**): The ET-state is defined as the *noncoupled* state with kinetically-saturating concentrations of O2, respiratory substrate and optimum *exogenous* uncoupler concentration for maximum O2 flux, as an estimate of ET-capacity. Inhibition of respiration is observed at higher than optimum uncoupler concentrations. As a consequence of the nearly collapsed protonmotive force, the driving force is insufficient for phosphorylation, and *J*P» = 0.

**ROX state and *Rox*:** Besides the three fundamental coupling states of mitochondrial preparations, the state of residual oxygen consumption, ROX, is relevant to assess respiratory function. ROX is not a coupling state. The rate of residual oxygen consumption, *Rox*, is defined

as O2 consumption due to oxidative side reactions remaining after inhibition of ET—with rotenone, malonic acid and antimycin A. Cyanide and azide not only inhibit CIV but also

several peroxidases involved in *Rox*. ROX represents a baseline that is used to correct mitochondrial respiration in defined coupling states. *Rox* is not necessarily equivalent to non- mitochondrial respiration, considering oxygen-consuming reactions in mitochondria not related to ET—such as oxygen consumption in reactions catalyzed by monoamine oxidases (type A and B), monooxygenases (cytochrome P450 monooxygenases), dioxygenase (sulfur dioxygenase and trimethyllysine dioxygenase), and several hydoxylases. Mitochondrial preparations, especially those obtained from liver, may be contaminated by peroxisomes. This fact makes the exact determination of mitochondrial oxygen consumption and mitochondria- associated generation of reactive oxygen species complicated (Schönfeld *et al.* 2009). The dependence of ROX-linked oxygen consumption needs to be studied in detail together with non-ET enzyme activities, availability of specific substrates, oxygen concentration, and electron leakage leading to the formation of reactive oxygen species.

*2.3. Classical terminology for isolated mitochondria*

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

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

761

762

763

Table 3. Metabolic states of mitochondria (Chance and

Williams, 1956; Table V).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **State** | **[O2]** | **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 |

764

765

766

767

768

769

770

771

772

773

774

775

776

777

778

779

780

781

782

783

784

785

786

787

788

789

790

791

792

793

794

795

796

797

798

799

800

801

802

803

804

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

**State 2** is induced by addition of a ‘high’ concentration of ADP (typically 100 to 300

µM), which stimulates respiration transiently on the basis of endogenous fuel substrates and phosphorylates only a small portion of the added ADP. State 2 is then obtained at a low respiratory activity limited by exhausted endogenous fuel substrate availability (**Table 3**). If addition of specific inhibitors of respiratory complexes—such as rotenone—does not cause a further decline of oxygen consumption, State 2 is equivalent to the state of residual oxygen consumption, ROX (See below.). If inhibition is observed, undefined endogenous fuel substrates are a confounding factor of pathway control, contributing to the effect of subsequently externally added substrates and inhibitors. In contrast to the original protocol, an alternative sequence of titration steps is frequently applied, in which the alternative ‘State 2’ has an entirely different meaning, when this second state is induced by addition of fuel substrate without ADP (LEAK-state; in contrast to State 2 defined in **Table 1** as a ROX state), followed by addition of ADP.

**State 3** is the state stimulated by addition of fuel substrates while the ADP concentration is still high (**Table 3**) and supports coupled energy transformation through oxidative phosphorylation. 'High ADP' is a concentration of ADP specifically selected to allow the measurement of State 3 to State 4 transitions of isolated mitochondria in a closed respirometric chamber. Repeated ADP titration re-establishes State 3 at ‘high ADP’. Starting at oxygen concentrations near air-saturation (ca. 200 µM O2 at sea level and 37 °C), the total ADP concentration added must be low enough (typically 100 to 300 µM) to allow phosphorylation to ATP at a coupled rate of oxygen consumption that does not lead to oxygen depletion during the transition to State 4. In contrast, kinetically-saturating ADP concentrations usually are 10- fold higher than 'high ADP', *e.g.,* 2.5 mM in isolated mitochondria. The abbreviation State 3u is occasionally used in bioenergetics, to indicate the state of respiration after titration of an uncoupler, without sufficient emphasis on the fundamental difference between OXPHOS- capacity (*well-coupled* with an *endogenous* uncoupled component) and ET-capacity (*noncoupled*).

**State 4** is a LEAK-state that is obtained only if the mitochondrial preparation is intact and well-coupled. Depletion of ADP by phosphorylation to ATP leads to a decline in the rate of oxygen consumption in the transition from State 3 to State 4. Under these conditions of State

4, a maximum protonmotive force and high ATP/ADP ratio are maintained. For calculation of P»/O2 ratios the gradual decline of *Y*P»/O2 towards diminishing [ADP] at State 4 must be taken into account (Gnaiger 2001). State 4 respiration, *L*T (**Table 1**), reflects intrinsic proton leak and intrinsic ATP hydrolysis activity. Oxygen consumption in State 4 is an overestimation of LEAK-respiration if the contaminating ATP hydrolysis activity recycles some ATP to ADP, *J*P«, which stimulates respiration coupled to phosphorylation, *J*P» > 0. This can be tested by inhibition of the phosphorylation-pathway using oligomycin, ensuring that *J*P» = 0 (State 4o).

805

806

807

808

809

810

811

812

813

814

815

816

817

818

819

820

821

822

823

824

825

826

827

828

829

830

831

832

833

834

835

836

837

838

839

840

841

842

843

844

845

846

847

848

849

850

851

852

853

854

855

856

Alternatively, sequential ADP titrations re-establish State 3, followed by State 3 to State 4 transitions while sufficient oxygen is available. Anoxia may be reached, however, before exhaustion of ADP (State 5).

**State 5** is the state after exhaustion of oxygen in a closed respirometric chamber. Diffusion of oxygen from the surroundings into the aqueous solution may be a confounding factor preventing complete anoxia (Gnaiger 2001). Chance and Williams (1955) provide an alternative definition of State 5, which gives it the different meaning of ROX versus anoxia:

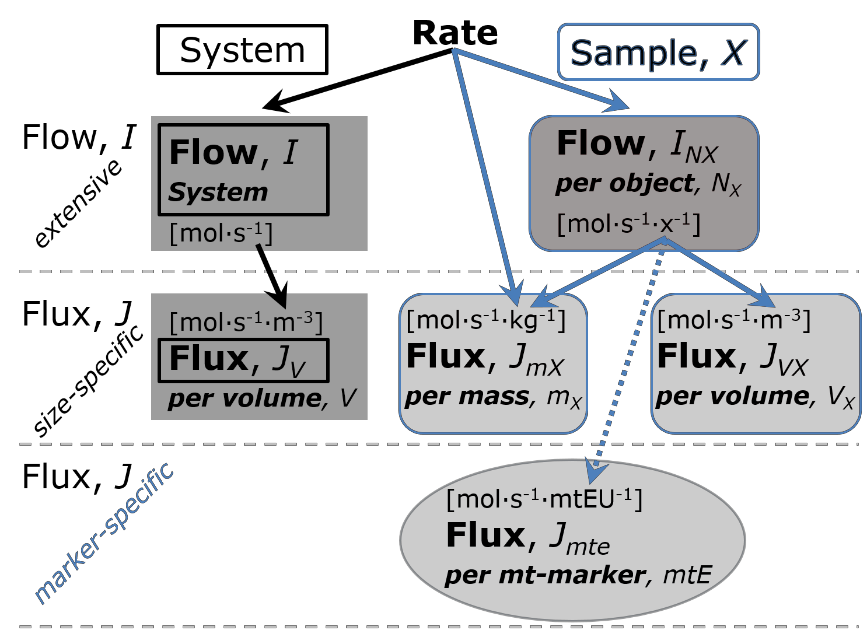
‘State 5 may be obtained by antimycin A treatment or by anaerobiosis’.

In **Table 3**, only States 3 and 4 (and ‘State 2’ in the alternative protocol: addition of fuel substrates without ADP; not included in the table) are coupling control states, with the restriction that O2 flux in State 3 may be limited kinetically by non-saturating ADP concentrations (**Table 1**).

**3. Normalization: fluxes and flows**

*3.1. Normalization: system or sample*

The term *rate* is not sufficiently defined to be useful for reporting data (**Fig. 6**). The inconsistency of the meanings of rate becomes fully apparent when considering Galileo Galilei’s famous principle, that ‘bodies of different weight all fall at the same rate (have a constant acceleration)’ (Coopersmith 2010).

**Fig. 6. Different meanings of *rate* may lead to confusion, if the normalization is not sufficiently specified.** Results are frequently expressed as mass- specific *flux*, *JmX*, per mg protein, dry or wet weight (mass). Cell volume, *V*cell, may be used for normalization (volume-specific flux, *JV*cell), which must be clearly distinguished from flow per cell, *IN*cell, or flux, *JV*, expressed for methodological reasons per volume of the measurement system. For details see **Table 4**.

**Flow per system, *I*:** In a generalization of electrical terms, flow as an extensive quantity

(*I*; per system) is distinguished from flux as a size-specific quantity (*J*; per system size) (**Fig.**

**6**). Electric current is flow, *I*el [A ≡ C∙s-1] per system (extensive quantity). When dividing this extensive quantity by system size (cross-sectional area of a ‘wire’), a size-specific quantity is

obtained, which is flux (current density), ***J***el [A∙m-2 = C∙s-1∙m-2].

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

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

857

858

859

860

861

862

863

864

865

866

867

868

869

870

871

872

873

874

875

876

877

878

879

880

881

882

883

884

885

886

887

888

889

890

891

892

893

894

895

896

897

898

899

900

901

902

903

904

905

906

907

interacting homogenous subsystems. Tissue-specific quantities (related to the *sample* in contrast to the *system*) are of fundamental interest in comparative mitochondrial physiology, where *specific* refers to the *type of the sample* rather than *mass of the system*. The term *specific*, therefore, must be clarified; *sample*-specific, *e.g.*, muscle mass-specific normalization, is distinguished from *system*-specific quantities (mass or volume; **Fig. 6**).

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

Fluxes are *vectors*, if they have *spatial* geometric direction in addition to magnitude. Electric charge per unit time is electric flow or current, *I*el = d*Q*el∙d*t*-1 [A]. When expressed per unit cross-sectional area, *A* [m2], a vector flux is obtained, which is current density or surface- density of flow) perpendicular to the direction of flux, ***J***el = *I*el∙*A*-1 [A∙m-2] (Cohen et al. 2008). For all transformations *flows, I*tr*,* are defined as extensive quantities. Vector and scalar *fluxes* are obtained as ***J***tr = *I*tr∙*A*-1 [mol∙s-1∙m-2] and *J*tr = *I*tr∙*V*-1 [mol∙s-1∙m-3], expressing flux as an area- specific vector or volume-specific vectorial or scalar quantity, respectively (Gnaiger 1993b).

We suggest to define: (*1*) *vectoral* fluxes, which are translocations as functions of *gradients* with direction in geometric space in continuous systems; (*2*) *vectorial* fluxes, which describe translocations in discontinuous systems and are restricted to information on *compartmental differences* (**Fig. 2**, transmembrane proton flux); and (*3*) *scalar* fluxes, which are transformations in a *homogenous* system (**Fig. 2**, catabolic O2 flux, *J*kO2).

Vectorial transmembrane proton fluxes, *J*mH+pos and *J*mH+neg, are analyzed in a heterogenous compartmental system as a quantity with *directional* but not *spatial* information.

Translocation of protons across the mtIM has a defined direction, either from the negative compartment (matrix space; negative, neg–compartment) to the positive compartment (inter- membrane space; positive, pos–compartment) or *vice versa* (**Fig. 2**). The arrows defining the direction of the translocation between the two compartments may point upwards or downwards, right or left, without any implication that these are actual directions in space. The pos– compartment is neither above nor below the neg–compartment in a spatial sense, but can be visualized arbitrarily in a figure in the upper position (**Fig. 2**). In general, the *compartmental direction* of vectorial translocation from the neg–compartment to the pos–compartment is defined by assigning the initial and final state as *ergodynamic compartments*, H+neg → H+ os or

p

0 = -1 H+neg+1 H+pos, related to work (erg = work) that must be performed to lift the proton from a lower to a higher electrochemical potential or from the lower to the higher ergodynamic

compartment (Gnaiger 1993b).

In analogy to *vectorial* translocation, the direction of a *scalar* chemical reaction, A → B or 0 = -1 A+1 B, is defined by assigning substrates and products, A and B, as ergodynamic compartments. O2 is defined as a substrate in respiratory O2 consumption, which together with the fuel substrates comprises the substrate compartment of the catabolic reaction (**Fig. 2**). Volume-specific scalar O2 flux is coupled to vectorial translocation, yielding the H+pos/O2 ratio (**Fig. 1**).

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

**System-specific flux, *JV*,O2**: The experimental system (experimental chamber) is part of the measurement apparatus, separated from the environment as an isolated, closed, open, isothermal or non-isothermal system (**Table 4**). On another level, we distinguish between (*1*) the *system* with volume *V* and mass *m* defined by the system boundaries, and (*2*) the *sample* or *objects* with volume *VX* and mass *mX* which are enclosed in the experimental chamber (**Fig. 6**). Metabolic O2 flow per object, *I*O2/*X*, increases as the mass of the object is increased. Sample mass-specific O2 flux, *J*O2/*mX* should be independent of the mass of the sample studied in the

908

909

910

911

912

913

914

915

916

917

918

919

920

921

922

923

924

925

926

927

928

929

930

931

932

933

934

935

936

937

938

939

940

941

942

943

944

945

946

947

948

949

950

951

952

953

954

955

956

957

958

instrument chamber, but system volume-specific O2 flux, *JV*,O2 (per volume of the instrument chamber), should increase in direct proportion to the mass of the sample in the chamber. Whereas *JV*,O2 depends on mass-concentration of the sample in the chamber, it should be independent of the chamber (system) volume at constant sample mass. There are practical limitations to increase the mass-concentration of the sample in the chamber, when one is concerned about crowding effects and instrumental time resolution.

When the reactor volume does not change during the reaction, which is typical for liquid phase reactions, the volume-specific *flux of a chemical reaction* r is the time derivative of the advancement of the reaction per unit volume, *JV*,rB = drξB/d*t*∙*V*-1 [(mol∙s•1)∙L•1]. The *rate of concentration change* is d*c*B/d*t* [(mol∙L•1)∙s•1], where concentration is *c*B = *n*B/*V*. There is a difference between (*1*) *JV*,rO2 [mol∙s•1∙L•1] and (*2*) rate of concentration change [mol∙L•1∙s•1]. These merge to a single expression only in closed systems. In open systems, external fluxes (such as O2 supply) are distinguished from internal transformations (catabolic flux, O2 consumption). In a closed system, external flows of all substances are zero and O2 consumption (internal flow of catabolic reactions k), *I*kO2 [pmol∙s-1], causes a decline of the amount of O2 in the system, *n*O2 [nmol]. Normalization of these quantities for the volume of the system, *V* [L ≡ dm3], yields volume-specific O2 flux, *JV*,kO2 = *I*kO2/*V* [nmol∙s-1∙L-1], and O2 concentration, [O2] or *c*O2 = *n*O2/*V* [µmol∙L•1 = µM = nmol∙mL•1]. Instrumental background O2 flux is due to external flux into a non-ideal closed respirometer; then total volume-specific flux has to be corrected for instrumental background O2 flux—O2 diffusion into or out of the instrumental chamber. *JV*,kO2 is relevant mainly for methodological reasons and should be compared with the accuracy of instrumental resolution of background-corrected flux, *e.g.*, ±1 nmol∙s•1∙L-1 (Gnaiger 2001).

‘Metabolic’ or catabolic indicates O2 flux, *J*kO2, corrected for: (*1*) instrumental background O2 flux; (*2*) chemical background O2 flux due to autoxidation of chemical components added to the incubation medium; and (*3*) *Rox* for O2-consuming side reactions unrelated to the catabolic pathway k.

*3.3. Normalization: per sample*

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

**Sample concentration, *CmX*:** Normalization for sample concentration is required to report respiratory data. Considering a tissue or cells as the sample, *X*, the sample mass is *mX* [mg], which is frequently measured as wet or dry weight, *W*w or *W*d [mg], or as amount of tissue or cell protein, *m*Protein. In the case of permeabilized tissues, cells, and homogenates, the sample concentration, *CmX* = *mX*/*V* [g∙L-1 = mg∙mL•1], is the mass of the subsample of tissue that is transferred into the instrument chamber.

**Mass-specific flux, *J*O2/*mX***: Mass-specific flux is obtained by expressing respiration per mass of sample, *mX* [mg]. *X* is the type of sample—isolated mitochondria, tissue homogenate, permeabilized fibres or cells. Volume-specific flux is divided by mass concentration of *X*, *J*O2*/mX*

= *JV*,O2/*CmX*; or flow per cell is divided by mass per cell, *J*O2*/m*cell = *I*O2/cell/*M*cell. If mass-specific

O2 flux is constant and independent of sample size (expressed as mass), then there is no interaction between the subsystems. A 1.5 mg and a 3.0 mg muscle sample respires at identical

mass-specific flux. Mass-specific O2 flux, however, may change with the mass of a tissue sample, cells or isolated mitochondria in the measuring chamber, in which the nature of the

959

960

961

962

963

964

965

966

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Expression** | **Symbol** | **Definition** | **Unit** | **Notes** |
| **Sample** | | | | |
| identity of sample | *X* | object: cell, tissue,  animal, patient |  |  |
| number of sample entities *X* | *NX* | number of objects | x |  |
| mass of sample *X* | *mX* |  | kg | 1 |
| mass of object *X* | *MX* | *MX* = *mX*∙*NX*-1 | kg∙x•1 | 1 |
| **Mitochondria** | | | | |
| mitochondria | mt | *X* = mt |  |  |
| amount of mt-elements | *mtE* | quantity of mt-marker | mtEU |  |
| **Concentrations** | | | | |
| object number concentration | *CNX* | *CNX* = *NX*∙*V*-1 | x∙m•3 | 2 |
| sample mass concentration | *CmX* | *CmX* = *mX*∙*V*-1 | kg∙m•3 |  |
| mitochondrial concentration | *CmtE* | *CmtE* = *mtE*∙*V*-1 | mtEU∙m•3 | 3 |
| specific mitochondrial density | *DmtE* | *DmtE* = *mtE*∙*mX*-1 | mtEU∙kg•1 | 4 |
| mitochondrial content,  *mtE* per object *X* | *mtEX* | *mtEX* = *mtE*∙*NX*-1 | mtEU∙x•1 | 5 |
| **O2 flow and flux** |  |  |  | 6 |
| flow, system | *I*O2 | internal flow | mol∙s-1 | 7 |
| volume-specific flux | *JV,*O2 | *JV,*O2 = *I*O2∙*V*-1 | mol∙s-1∙m-3 | 8 |
| flow per object *X* | *I*O2*/X* | -1 | -1 -1 | 9 |
| mass-specific flux | *J*O2/*mX* | *J*O2/*mX* = *JV,*O2∙*CmX*-1 | mol∙s-1∙kg-1 |  |
| mitochondria-specific flux | *J*O2/*mtE* | *J*O2/*mtE* = *JV,*O2∙*CmtE*-1 | mol∙s-1∙mtEU-1 | 10 |

interaction becomes an issue. Therefore, cell density must be optimization, particularly in experiments carried out in wells, considering the confluency of the cell monolayer or clumps of cells (Salabei *et al.* 2014).

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

Table 4. Sample concentrations and normalization of flux.

*I*O2*/X* = *JV,*O2∙*CNX*

mol∙s ∙x

967

968

969

970

971

972

973

974

975

976

977

1 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.

2 In case sample X = cells, the object number concentration is CNcell = Ncell∙V-1, and volume may be expressed in [dm3 ≡ L] or [cm3 = mL]. See Table 5 for different object types.

3 mt-concentration is an experimental variable, dependent on sample concentration: (1) CmtE = mtE∙V•1; (2) CmtE = mtEX∙CNX; (3) CmtE = CmX∙DmtE.

4 If the amount of mitochondria, mtE, is expressed as mitochondrial mass, then DmtE is the mass fraction of mitochondria in the sample. If mtE is expressed as mitochondrial volume, Vmt, and the

mass of sample, mX, is replaced by volume of sample, VX, then DmtE is the volume fraction of mitochondria in the sample.

-1 -1

978

5 mtEX = mtE∙NX

= CmtE∙CNX .

979

980

981

982

983

6 O2 can be replaced by other chemicals B to study different reactions, e.g., ATP, H2O2, or compartmental translocations, e.g., Ca2+.

7 *I*O2 and V are defined per instrument chamber as a system of constant volume (and constant temperature), which may be closed or open. *I*O2 is abbreviated for *I*rO2, i.e., the metabolic or internal O2 flow of the chemical reaction r in which O2 is consumed, hence the negative stoichiometric

-1

984

number, νO2 = -1. *I*rO2 = dr*n*O2/d*t*∙νO2

. If r includes all chemical reactions in which O2 participates, then

985

dr*n*O2 = d*n*O2 – de*n*O2, where d*n*O2 is the change in the amount of O2 in the instrument chamber and de*n*O2

986

987

988

989

990

is the amount of O2 added externally to the system. At steady state, by definition d*n*O2 = 0, hence dr*n*O2

= –de*n*O2.

8 *JV,*O2 is an experimental variable, expressed per volume of the instrument chamber.

9 *I*O2*/X* is a physiological variable, depending on the size of entity X.

10 There are many ways to normalize for a mitochondrial marker, that are used in different experimental

-1 -1 •1

991

approaches: (*1*) *J*O2/*mtE* = *JV,*O2∙*CmtE*-1; (*2*) *J*O2/*mtE* = *JV,*O2∙*CmX*

∙*DmtE*

= *J*O2*/mX*∙*DmtE*

; (*3*) *J*O2/*mtE* =

992

993

994

*JV,*O2∙*CNX*•1∙*mtEX*•1 = *I*O2*/X*∙*mtEX*-1; (*4*) *J*O2/*mtE* = *I*O2∙*mtE*-1. The mt-elemental unit [mtEU] varies between different mt-markers.

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

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Identity of sample** | *X* | *NX* | **Mass*a*** | **Volume** | **mt-Marker** |
| mitochondrial preparation | Mtprep | [x] | [kg] | [m3] | [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 |
| intact cell | ce | *N*ce | *M*ce | *V*ce | *mtE*ce |
| Organism | org | *N*org | *M*org | *V*org |  |

995

996

997

998

999

1000

1001

1002

1003

1004

1005

1006

1007

1008

1009

1010

1011

1012

1013

1014

1015

1016

1017

1018

1019

1020

1021

1022

1023

1024

1025

1026

1027

1028

***a*** Instead of mass, frequently the wet weight or dry weight is stated, *W*w or *W*d.

*mX* is mass of the sample [kg], *MX* is mass of the object [kg∙x-1].

**Flow per object, *I*O2/*X*:** A special case of normalization is encountered in respiratory studies with permeabilized (or intact) cells. If respiration is expressed per cell, the O2 flow per measurement system is replaced by the O2 flow per cell, *I*O2/cell (**Table 4**). O2 flow can be calculated from volume-specific O2 flux, *JV,*O2 [nmol∙s-1∙L-1] (per *V* of the measurement chamber [L]), divided by the number concentration of cells, *CN*ce *= N*ce*/V* [cell∙L•1], where *N*ce is the number of cells in the chamber. Cellular O2 flow can be compared between cells of identical size. To take into account changes and differences in cell size, normalization is required to obtain cell size-specific or mitochondrial marker-specific O2 flux (Renner *et al.* 2003).

The complexity changes when the sample is a whole organism studied as an experimental model. The scaling law in respiratory physiology reveals a strong interaction of O2 consumption and individual body mass of an organism, since *basal* metabolic rate (flow) does not increase linearly with body mass, whereas *maximum* mass-specific O2 flux, 𝑉̇O2max or 𝑉̇O2peak, is approximately constant across a large range of individual body mass (Weibel and Hoppeler

2005), with individuals, breeds, and species deviating substantially from this relationship.

𝑉̇O2peak of human endurance athletes is 60 to 80 mL O2·min•1·kg•1 body mass, converted to

*JM*,O2peak of 45 to 60 nmol·s-1·g-1 (Gnaiger 2014; **Table 6**).

*3.4. Normalization for mitochondrial content*

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

Part of the mitochondrial content of a tissue is lost during preparation of isolated mitochondria. The fraction of mitochondria in the isolate is expressed as mitochondrial recovery. At a high mitochondrial recovery the sample of isolated mitochondria is more representative of the total mitochondrial population than in preparations characterized by low

1029

1030

1031

1032

1033

1034

1035

1036

1037

1038

1039

1040

1041

1042

1043

1044

1045

1046

1047

1048

1049

1050

1051

1052

1053

1054

1055

1056

1057

1058

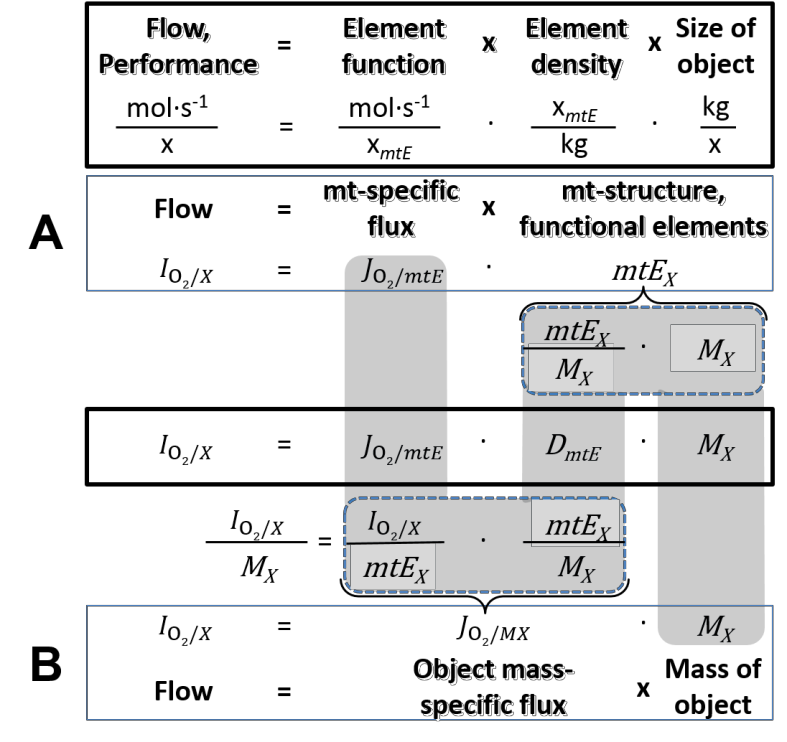
1059

1060

1061

recovery. Determination of the mitochondrial recovery and yield is based on measurement of the concentration of a mitochondrial marker in the tissue homogenate, *CmtE*,thom, which simultaneously provides information on the specific mitochondrial density in the sample.

Normalization is a problematic subject; it is essential to consider the question of the study. If the study aims at comparing tissue performance—such as the effects of a treatment on a specific tissue, then normalization can be successful, using tissue mass or protein content, for example. However, if the aim is to find differences on mitochondrial function independent of mitochondrial density (**Table 4**), then normalization to a mitochondrial marker is imperative (**Fig. 7**). One cannot assume that quantitative changes in various markers—such as mitochondrial proteins—necessarily occur in parallel with one another. It should be established that the marker chosen is not selectively altered by the performed treatment. In conclusion, the normalization must reflect the question under investigation to reach a satisfying answer. On the other hand, the goal of comparing results across projects and institutions requires standardization on normalization for entry into a databank.



**Fig. 7. Structure-function analysis of performance of an organism, organ or tissue, or a cell (sample entity, *X*). O2 flow, *I*O2/*X*, is the product of performance per functional element (element function, mitochondria-specific flux), element density (mitochondrial density, *DmtE*), and size of entity *X* (mass, *MX*).** (**A**) Structured analysis: performance is the product of mitochondrial *function* (mt-specific flux) and *structure* (functional elements; *DmtE* times mass of *X*). (**B**) Unstructured analysis: performance is the product of *entity mass-specific flux*, *J*O2/*MX*

= *I*O2/*X*/*MX* = *I*O2/*mX* [mol∙s-1∙kg-1] and *size of entity*, expressed as mass of *X*; *MX* = *mX*∙*NX*-1

[kg∙x•1]. See **Table 4** for further explanation of quantities and units. Modified from Gnaiger

(2014).

**Mitochondrial concentration, *CmtE*, and mitochondrial markers:** Mitochondrial organelles comprise a dynamic cellular reticulum in various states of fusion and fission. Hence, the definition of an "amount" of mitochondria is often misconceived: mitochondria cannot be counted reliably as a number of occurring elements. Therefore, quantification of the "amount" of mitochondria depends on the measurement of chosen mitochondrial markers. ‘Mitochondria are the structural and functional elemental units of cell respiration’ (Gnaiger 2014). The quantity of a mitochondrial marker can reflect the amount of *mitochondrial elements*, *mtE*,

1062

1063

1064

1065

1066

1067

1068

1069

1070

1071

1072

1073

1074

1075

1076

1077

1078

1079

1080

1081

1082

1083

1084

1085

1086

1087

1088

1089

1090

1091

1092

1093

1094

1095

1096

1097

1098

1099

1100

1101

1102

1103

1104

1105

1106

1107

1108

1109

1110

1111

1112

expressed in various mitochondrial elemental units [mtEU] specific for each measured mt- marker (**Table 4**). However, since mitochondrial quality may change in response to stimuli— particularly in mitochondrial dysfunction and after exercise training (Pesta *et al.* 2011; Campos *et al.* 2017)—some markers can vary while others are unchanged: (*1*) Mitochondrial volume and membrane area are structural markers, whereas mitochondrial protein mass is frequently used as a marker for isolated mitochondria. (*2*) Molecular and enzymatic mitochondrial markers (amounts or activities) can be selected as matrix markers, *e.g.*, citrate synthase activity, mtDNA; mtIM-markers, *e.g.*, cytochrome *c* oxidase activity, *aa*3 content, cardiolipin, or mtOM-markers, *e.g.*, TOM20. (*3*) Extending the measurement of mitochondrial marker enzyme activity to mitochondrial pathway capacity, ET- or OXPHOS-capacity can be considered as an integrative functional mitochondrial marker.

Depending on the type of mitochondrial marker, the mitochondrial elements, *mtE*, are expressed in marker-specific units. Mitochondrial concentration in the measurement chamber and the tissue of origin are quantified as (*1*) a quantity for normalization in functional analyses, *CmtE*, and (*2*) a physiological output that is the result of mitochondrial biogenesis and degradation, *DmtE*, respectively (**Table 4**). It is recommended, therefore, to distinguish *experimental mitochondrial concentration*, *CmtE* = *mtE*/*V* and *physiological mitochondrial density*, *DmtE* = *mtE*/*mX*. Then mitochondrial density is the amount of mitochondrial elements per mass of tissue, which is a biological variable (**Fig. 7**). The experimental variable is mitochondrial density multiplied by sample mass concentration in the measuring chamber, *CmtE*

= *DmtE*∙*CmX*, or mitochondrial content multiplied by sample number concentration, *CmtE* =

*mtEX*∙*CNX* (**Table 4**).

**Mitochondria-specific flux, *J*O2/*mtE***: Volume-specific metabolic O2 flux depends on: (*1*) the sample concentration in the volume of the instrument chamber, *CmX*, or *CNX*; (*2*) the mitochondrial density in the sample, *DmtE* = *mtE*/*mX* or *mtEX* = *mtE*/*NX*; and (*3*) the specific mitochondrial activity or performance per elemental mitochondrial unit, *J*O2/*mtE* = *JV*,O2/*CmtE* [mol∙s-1∙mtEU-1] (**Table 4**). Obviously, the numerical results for *J*O2/*mtE* vary with the type of mitochondrial marker chosen for measurement of *mtE* and *CmtE* = *mtE*/*V* [mtEU∙m-3].

*3.5. Evaluation of mitochondrial markers*

Different methods are implicated in the quantification of mitochondrial markers and have different strengths. Some problems are common for all mitochondrial markers, *mtE*: (*1*) Accuracy of measurement is crucial, since even a highly accurate and reproducible measurement of O2 flux results in an inaccurate and noisy expression normalized for a biased and noisy measurement of a mitochondrial marker. This problem is acute in mitochondrial respiration because the denominators used (the mitochondrial markers) are often small moieties of which accurate and precise determination is difficult. This problem can be avoided when O2 fluxes measured in substrate-uncoupler-inhibitor titration protocols are normalized for flux in a defined respiratory reference state, which is used as an *internal* marker and yields flux control ratios, *FCR*s. *FCR*s are independent of any *externally* measured markers and, therefore, are statistically robust, considering the limitations of ratios in general (Jasienski and Bazzaz 1999). *FCR*s indicate qualitative changes of mitochondrial respiratory control, with highest quantitative resolution, separating the effect of mitochondrial density or concentration on *J*O2/*mX* and *I*O2/*X* from that of function per elemental mitochondrial marker, *J*O2/*mtE* (Pesta *et al.* 2011; Gnaiger 2014). (*2*) If mitochondrial quality does not change and only the amount of mitochondria varies as a determinant of mass-specific flux, any marker is equally qualified in principle; then in practice selection of the optimum marker depends only on the accuracy and precision of measurement of the mitochondrial marker. (*3*) If mitochondrial flux control ratios change, then there may not be any best mitochondrial marker. In general, measurement of multiple mitochondrial markers enables a comparison and evaluation of normalization for a

1113

1114

1115

1116

1117

1118

1119

1120

1121

1122

1123

1124

1125

1126

1127

1128

1129

1130

1131

1132

1133

1134

1135

1136

1137

1138

1139

1140

1141

1142

1143

1144

1145

1146

1147

1148

1149

1150

1151

1152

1153

1154

1155

1156

1157

1158

1159

1160

1161

1162

1163

variety of mitochondrial markers. Particularly during postnatal development, the activity of marker enzymes—such as cytochrome *c* oxidase and citrate synthase—follows different time courses (Drahota *et al.* 2004). Evaluation of mitochondrial markers in healthy controls is insufficient for providing guidelines for application in the diagnosis of pathological states and specific treatments.

In line with the concept of the respiratory control ratio (Chance and Williams 1955a), the most readily used normalization is that of flux control ratios and flux control factors (Gnaiger

2014). Selection of the state of maximum flux in a protocol as the reference state has the advantages of: (*1*) internal normalization; (*2*) statistical linearization of the response in the range of 0 to 1; and (*3*) consideration of maximum flux for integrating a large number of elemental steps in the OXPHOS- or ET-pathways. This reduces the risk of selecting a functional marker that is specifically altered by the treatment or pathology, yet increases the chance that the highly integrative pathway is disproportionately affected, *e.g.,* the OXPHOS- rather than ET-pathway in case of an enzymatic defect in the phosphorylation-pathway. In this case, additional information can be obtained by reporting flux control ratios based on a reference state which indicates stable tissue-mass specific flux. Stereological determination of mitochondrial content via two-dimensional transmission electron microscopy can have limitations due to the dynamics of mitochondrial size (Meinild Lundby *et al.* 2017). Accurate determination of three- dimensional volume by two-dimensional microscopy can be both time consuming and statistically challenging (Larsen *et al.* 2012).

The validity of using mitochondrial marker enzymes (citrate synthase activity, Complex I–IV amount or activity) for normalization of flux is limited in part by the same factors that apply to flux control ratios. Strong correlations between various mitochondrial markers and citrate synthase activity (Reichmann *et al.* 1985; Boushel *et al.* 2007; Mogensen *et al.* 2007) are expected in a specific tissue of healthy subjects and in disease states not specifically targeting citrate synthase. Citrate synthase activity is acutely modifiable by exercise (Tonkonogi *et al.* 1997; Leek *et al.* 2001). Evaluation of mitochondrial markers related to a selected age and sex cohort cannot be extrapolated to provide recommendations for normalization in respirometric diagnosis of disease, in different states of development and ageing, different cell types, tissues, and species. mtDNA normalized to nDNA via qPCR is correlated to functional mitochondrial markers including OXPHOS- and ET-capacity in some cases (Puntschart *et al.* 1995; Wang *et al.* 1999; Menshikova *et al.* 2006; Boushel *et al.* 2007), but lack of such correlations have been reported (Menshikova *et al.* 2005; Schultz and Wiesner

2000; Pesta *et al.* 2011). Several studies indicate a strong correlation between cardiolipin content and increase in mitochondrial function with exercise (Menshikova *et al.* 2005; Menshikova *et al.* 2007; Larsen *et al.* 2012; Faber *et al.* 2014), but its use as a general mitochondrial biomarker in disease remains questionable.

*3.6. Conversion: units*

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

Although volume is expressed as m3 using the *SI* base unit, the litre [dm3] is a conventional unit of volume for concentration and is used for most solution chemical kinetics.

If one multiplies *I*O2/cell by *CN*cell, then the result will not only be the amount of O2 [mol] consumed per time [s-1] in one litre [L•1], but also the change in the concentration of oxygen per second (for any volume of an ideally closed system). This is ideal for kinetic modeling as it

1164

1165

1166

1167

1168

1169

1170

1171

1172

1173

1174

blends with chemical rate equations where concentrations are typically expressed in mol∙L-1 (Wagner *et al.* 2011). In studies of multinuclear cells—such as differentiated skeletal muscle cells—it is easy to determine the number of nuclei but not the total number of cells. A generalized concept, therefore, is obtained by substituting cells by nuclei as the sample entity. This does not hold, however, for enucleated platelets.

Table 6. Conversion of various units used in respirometry and ergometry. e- is the number of electrons or reducing equivalents. zB is the charge number of entity B.

|  |  |  |  |
| --- | --- | --- | --- |
| 1 Unit x | Multiplication factor | *SI*-unit | Note |
| ng.atom O∙s-1 (2 e-) | 0.5 | nmol O2∙s•1 |  |
| ng.atom O∙min-1 (2 e-) | 8.33 | pmol O2∙s•1 |  |
| natom O∙min-1 (2 e-) | 8.33 | pmol O2∙s•1 |  |
| nmol O2∙min-1 (4 e-) | 16.67 | pmol O2∙s•1 |  |
| nmol O2∙h-1 (4 e-) | 0.2778 | pmol O2∙s•1 |  |
| mL O2∙min-1 at STPD*a* | 0.744 | µmol O2∙s•1 | 1 |
| W = J/s at -470 kJ/mol O2 | -2.128 | µmol O2∙s•1 |  |
| mA = mC∙s-1 (*z*H+ = 1) | 10.36 | nmol H+∙s•1 | 2 |
| mA = mC∙s-1 (*z*O2 = 4) | 2.59 | nmol O2∙s•1 | 2 |
| nmol H+∙s•1 (*z*H+ = 1) | 0.09649 | mA | 3 |
| nmol O2∙s•1 (*z*O2 = 4) | 0.38594 | mA | 3 |

1175

1176

1177

1178

1179

1180

1181

1182

1183

1184

1185

1186

1187

1188

1189

1190

1191

1192

1193

1194

1195

1196

1197

1198

1199

1200

1 At standard temperature and pressure dry (STPD: 0 °C = 273.15 K and 1 atm =

101.325 kPa = 760 mmHg), the molar volume of an ideal gas, *V*m, and *V*m,O2 is

22.414 and 22.392 L∙mol-1, respectively. Rounded to three decimal places, both values yield the conversion factor of 0.744. For comparison at NTPD (20 °C),

*V*m,O2 is 24.038 L∙mol-1. Note that the *SI* standard pressure is 100 kPa.

2 The multiplication factor is 106/(*z*B∙*F*).

3 The multiplication factor is *z*B∙*F*/106.

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

ET-capacity in human cell types including HEK 293, primary HUVEC and fibroblasts ranges from 50 to 180 amol∙s-1∙cell-1, measured in intact cells in the noncoupled state (see Gnaiger 2014). At 100 amol∙s-1∙cell-1 corrected for *Rox*, the current across the mt-membranes,

1201

1202

1203

1204

1205

1206

1207

*I*H+***e***, approximates 193 pA∙cell-1 or 0.2 nA per cell. See Rich (2003) for an extension of quantitative bioenergetics from the molecular to the human scale, with a transmembrane proton flux equivalent to 520 A in an adult at a catabolic power of •110 W. Modelling approaches illustrate the link between protonmotive force and currents (Willis *et al.* 2016).

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

1208

1209

1210

1211

1212

1213

1214

1215

1216

1217

1218

1219

1220

1221

1222

1223

1224

1225

1226

1227

1228

1229

1230

1231

1232

1233

1234

1 pmol: picomole = 10-12 mol 4 nmol: nanomole = 10-9 mol

|  |  |  |  |
| --- | --- | --- | --- |
| Name | Frequently used unit | Equivalent unit | Note |
| volume-specific flux, *JV,*O2 | pmol∙s-1∙mL-1 | nmol∙s-1∙L-1 | 1 |
|  | mmol∙s-1∙L-1 | mol∙s-1∙m-3 |  |
| cell-specific flow, *I*O2/cell | pmol∙s-1∙10-6 cells | amol∙s-1∙cell-1 | 2 |
|  | pmol∙s-1∙10-9 cells | zmol∙s-1∙cell-1 | 3 |
| cell number concentration, *CN*ce | 106 cells∙mL-1 | 109 cells∙L-1 |  |
| mitochondrial protein concentration, *CmtE* | 0.1 mg∙mL-1 | 0.1 g∙L-1 |  |
| mass-specific flux, *J*O2*/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 | m3 (1,000 kg) |  |
|  | L | dm3 (kg) |  |
|  | mL | cm3 (g) |  |
|  | µL | mm3 (mg) |  |
|  | fL | µm3 (pg) | 5 |
| amount of substance concentration | M = mol∙L-1 | mol∙dm-3 |  |

2 amol: attomole = 10-18 mol 5 fL: femtolitre = 10-15 L

3 zmol: zeptomole = 10-21 mol

We consider isolated mitochondria as powerhouses and proton pumps as molecular machines to relate experimental results to energy metabolism of the intact cell. The cellular P»/O2 based on oxidation of glycogen is increased by the glycolytic (fermentative) substrate- level phosphorylation of 3 P»/Glyc or 0.5 mol P» for each mol O2 consumed in the complete oxidation of a mol glycosyl unit (Glyc). Adding 0.5 to the mitochondrial P»/O2 ratio of 5.4 yields a bioenergetic cell physiological P»/O2 ratio close to 6. Two NADH equivalents are formed during glycolysis and transported from the cytosol into the mitochondrial matrix, either by the malate-aspartate shuttle or by the glycerophosphate shuttle resulting in different theoretical yields of ATP generated by mitochondria, the energetic cost of which potentially must be taken into account. Considering also substrate-level phosphorylation in the TCA cycle, this high P»/O2 ratio not only reflects proton translocation and OXPHOS studied in isolation, but integrates mitochondrial physiology with energy transformation in the living cell (Gnaiger

1993a).

**4. Conclusions**

MitoEAGLE can serve as a gateway to better diagnose mitochondrial respiratory defects linked to genetic variation, age-related health risks, sex-specific mitochondrial performance, lifestyle with its effects on degenerative diseases, and thermal and chemical environment. The present recommendations on coupling control states and rates, linked to the concept of the

1235

1236

1237

1238

1239

1240

1241

1242

1243

1244

1245

1246

1247

1248

1249

1250

1251

1252

1253

1254

1255

1256

1257

1258

1259

1260

1261

1262

1263

1264

1265

1266

1267

1268

1269

1270

1271

1272

1273

1274

1275

1276

1277

1278

1279

1280

1281

1282

1283

1284

protonmotive force, are focused on studies with mitochondrial preparations. These will be extended in a series of reports on pathway control of mitochondrial respiration, respiratory states in intact cells, and harmonization of experimental procedures.

The optimal choice for expressing mitochondrial and cell respiration (**Box 3**) as O2 flow per biological system, and normalization for specific tissue-markers (volume, mass, protein)

and mitochondrial markers (volume, protein, content, mtDNA, activity of marker enzymes, respiratory reference state) is guided by the scientific question under study. Interpretation of the obtained data depends critically on appropriate normalization, and therefore reporting rates merely as nmol∙s-1 is discouraged, since it restricts the analysis to intra-experimental comparison of relative (qualitative) differences. Expressing O2 consumption per cell may not be possible when dealing with tissues. For studies with mitochondrial preparations, we recommend that normalizations be provided as far as possible: (*1*) on a per cell basis as O2 flow (a biophysical normalization); (*2*) per g cell or tissue protein, or per cell or tissue mass as mass- specific O2 flux (a cellular normalization); and (*3*) per mitochondrial marker as mt-specific flux (a mitochondrial normalization). With information on cell size and the use of multiple normalizations, maximum potential information is available (Renner *et al.* 2003; Wagner *et al.*

2011; Gnaiger 2014).

Total mitochondrial protein is frequently applied as a mitochondrial marker restricted to isolated mitochondria. The mitochondrial recovery and yield, and experimental criteria for evaluation of purity versus integrity should be reported. Mitochondrial markers—such as citrate synthase activity as an enzymatic matrix marker—provide a link to the tissue of origin on the basis of calculating the mitochondrial recovery, *i.e.*, the fraction of mitochondrial marker obtained from a unit mass of tissue.

**Box 3: Mitochondrial and cell respiration**

Mitochondrial and cell respiration is the process of exergonic and exothermic energy transformation in which scalar redox reactions are coupled to vectorial ion translocation across a semipermeable membrane, which separates the small volume of a bacterial cell or mitochondrion from the larger volume of its surroundings. The electrochemical exergy can be partially conserved in the phosphorylation of ADP to ATP or in ion pumping, or dissipated in an electrochemical short-circuit. Respiration is thus clearly distinguished from fermentation as the counterpart of cellular core energy metabolism. Respiration is separated in mitochondrial preparations from the partial contribution of fermentative pathways of the intact cell. Residual oxygen consumption—as measured after inhibition of mitochondrial electron transfer—does not belong to the class of catabolic reactions and is, therefore, subtracted from total oxygen consumption to obtain baseline-corrected respiration.

Terms and symbols are summarized in **Table 8**. Their use will facilitate transdisciplinary communication and support further developments towards a consistent theory of bioenergetics and mitochondrial physiology. Technical terms related to and defined with normal words can be used as index terms in databases, support the creation of ontologies towards semantic information processing (MitoPedia), and help in communicating analytical findings as impactful data-driven stories. ‘*Making data available without making it understandable may be worse than not making it available at all*’ (National Academies of Sciences, Engineering, and Medicine 2018). This is a call to carefully contribute to FAIR principles (Findable, Accessible, Interoperable, Reusable) for the sharing of scientific data.

1286

Table 8. Terms, symbols, and units.

1287

1288

1289

1290

1291

1292

1293

1294

1295

1296

1297

1298

1299

1300

1301

1302

1303

1304

1305

1306

1307

1308

1309

1310

1311

1312

1313

1314

1315

1316

1317

1318

1319

1320

1321

1322

1323

1324

1325

1326

1327

1328

**Term Symbol Unit Links and comments**

alternative quinol oxidase AOX Fig. 1 amount of substance B *n*B [mol]

Complexes I to IV CI to CIV respiratory ET Complexes; Fig. 1

concentration of substance B *c*B = *n*B∙*V*•1; [B] [mol∙m•3] Box 2 electron transfer system ETS Fig. 1, Fig. 4

flow, for substance B *I*B [mol∙s•1] system-related extensive quantity; Fig. 6 flux, for substance B *J*B *varies* size-specific quantitiy; Fig. 6

inorganic phosphate Pi Fig. 2

LEAK LEAK Tab. 1, Fig. 4

mass of sample *X mX* [kg] Tab. 4

mass of entity *X MX* [kg] mass of object *X*; Tab. 4

MITOCARTA [https://www.broadinstitute.org/scientific- community/science/programs/meta bolic-disease- program/publications/mitocarta/mit ocarta-in-0](https://www.broadinstitute.org/scientific-community/science/programs/metabolic-disease-program/publications/mitocarta/mitocarta-in-0)

MitoPedia <http://www.bioblast.at/index.php/MitoPedia>

mitochondria or mitochondrial mt Box 1 mitochondrial DNA mtDNA Box 1 mitochondrial concentration *CmtE* = *mtE*∙*V*-1 [mtEU∙m•3] Tab. 4 mitochondrial content *mtEX* = *mtE*∙*NX*-1 [mtEU∙x•1] Tab. 4

mitochondrial elemental unit mtEU *varies* Tab. 4, specific units for mt-marker

mitochondrial inner membrane mtIM MIM is widely used; the first M is replaced by mt; Box 1

mitochondrial outer membrane mtOM MOM is widely used; the first M is replaced by mt; Box 1

mitochondrial recovery *YmtE* fraction of *mtE* recovered in sample from the tissue of origin

mitochondrial yield *YmtE*/*m YmtE*/*m* = *YmtE* ∙ *DmtE*

negative neg Fig. 2 number concentration of *X CNX* [x∙m-3] Tab. 4 number of entities *X NX* [x] Tab. 4, Fig. 7 number of entity B *N*B [x] Tab. 4 oxidative phosphorylation OXPHOS Tab. 1, Fig. 4 oxygen concentration *c*O2 = *n*O2∙*V*•1; [O2] [mol∙m•3] Section 3.2 phosphorylation of ADP to ATP P» Section 2.2

positive pos Fig. 2

1329

1330

proton in the negative compartment H+

proton in the positive compartment H+

neg

pos

Fig. 2

Fig. 2

1331

1332

1333

1334

1335

1336

1337

1338

1339

1340

1341

1342

1343

1344

1345

rate of electron transfer in ET state *E* ET-capacity; Tab. 1 rate of LEAK respiration *L* Tab. 1

rate of oxidative phosphorylation *P* OXPHOS capacity; Tab. 1 rate of residual oxygen consumption *Rox* Tab. 1

residual oxygen consumption ROX Tab. 1 specific mitochondrial density *DmtE* = *mtE*∙*mX*-1 [mtEU∙kg•1] Tab. 7 volume *V* [m-3]

weight, dry weight *W*d [kg] used as mass of sample *X*; Fig. 6 weight, wet weight *W*w [kg] used as mass of sample *X*; Fig. 6

**Acknowledgements**

We thank M. Beno for management assistance. Supported by COST Action CA15203

MitoEAGLE and K-Regio project MitoFit (E.G.).

1347

1348

1349

1350

1351

1352

1353

1354

1355

1356

1357

1358

1359

1360

1361

1362

1363

1364

1365

1366

1367

1368

1369

1370

1371

1372

1373

1374

1375

1376

1377

1378

1379

1380

1381

1382

1383

1384

1385

1386

1387

1388

1389

1390

1391

1392

1393

1394

1395

1396

1397

1398

1399

1400

1401

1402

1403

1404

1405

1406

1407

**Competing financial interests:** E.G. is founder and CEO of Oroboros Instruments, Innsbruck, Austria.

**5. References**

Altmann R (1894) Die Elementarorganismen und ihre Beziehungen zu den Zellen. Zweite vermehrte Auflage.

Verlag Von Veit & Comp, Leipzig:160 pp.

Beard DA (2005) A biophysical model of the mitochondrial respiratory system and oxidative phosphorylation.

PLoS Comput Biol 1(4):e36.

Benda C (1898) Weitere Mitteilungen über die Mitochondria. Verh Dtsch Physiol Ges:376-83. Birkedal R, Laasmaa M, Vendelin M (2014) The location of energetic compartments affects energetic

communication in cardiomyocytes. Front Physiol 5:376.

Breton S, Beaupré HD, Stewart DT, Hoeh WR, Blier PU (2007) The unusual system of doubly uniparental inheritance of mtDNA: isn't one enough? Trends Genet 23:465-74.

Brown GC (1992) Control of respiration and ATP synthesis in mammalian mitochondria and cells. Biochem J

284:1-13.

Calvo SE, Klauser CR, Mootha VK (2016) MitoCarta2.0: an updated inventory of mammalian mitochondrial proteins. Nucleic Acids Research 44:D1251-7.

Calvo SE, Julien O, Clauser KR, Shen H, Kamer KJ, Wells JA, Mootha VK (2017) Comparative analysis of mitochondrial N-termini from mouse, human, and yeast. Mol Cell Proteomics 16:512-23.

Campos JC, Queliconi BB, Bozi LHM, Bechara LRG, Dourado PMM, Andres AM, Jannig PR, Gomes KMS, Zambelli VO, Rocha-Resende C, Guatimosim S, Brum PC, Mochly-Rosen D, Gottlieb RA, Kowaltowski AJ,

Ferreira JCB (2017) Exercise reestablishes autophagic flux and mitochondrial quality control in heart failure.

Autophagy 13:1304-317.

Canton M, Luvisetto S, Schmehl I, Azzone GF (1995) The nature of mitochondrial respiration and discrimination between membrane and pump properties. Biochem J 310:477-81.

Chance B, Williams GR (1955a) Respiratory enzymes in oxidative phosphorylation. I. Kinetics of oxygen utilization. J Biol Chem 217:383-93.

Chance B, Williams GR (1955b) Respiratory enzymes in oxidative phosphorylation: III. The steady state. J Biol

Chem 217:409-27.

Chance B, Williams GR (1955c) Respiratory enzymes in oxidative phosphorylation. IV. The respiratory chain. J Biol Chem 217:429-38.

Chance B, Williams GR (1956) The respiratory chain and oxidative phosphorylation. Adv Enzymol Relat Subj

Biochem 17:65-134.

Cobb LJ, Lee C, Xiao J, Yen K, Wong RG, Nakamura HK, Mehta HH, Gao Q, Ashur C, Huffman DM, Wan J, Muzumdar R, Barzilai N, Cohen P (2016) Naturally occurring mitochondrial-derived peptides are age- dependent regulators of apoptosis, insulin sensitivity, and inflammatory markers. Aging (Albany NY) 8:796-

809.

Cohen ER, Cvitas T, Frey JG, Holmström B, Kuchitsu K, Marquardt R, Mills I, Pavese F, Quack M, Stohner J, Strauss HL, Takami M, Thor HL (2008) Quantities, units and smbols in physical chemistry, IUPAC Green Book, 3rd Edition, 2nd Printing, IUPAC & RSC Publishing, Cambridge.

Cooper H, Hedges LV, Valentine JC, eds (2009) The handbook of research synthesis and meta-analysis. Russell

Sage Foundation.

Coopersmith J (2010) Energy, the subtle concept. The discovery of Feynman’s blocks from Leibnitz to Einstein.

Oxford University Press:400 pp.

Cummins J (1998) Mitochondrial DNA in mammalian reproduction. Rev Reprod 3:172-82.

Dai Q, Shah AA, Garde RV, Yonish BA, Zhang L, Medvitz NA, Miller SE, Hansen EL, Dunn CN, Price TM (2013) A truncated progesterone receptor (PR-M) localizes to the mitochondrion and controls cellular respiration. Mol Endocrinol 27:741-53.

Divakaruni AS, Brand MD (2011) The regulation and physiology of mitochondrial proton leak. Physiology

(Bethesda) 26:192-205.

Doerrier C, Garcia-Souza LF, Krumschnabel G, Wohlfarter Y, Mészáros AT, Gnaiger E (2018) High-Resolution FluoRespirometry and OXPHOS protocols for human cells, permeabilized fibres from small biopsies of muscle and isolated mitochondria. Methods Mol. Biol. (in press)

Doskey CM, van ‘t Erve TJ, Wagner BA, Buettner GR (2015) Moles of a substance per cell is a highly informative dosing metric in cell culture. PLOS ONE 10:e0132572.

Drahota Z, Milerová M, Stieglerová A, Houstek J, Ostádal B (2004) Developmental changes of cytochrome *c*

oxidase and citrate synthase in rat heart homogenate. Physiol Res 53:119-22.

Duarte FV, Palmeira CM, Rolo AP (2014) The role of microRNAs in mitochondria: small players acting wide.

Genes (Basel) 5:865-86.

1408

1409

1410

1411

1412

1413

1414

1415

1416

1417

1418

1419

1420

1421

1422

1423

1424

1425

1426

1427

1428

1429

1430

1431

1432

1433

1434

1435

1436

1437

1438

1439

1440

1441

1442

1443

1444

1445

1446

1447

1448

1449

1450

1451

1452

1453

1454

1455

1456

1457

1458

1459

1460

1461

1462

1463

1464

1465

1466

1467

1468

1469

Ernster L, Schatz G (1981) Mitochondria: a historical review. J Cell Biol 91:227s-55s.

Estabrook RW (1967) Mitochondrial respiratory control and the polarographic measurement of ADP:O ratios.

Methods Enzymol 10:41-7.

Faber C, Zhu ZJ, Castellino S, Wagner DS, Brown RH, Peterson RA, Gates L, Barton J, Bickett M, Hagerty L, Kimbrough C, Sola M, Bailey D, Jordan H, Elangbam CS (2014) Cardiolipin profiles as a potential biomarker of mitochondrial health in diet-induced obese mice subjected to exercise, diet-restriction and ephedrine treatment. J Appl Toxicol 34:1122-9.

Fell D (1997) Understanding the control of metabolism. Portland Press.

Garlid KD, Beavis AD, Ratkje SK (1989) On the nature of ion leaks in energy-transducing membranes. Biochim

Biophys Acta 976:109-20.

Garlid KD, Semrad C, Zinchenko V. Does redox slip contribute significantly to mitochondrial respiration? In: Schuster S, Rigoulet M, Ouhabi R, Mazat J-P, eds (1993) Modern trends in biothermokinetics. Plenum Press, New York, London:287-93.

Gerö D, Szabo C (2016) Glucocorticoids suppress mitochondrial oxidant production via upregulation of uncoupling protein 2 in hyperglycemic endothelial cells. PLoS One 11:e0154813.

Gnaiger E. Efficiency and power strategies under hypoxia. Is low efficiency at high glycolytic ATP production a paradox? In: Surviving Hypoxia: Mechanisms of Control and Adaptation. Hochachka PW, Lutz PL, Sick T, Rosenthal M, Van den Thillart G, eds (1993a) CRC Press, Boca Raton, Ann Arbor, London, Tokyo:77-109.

Gnaiger E (1993b) Nonequilibrium thermodynamics of energy transformations. Pure Appl Chem 65:1983-2002. Gnaiger E (2001) Bioenergetics at low oxygen: dependence of respiration and phosphorylation on oxygen and

adenosine diphosphate supply. Respir Physiol 128:277-97.

Gnaiger E (2009) Capacity of oxidative phosphorylation in human skeletal muscle. New perspectives of mitochondrial physiology. Int J Biochem Cell Biol 41:1837-45.

Gnaiger E (2014) Mitochondrial pathways and respiratory control. An introduction to OXPHOS analysis. 4th ed.

Mitochondr Physiol Network 19.12. Oroboros MiPNet Publications, Innsbruck:80 pp.

Gnaiger E, Méndez G, Hand SC (2000) High phosphorylation efficiency and depression of uncoupled respiration in mitochondria under hypoxia. Proc Natl Acad Sci USA 97:11080-5.

Greggio C, Jha P, Kulkarni SS, Lagarrigue S, Broskey NT, Boutant M, Wang X, Conde Alonso S, Ofori E, Auwerx J, Cantó C, Amati F (2017) Enhanced respiratory chain supercomplex formation in response to exercise in human skeletal muscle. Cell Metab 25:301-11.

Hinkle PC (2005) P/O ratios of mitochondrial oxidative phosphorylation. Biochim Biophys Acta 1706:1-11. Hofstadter DR (1979) Gödel, Escher, Bach: An eternal golden braid. A metaphorical fugue on minds and

machines in the spirit of Lewis Carroll. Harvester Press:499 pp.

Illaste A, Laasmaa M, Peterson P, Vendelin M (2012) Analysis of molecular movement reveals latticelike obstructions to diffusion in heart muscle cells. Biophys J 102:739-48.

Jasienski M, Bazzaz FA (1999) The fallacy of ratios and the testability of models in biology. Oikos 84:321-26. Jepihhina N, Beraud N, Sepp M, Birkedal R, Vendelin M (2011) Permeabilized rat cardiomyocyte response

demonstrates intracellular origin of diffusion obstacles. Biophys J 101:2112-21.

Klepinin A, Ounpuu L, Guzun R, Chekulayev V, Timohhina N, Tepp K, Shevchuk I, Schlattner U, Kaambre T (2016) Simple oxygraphic analysis for the presence of adenylate kinase 1 and 2 in normal and tumor cells. J Bioenerg Biomembr 48:531-48.

Klingenberg M (2017) UCP1 - A sophisticated energy valve. Biochimie 134:19-27.

Koit A, Shevchuk I, Ounpuu L, Klepinin A, Chekulayev V, Timohhina N, Tepp K, Puurand M,Truu L, Heck K, Valvere V, Guzun R, Kaambre T (2017) Mitochondrial respiration in human colorectal and breast cancer clinical material is regulated differently. Oxid Med Cell Longev 1372640.

Komlódi T, Tretter L (2017) Methylene blue stimulates substrate-level phosphorylation catalysed by succinyl- CoA ligase in the citric acid cycle. Neuropharmacology 123:287-98.

Lane N (2005) Power, sex, suicide: mitochondria and the meaning of life. Oxford University Press:354 pp. Larsen S, Nielsen J, Neigaard Nielsen C, Nielsen LB, Wibrand F, Stride N, Schroder HD, Boushel RC, Helge

JW, Dela F, Hey-Mogensen M (2012) Biomarkers of mitochondrial content in skeletal muscle of healthy

young human subjects. J Physiol 590:3349-60.

Lee C, Zeng J, Drew BG, Sallam T, Martin-Montalvo A, Wan J, Kim SJ, Mehta H, Hevener AL, de Cabo R, Cohen P (2015) The mitochondrial-derived peptide MOTS-c promotes metabolic homeostasis and reduces obesity and insulin resistance. Cell Metab 21:443-54.

Lee SR, Kim HK, Song IS, Youm J, Dizon LA, Jeong SH, Ko TH, Heo HJ, Ko KS, Rhee BD, Kim N, Han J (2013) Glucocorticoids and their receptors: insights into specific roles in mitochondria. Prog Biophys Mol Biol 112:44-54.

Leek BT, Mudaliar SR, Henry R, Mathieu-Costello O, Richardson RS (2001) Effect of acute exercise on citrate synthase activity in untrained and trained human skeletal muscle. Am J Physiol Regul Integr Comp Physiol

280:R441-7.

Lemieux H, Blier PU, Gnaiger E (2017) Remodeling pathway control of mitochondrial respiratory capacity by temperature in mouse heart: electron flow through the Q-junction in permeabilized fibers. Sci Rep 7:2840.

1470

1471

1472

1473

1474

1475

1476

1477

1478

1479

1480

1481

1482

1483

1484

1485

1486

1487

1488

1489

1490

1491

1492

1493

1494

1495

1496

1497

1498

1499

1500

1501

1502

1503

1504

1505

1506

1507

1508

1509

1510

1511

1512

1513

1514

1515

1516

1517

1518

1519

1520

1521

1522

1523

1524

1525

1526

1527

1528

1529

Lenaz G, Tioli G, Falasca AI, Genova ML (2017) Respiratory supercomplexes in mitochondria. In: Mechanisms of primary energy trasduction in biology. M Wikstrom (ed) Royal Society of Chemistry Publishing, London, UK:296-337.

Margulis L (1970) Origin of eukaryotic cells. New Haven: Yale University Press.

Meinild Lundby AK, Jacobs RA, Gehrig S, de Leur J, Hauser M, Bonne TC, Flück D, Dandanell S, Kirk N, Kaech A, Ziegler U, Larsen S, Lundby C (2018) Exercise training increases skeletal muscle mitochondrial volume density by enlargement of existing mitochondria and not de novo biogenesis. Acta Physiol 222, e12905.

Menshikova EV, Ritov VB, Fairfull L, Ferrell RE, Kelley DE, Goodpaster BH (2006) Effects of exercise on mitochondrial content and function in aging human skeletal muscle. J Gerontol A Biol Sci Med Sci 61:534-

40.

Menshikova EV, Ritov VB, Ferrell RE, Azuma K, Goodpaster BH, Kelley DE (2007) Characteristics of skeletal muscle mitochondrial biogenesis induced by moderate-intensity exercise and weight loss in obesity. J Appl Physiol (1985) 103:21-7.

Menshikova EV, Ritov VB, Toledo FG, Ferrell RE, Goodpaster BH, Kelley DE (2005) Effects of weight loss and physical activity on skeletal muscle mitochondrial function in obesity. Am J Physiol Endocrinol Metab

288:E818-25.

Miller GA (1991) The science of words. Scientific American Library New York:276 pp.

Mitchell P (1961) Coupling of phosphorylation to electron and hydrogen transfer by a chemi -osmotic type of mechanism. Nature 191:144-8.

Mitchell P (2011) Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. Biochim Biophys

Acta Bioenergetics 1807:1507-38.

Mogensen M, Sahlin K, Fernström M, Glintborg D, Vind BF, Beck-Nielsen H, Højlund K (2007) Mitochondrial respiration is decreased in skeletal muscle of patients with type 2 diabetes. Diabetes 56:1592-9.

Mohr PJ, Phillips WD (2015) Dimensionless units in the SI. Metrologia 52:40-7.

Moreno M, Giacco A, Di Munno C, Goglia F (2017) Direct and rapid effects of 3,5-diiodo-L-thyronine (T2).

Mol Cell Endocrinol 7207:30092-8.

Morrow RM, Picard M, Derbeneva O, Leipzig J, McManus MJ, Gouspillou G, Barbat-Artigas S, Dos Santos C, Hepple RT, Murdock DG, Wallace DC (2017) Mitochondrial energy deficiency leads to hyperproliferation of skeletal muscle mitochondria and enhanced insulin sensitivity. Proc Natl Acad Sci U S A 114:2705-10.

Murley A, Nunnari J (2016) The emerging network of mitochondria-organelle contacts. Mol Cell 61:648-53. National Academies of Sciences, Engineering, and Medicine (2018) International coordination for science data

infrastructure: Proceedings of a workshop—in brief. Washington, DC: The National Academies Press. doi:

https://doi.org/10.17226/25015.

Paradies G, Paradies V, De Benedictis V, Ruggiero FM, Petrosillo G (2014) Functional role of cardiolipin in mitochondrial bioenergetics. Biochim Biophys Acta 1837:408-17.

Pesta D, Gnaiger E (2012) High-Resolution Respirometry. OXPHOS protocols for human cells and permeabilized fibres from small biopsies of human muscle. Methods Mol Biol 810:25-58.

Pesta D, Hoppel F, Macek C, Messner H, Faulhaber M, Kobel C, Parson W, Burtscher M, Schocke M, Gnaiger E (2011) Similar qualitative and quantitative changes of mitochondrial respiration following strength and endurance training in normoxia and hypoxia in sedentary humans. Am J Physiol Regul Integr Comp Physiol

301:R1078–87.

Price TM, Dai Q (2015) The role of a mitochondrial progesterone receptor (PR-M) in progesterone action.

Semin Reprod Med 33:185-94.

Puchowicz MA, Varnes ME, Cohen BH, Friedman NR, Kerr DS, Hoppel CL (2004) Oxidative phosphorylation analysis: assessing the integrated functional activity of human skeletal muscle mitochondria – case studies. Mitochondrion 4:377-85. Puntschart A, Claassen H, Jostarndt K, Hoppeler H, Billeter R (1995) mRNAs of enzymes involved in energy metabolism and mtDNA are increased in endurance-trained athletes. Am J Physiol 269:C619-25.

Quiros PM, Mottis A, Auwerx J (2016) Mitonuclear communication in homeostasis and stress. Nat Rev Mol

Cell Biol 17:213-26.

Reichmann H, Hoppeler H, Mathieu-Costello O, von Bergen F, Pette D (1985) Biochemical and ultrastructural changes of skeletal muscle mitochondria after chronic electrical stimulation in rabbits. Pflugers Arch 404:1-

9.

Renner K, Amberger A, Konwalinka G, Gnaiger E (2003) Changes of mitochondrial respiration, mitochondrial content and cell size after induction of apoptosis in leukemia cells. Biochim Biophys Acta 1642:115-23.

Rich P (2003) Chemiosmotic coupling: The cost of living. Nature 421:583.

Rostovtseva TK, Sheldon KL, Hassanzadeh E, Monge C, Saks V, Bezrukov SM, Sackett DL (2008) Tubulin binding blocks mitochondrial voltage-dependent anion channel and regulates respiration. Proc Natl Acad Sci USA 105:18746-51.

1530

1531

1532

1533

1534

1535

1536

1537

1538

1539

1540

1541

1542

1543

1544

1545

1546

1547

1548

1549

1550

1551

1552

1553

1554

1555

1556

1557

1558

1559

1560

1561

1562

1563

1564

1565

1566

1567

1568

1569

1570

1571

Rustin P, Parfait B, Chretien D, Bourgeron T, Djouadi F, Bastin J, Rötig A, Munnich A (1996) Fluxes of nicotinamide adenine dinucleotides through mitochondrial membranes in human cultured cells. J Biol Chem

271:14785-90.

Saks VA, Veksler VI, Kuznetsov AV, Kay L, Sikk P, Tiivel T, Tranqui L, Olivares J, Winkler K, Wiedemann F, Kunz WS (1998) Permeabilised cell and skinned fiber techniques in studies of mitochondrial function in

vivo. Mol Cell Biochem 184:81-100.

Salabei JK, Gibb AA, Hill BG (2014) Comprehensive measurement of respiratory activity in permeabilized cells using extracellular flux analysis. Nat Protoc 9:421-38.

Sazanov LA (2015) A giant molecular proton pump: structure and mechanism of respiratory complex I. Nat Rev

Mol Cell Biol 16:375-88.

Schneider TD (2006) Claude Shannon: biologist. The founder of information theory used biology to formulate the channel capacity. IEEE Eng Med Biol Mag 25:30-3.

Schönfeld P, Dymkowska D, Wojtczak L (2009) Acyl-CoA-induced generation of reactive oxygen species in mitochondrial preparations is due to the presence of peroxisomes. Free Radic Biol Med 47:503-9.

Schultz J, Wiesner RJ (2000) Proliferation of mitochondria in chronically stimulated rabbit skeletal muscle-- transcription of mitochondrial genes and copy number of mitochondrial DNA. J Bioenerg Biomembr 32:627-

34.

Simson P, Jepihhina N, Laasmaa M, Peterson P, Birkedal R, Vendelin M (2016) Restricted ADP movement in cardiomyocytes: Cytosolic diffusion obstacles are complemented with a small number of open mitochondrial voltage-dependent anion channels. J Mol Cell Cardiol 97:197-203.

Stucki JW, Ineichen EA (1974) Energy dissipation by calcium recycling and the efficiency of calcium transport in rat-liver mitochondria. Eur J Biochem 48:365-75.

Tonkonogi M, Harris B, Sahlin K (1997) Increased activity of citrate synthase in human skeletal muscle after a single bout of prolonged exercise. Acta Physiol Scand 161:435-6.

Waczulikova I, Habodaszova D, Cagalinec M, Ferko M, Ulicna O, Mateasik A, Sikurova L, Ziegelhöffer A (2007) Mitochondrial membrane fluidity, potential, and calcium transients in the myocardium from acute diabetic rats. Can J Physiol Pharmacol 85:372-81.

Wagner BA, Venkataraman S, Buettner GR (2011) The rate of oxygen utilization by cells. Free Radic Biol Med

51:700-712.

Wang H, Hiatt WR, Barstow TJ, Brass EP (1999) Relationships between muscle mitochondrial DNA content, mitochondrial enzyme activity and oxidative capacity in man: alterations with disease. Eur J Appl Physiol Occup Physiol 80:22-7.

Watt IN, Montgomery MG, Runswick MJ, Leslie AG, Walker JE (2010) Bioenergetic cost of making an adenosine triphosphate molecule in animal mitochondria. Proc Natl Acad Sci U S A 107:16823-7.

Weibel ER, Hoppeler H (2005) Exercise-induced maximal metabolic rate scales with muscle aerobic capacity. J Exp Biol 208:1635–44.

White DJ, Wolff JN, Pierson M, Gemmell NJ (2008) Revealing the hidden complexities of mtDNA inheritance.

Mol Ecol 17:4925–42.

Wikström M, Hummer G (2012) Stoichiometry of proton translocation by respiratory complex I and its mechanistic implications. Proc Natl Acad Sci U S A 109:4431-6.

Willis WT, Jackman MR, Messer JI, Kuzmiak-Glancy S, Glancy B (2016) A simple hydraulic analog model of oxidative phosphorylation. Med Sci Sports Exerc 48:990-1000.