Consortium communication

Mitochondrial physiology 3. Mitochondrial markers

MitoEAGLE Task Group*

Living Communication: extended resource of **Mitochondrial respiratory states and rates. Nat Metab** (Gnaiger *et al*, in review); from Gnaiger *et al* (2020) Bioenerg Commun 2020.1.

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Abstract

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When discussing concepts of normalization, it is essential to consider the question posed by the study. If the study aims at comparing tissue performance—such as the effects of a treatment on a specific tissue, then normalization for tissue mass or protein content is appropriate. However, if the aim is to find differences in mitochondrial function independent of mitochondrial density (**Table 4**), then normalization to a mitochondrial marker is imperative (**Figure 5**). 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.

For comments

Isolated mitochondria

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 that 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 the isolation protocols utilized. 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 can be used strategically for isolation of mitochondrial subpopulations.

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

Mitochondrial concentration, C_{mtE}, and mitochondrial markers

Mitochondrial organelles compose 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 elementary components. Therefore, quantification of the amount of mitochondria depends on the measurement of chosen mitochondrial markers. '*Mitochondria are the structural and functional elementary units of cell respiration*' (Gnaiger 2020). The quantity of a mitochondrial marker can reflect the amount of mitochondrial elementary components, *mtE*, expressed in various mitochondrial elementary units [mtEU] specific for each measured mt-marker (**Table 4**). However, since mitochondrial quality may change in response to stimuli—particularly in mitochondrial dysfunction (Campos *et al* 2017) and after exercise training (Pesta *et al* 2011) and during aging (Daum *et al* 2013)—some markers can vary while others are unchanged: (*1*) Mitochondrial volume and membrane area are structural markers, whereas mitochondrial protein mass is commonly 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.*, the voltage-dependent anion channel (VDAC), 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 elementary entity, *mtE*, is expressed in marker-specific units. Mitochondrial concentration in the instrumental chamber and mitochondrial density in the tissue of origin are quantified as (*1*) a quantity for normalization in functional analyses, C_{mtE}, and (*2*) a physiological output that is the result of mitochondrial biogenesis and degradation, D_{mtE}, respectively (**Table 4**). It is recommended, therefore, to distinguish *experimental mitochondrial concentration*, $C_{mtE} = mtE \cdot V^{-1}$ and *physiological mitochondrial density*, $D_{mtE} = mtE \cdot m_X^{-1}$. Then mitochondrial density is the amount of mitochondrial elementary components per mass of tissue, which is a biological variable (**Figure 5**). The experimental variable is mitochondrial density multiplied by sample mass concentration in the measuring chamber, $C_{mtE} = D_{mtE} \cdot C_{\underline{m}X}$, or mitochondrial content multiplied by sample number concentration, $C_{mtE} = mtE_X \cdot C_{\underline{N}X}$ (**Table 4**).

mt-Marker-specific flux, $J_{O_2/mtE}$

Volume-specific metabolic O_2 flux depends on: (*1*) the sample concentration in the volume of the instrumental chamber, $C_{\underline{m}X}$, or $C_{\underline{N}X}$; (*2*) the mitochondrial density in the sample, $D_{mtE} = mtE \cdot m_X^{-1}$ or $mtE_X = mtE \cdot N_X^{-1}$; and (*3*) the specific mitochondrial activity or performance per mitochondrial elementary marker, $J_{O_2/mtE} = J_{V,O_2} \cdot C_{mtE}^{-1}$ [mol·s^{-1}·mtEU^{-1}] (**Table 4**). Obviously, the numerical results for $J_{O_2/mtE}$ vary with the type of mitochondrial marker chosen for measurement of *mtE* and $C_{mtE} = mtE \cdot V^{-1}$ [mtEU·m^{-3}].

Different methods for the quantification of mitochondrial markers have different strengths and weaknesses. Some problems are common for all mitochondrial markers, *mtE*: (*1*) Accuracy of measurement is crucial, since even a highly accurate and reproducible measurement of chamber volume-specific O_2 flux results in an inaccurate and noisy expression if normalized by 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. In contrast, an *internal* marker is used when O_2 fluxes measured in substrate-uncoupler-inhibitor titration protocols are normalized for flux in a defined respiratory reference state within the assay, which yields flux control ratios, *FCR*s. *FCR*s are independent of 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 on $J_{O_2/\underline{m}X}$ and $I_{O_2/\underline{N}X}$ from that of function per

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mitochondrial elementary marker, $J_{O_2/mtE}$ (Pesta *et al* 2011; Gnaiger 2020). (*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 these 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.

Flux contral ratios and flux control factors

In line with the concept of the respiratory acceptor control ratio (Chance and Williams 1955a), the most readily applied normalization is that of flux control ratios and flux control factors (Gnaiger 2009; 2020). Then, instead of a specific mt-enzyme activity, the respiratory activity in a reference state serves as the *mtE*, yielding a dimensionless ratio of two fluxes measured consecutively in the same respirometric titration protocol. Selection of the state of maximum flux in a protocol as the reference state – *e.g.*, ET-state in L/E and P/E flux control ratios (Gnaiger 2009) – has the advantages of: (*1*) elimination of experimental variability in additional measurements, such as determination of enzyme activity or tissue mass; (*2*) statistically validated linearization of the response in the range of 0 to 1; and (*3*) consideration of maximum flux for integrating a large number of metabolic 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 that indicates stable tissue-mass specific flux.

Is there a best mitochondrial marker?

Stereological measurement of mitochondrial content via two-dimensional transmission electron microscopy is considered as the gold standard in determination of mitochondrial volume fractions in cells and tissues (Weibel, Hoppeler, 2005). Accurate determination of three-dimensional volume by two-dimensional microscopy, however, is both time consuming and statistically challenging (Larsen *et al* 2012). The validity of using mitochondrial marker enzymes (citrate synthase activity, CI to CIV 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 persons 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 aging, 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; Ehinger *et al* 2015), 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 it has not been evaluated as a general mitochondrial biomarker in disease. With no single best mitochondrial marker, a good strategy is to quantify several different biomarkers to minimize the decorrelating effects caused by diseases, treatments, or other factors.

For comments

162 Determination of multiple markers, particularly a matrix marker and a marker from the mtIM,
163 allows tracking changes in mitochondrial quality defined by their ratio.
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241
242 **Author contributions:** This manuscript developed as an open invitation to scientists and students to join
243 as coauthors in the bottom-up spirit of COST, based on a first draft written by the corresponding author,
244 who integrated coauthor contributions in a sequence of Open Access versions. Coauthors contributed to the
245 scope and quality of the manuscript, may have focused on a particular section, and are listed in alphabetical
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For comments

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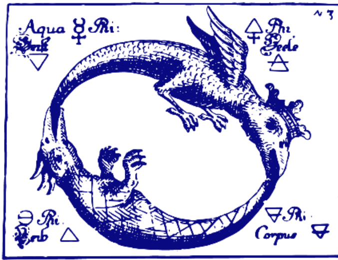

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