Strategies to detect mitochondrial oxidants

Alicia J. Kowaltowski

Department of Biochemistry, Institute of Chemistry, University of São Paulo, Av. Prof. Lineu Prestes, 748, City University, São Paulo, SP 05508-000, Brazil

ARTICLE INFO

Keywords: Mitochondria Oxidants Reactive oxygen species Probes

ABSTRACT

Mitochondrial oxidants (or reactive oxygen species) participate in a myriad of physiological and pathological processes. They are, however, quite hard to measure due to their chemical nature and specific subcellular location. Here, we review techniques to measure mitochondrial oxidants in biological systems as well as the results of their activity, highlighting conditions to be considered, controls and recommended practices. We will delineate experimental setups that use combined strategies to convincingly demonstrate the biological effects of mitochondrial oxidants, using the imperfect methodology available today.

1. Introduction

Mitochondria produce oxidants (or reactive oxygen species) such as the superoxide radical anion (O₂⁻) and hydrogen peroxide (H₂O₂) as a result of the immense variety of redox reactions that are located in this organelle [2,20,34,51]. Consequently, mitochondria also developed efficient antioxidant systems and regulatory pathways that modulate oxidant production [20,51]. The balance between production and removal determines oxidant release from the organelle, which is modified by the substrate in use, physiological energy metabolism regulation and a variety of pathological conditions [20,48,9]. Interestingly, a large body of data shows that oxidants not only lead to varying degrees of oxidative damage in mitochondria and in other cellular components (when antioxidant systems are overwhelmed), but also act as signaling molecules [21,45].

Given the varied and pivotal roles of mitochondrial oxidants, measuring these species, mitochondrial redox state and the consequences of oxidants in this organelle is an important element in many current scientific studies. Unfortunately, although the literature has described many tools to measure mitochondrial oxidants, none is foolproof or can be used in the absence of controls. Furthermore, most mitochondrial redox state measurement techniques are artifact-prone due to the reactive nature of the oxidants they measure and conditions specific to mitochondrial biology such as the presence of a variable inner membrane potential, changes in mitochondrial morphology and mass. The aim of this review is to highlight experimental conditions, controls and recommended practices when measuring mitochondrial redox state. We will start by quickly reviewing characteristics of mitochondrial oxidant production and removal. We will then address some important particularities of animal and cell culture models that pertain to studies of mitochondrial oxidants. Next, we will focus on techniques available, discussing their capabilities and limitations, as well as necessary controls. The review will end by discussing ideal strategy designs to study biological effects of mitochondrial oxidants using the imperfect techniques currently available.

2. Mitochondrial oxidants and antioxidants – a very brief overview

Characteristics of mitochondrial oxidant production and antioxidant mechanisms have been extensively reviewed elsewhere [2,20,34,51,9] and are not the aim of this review, which focuses instead on mitochondrial oxidant measurement techniques. It suffices to say here that many mitochondrial components, including electron transport chain complexes (such as complexes I, II and III) and redox enzymes (such as glycerol 3-phosphate, pyruvate, alfa-ketoglutarate, dihydroorotate, very long chain acyl-CoA dehydrogenases and the electron transfer flavoprotein) can generate oxidants such as O₂⁻ and H₂O₂ within mitochondria. The rate of oxidant production is modulated by oxygen consumption velocity in the organelle: higher mitochondrial oxygen consumption rates are generally, but not universally, associated with lower oxidant production [48], since intermediates generating these oxidants turn over more quickly, reverse electron transport is unfavorable, and local oxygen tensions are lower [51].

Superoxide radical anions generated in both the matrix and the intermembrane space are rapidly converted to H₂O₂ due to the presence of superoxide dismutases in both compartments [38]. Because mitochondria contain both labile iron and metal-containing enzymes, the highly reactive hydroxyl radical, generated from H₂O₂ via the Fenton reaction, has been shown to be produced in the organelle [53], although its short half-life hampers its detection in complex biological models. Since H₂O₂ has the ability to generate damaging hydroxyl...
rates [29,36,42]. Thus, the lack of NNT in C57BL/6J mice has a strong damage. A.J. Kowaltowski

![Fig. 1. Mitochondrial nicotinamide nucleotide transhydrogenase (NNT). NNT uses the inner membrane proton gradient as a driving force to transfer electrons to NADP⁺ from NADH, produced by many intramitochondrial enzymes including pyruvate, isocitrate, malate and α-ketoglutarate dehydrogenases (PDH, IDH, MDH and αKGDH, respectively). This NNT activity generates mitochondrial matrix NADPH coupled to the entry of a proton into the matrix, and is deficient in the widely-used C57BL/6J mouse. Intramitochondrial NADPH reduces oxidized glutathione and thioredoxin [GSSG and Trx(S-S)] to GSH and Trx (SH₂), which allows for the removal of H₂O₂ through the activities of glutathione and thioredoxin peroxidases. As a result, C57BL/6J mice are unable to adequately remove oxidants and prone to oxidative damage.](image)

radicals, these organelles have evolved efficient systems to remove peroxides [2,20,34,51], including catalases and thi peroxidases such as glutathione and thioredoxin peroxidases. Reduced glutathione and thioredoxin are necessary for the function of thiol peroxidases, and are maintained reduced by electrons donated from mitochondrial NADPH. Mitochondrial NADPH is mostly produced by the mitochondrial nicotinamide nucleotide transhydrogenase (NNT, Fig. 1), an inner membrane protein described in more detail below. Curiously, this enzyme is deficient in one of the most used laboratory animal rodent models.

3. Animal models

Animal models are often used to study the effects of disease, diet, aging or other interventions on mitochondrial oxidants in different tissues. Laboratory rodents constitute the bulk of these animal models and are also often used to obtain isolated mitochondria or tissue samples for in vitro redox state studies.

Some particularities regarding mitochondrial redox state should be considered when using laboratory rodents. The first pertains to the animal strain used. In 2005, while investigating reasons for impaired insulin secretion in the widely-used C57BL/6J mouse, Towe et al. [50] discovered this strain harbored a spontaneous missense mutation in exon 1 and a multi-exon deletion (exons 7–11) in the nuclear gene that encodes for NNT. NNT is an inner mitochondrial membrane protein which uses the proton gradient as a driving force to transfer electrons from NADH to NADP⁺ (Fig. 1). In the absence of this protein, NADP⁺ reduction in mitochondria is hampered, resulting in low NADPH availability for antioxidant systems, increased oxidized glutathione levels, poor ability to remove added oxidants and higher H₂O₂ release rates [29,36,42]. Thus, the lack of NNT in C57BL/6J mice has a strong impact on mitochondrial redox state.

Importantly, the C57BL/6J mouse is the most widely used inbred laboratory mouse strain today (https://www.jax.org/strain/000664, consulted September 2⁴th, 2018), and is also the background used to generate many genetically modified mouse strains, which means the redox effects in most of these studies should be analyzed in light of the hampered mitochondrial antioxidant system these animals have. Furthermore, mispairings and mislabeling of C57BL/6 mouse strains are not uncommon, and can result in widely different biological effects [5].

Given the wide use of this animal model, we don’t believe it is feasible to avoid it altogether, but recommend testing C57BL/6 mice for the presence of the Nnt mutation, as well as considering biological implications of the lack of NNT when using this widely-adopted spontaneous mutant. This characteristic of C57BL/6 mice also brings to light the peculiar nature of inbred laboratory animal strains and their tendency to develop specific genetic drifts over time, emphasizing the importance of using congenic animals to determine the effects of single gene modifications whenever possible.

Another aspect to keep in mind regarding animal studies and mitochondrial redox state is the diet used by these animals. Because of the central role of mitochondria in energy metabolism, mitochondrial oxidants are often studied following nutritional interventions such as caloric restriction or overnutrition. We find that many studies in which calories are restricted do not use supplemented diets to account for micronutrient intake decreases [10]. Non-supplemented diets can result in micronutrient malnutrition, including low levels of vitamins and minerals important to maintain redox balance such as copper, iron, manganese, selenium, riboflavin and thiamin. As a result, many studies that see changes in mitochondrial physiology with caloric restriction may in fact describe effects of lower micronutrient, and not caloric, intake [11].

Overnutrition studies such as studies involving high fat diets may also promote malnutrition due to decreases in total food intake promoted by fat-induced satiety. High fat dietary interventions in the literature are also confounded by the fact that the low fat diets they are compared to are quite variable, and often not a known-component diet which differs only in fat and energy contents, thus promoting changes in micronutrient availability [24]. Overall, dietary interventions should be formulated in animal redox studies so as not to involve unwanted changes in micronutrient content that lead to differences in electron transport chain function or impair the synthesis of antioxidant system components. Diets should also be checked to ensure no alterations in added antioxidant preservatives, such as butylated hydroxytoluene (BHT), occur within the experimental protocol.

4. Cell culture models

Cultured cells are often used in studies involving mitochondrial oxidant production, both for in situ experiments and as sources of isolated organelles or homogenates. A particularity of mammalian cell cultures that these cells are grown at oxygen concentrations similar to the solubility of oxygen in media (~200 μM), while the concentration of oxygen in mammalian tissues depends on proximity to blood vessels and diffusion across cell layers, and is thus usually much lower (~1–30 μM). Careful experiments in isolated mitochondria by Hoffman and Brookes [22] have determined that mitochondrial H₂O₂ release rates are highly sensitive to changes in oxygen concentrations within the expected physiological in vivo concentration range, while completely saturated at the concentrations in which cells are cultured. Experiments under more physiological oxygen concentration growth conditions are possible, although they require specialized incubators and are thus probably unrealistic for most studies. However, researchers should keep this important experimental difference in mind when designing protocols, and particularly when comparing in vivo animal tissue effects to those in cultured cells.

Another consideration in cell cultures pertains to the adaptations these cultures undergo during many generations of replication under laboratory conditions, which usually involve growth in large relative quantities of media with high glucose content. These high glucose levels allow the cells to generate ATP mainly through glucose fermentation to lactate, and many cell lines grown in this manner have significant loss of mitochondrial function, which can predictably affect mitochondrial redox state and signaling. This low mitochondrial activity in cell lines can be reversed by cultivating cells in non-fermentable substrates or galactose, which reduces glycolytic efficiency, thus stimulating...
mitochondria, H2O2 peroxide release can be measured in a relatively stable, membrane diffusible and long-lived [9]. Therefore this form of measurement is usually restricted to detection of mitochondrial H2O2.

When using isolated mitochondrial preparations or fractions of mitochondria, H2O2 peroxide release can be measured in a relatively straightforward manner using horseradish peroxidase and probes such as Amplex Red, which is oxidized to fluorescent resorufin [56]. Amplex Red has been widely adopted because it is stable and highly responsive. It produces fluorescence increases that correlate linearly with H2O2 additions within biological ranges around pH 7 [54]. Changes in mitochondrial energy metabolism are often accompanied by modifications in the local production of CO2 and lactate, among other effects that alter intracellular pH, which may modify DCF detection independently of the presence of oxidants. Overall, DCF is a questionable method to detect mitochondrial H2O2 release, and should not be used in isolation for such measurements.

An alternative system to measure mitochondrionally-generated H2O2 inside cells are membrane-permeable boronate esters attached to a fluorophore, which interact directly with intracellular H2O2 and other peroxides to form fluorescent products [25,32,57]. These compounds have the disadvantage of reacting with H2O2 with low second-order rate constants, that compete with endogenous detoxifying systems. However, even the small quantity of oxidants that escape from anti-oxidant systems and are detectable by boronate esters are already a strong indication of oxidant production. Mitochondrially-targeted boronate esters have been developed for detection within these organelles [15,17,57].

Dihydroethidium and dihydrorhodamine 123 are also often-used probes to measure oxidants in the cytosol. Dihydroethidium specifically generates 2-hydroxyethidium when oxidized by O2•-, which could allow it to be an oxidant-specific probe. However, other fluorescent dihydroethidium products, particularly ethidium, are also formed in vivo, so total fluorescence is not an indicator of O2•-. These different fluorescent products can be separated by HPLC for specific measurements [19]. Interestingly, dihydroethidium and dihydrorhodamine 123 oxidation products are accumulated in mitochondria when oxidized, since they have positive charges in this form [43,7], and therefore their use will also require the considerations we will discuss under the next topic for mitochondrially-accumulated probes.

Another class of probes to detect oxidants in cells are genetically encoded fluorescent proteins which are sensitive to oxidant levels, generally by means of structural modifications involving disulfides (reviewed in detail by [31]). Examples of these proteins are redox-sensitive green fluorescent proteins (roGFPs), and HyPer, which have the advantage of being ratiometric probes, thus eliminating concerns regarding expression levels and unequal probe distribution. These proteins can be targeted to specific intracellular compartments, and molecularly engineered for different types of detection. Expression of fluorescent proteins in small intracellular spaces (such as the mitochondrial intermembrane space) can hamper the detection of fluorescence, but oxidized and reduced forms of these proteins can also be separated in gels and quantified, overcoming this caveat [23]. Recently, peroxiredoxins in combination with GFP and YFPs have also been proposed to be good scaffolds for the design of intracellular peroxide probes [52]. While encoded protein probes are very interesting and bring exciting new possibilities, some care must be expended when considering studies with them: (i) All fluorescent proteins can undergo photobleaching and produce oxidants under illumination [39]. (ii) As mentioned previously, accumulation of these probes in specific environments can affect whole cell fluorescence. (iii) Some of these proteins are pH-sensitive [41], and changes in pH have been erroneously interpreted as changes in O2•- levels in the past [44]. These caveats, as well as possible lack of selectivity to specific oxidants, should be considered when using encoded protein sensors to measure mitochondrially-generated oxidants.

Another class of probes used to measure changes in oxidant levels are cationic probes which accumulate in mitochondria by means of their inner membrane potential [16,57], including dihidrroethidium, Mitotracker Red CMX Ros and MitoSX. A first point to consider when using cationic probes is that their accumulation in mitochondria is dependent on both the plasma membrane potential and the mitochondrial inner membrane potential [6]. Mitochondrial matrix concentrations of the probe at 37°C can thus be estimated using the Nernst equation:

\[
[\text{Probe}^+_{\text{matrix}}] = \left[\text{Probe}^+_{\text{extracellular}}\right] \times 10^{(\Delta \Psi m + \Delta \Psi p)/61.5}
\]

were [\text{Probe}^+_{\text{matrix}}] is the probe concentration in the mitochondrial matrix, [\text{Probe}^+_{\text{extracellular}}] is the concentration of the probe added to the extracellular medium, \Delta \Psi m is the mitochondrial inner membrane potential.
accumulated probes is that fluorescence responses are also affected by mitochondrial size and shape [26]. Mitochondria have been recently found to be highly dynamic, changing size and shape continuously, in a manner altered by many redox-sensitive factors and nutrient availability (reviewed by [27]). As a result, organelle morphology and dynamics should also be considered when using mitochondrially-accumulated oxidant probes (Fig. 2). A suggested control that helps to normalize both for changes in membrane potentials and size and shape is to use an oxidant-insensitive mitochondrially-accumulated probe as a normalizing factor when analyzing whole cell fluorescence (cytometry or microscopy) or mitochondrial fluorescence (confocal localized microscopy). Overall, we suggest this class of probes has too many artifacts associated with them to be used in isolation as evidence of mitochondrial oxidant production (summarized in Fig. 2). While they can provide a useful suggestion of changes in mitochondrial oxidants, other techniques and experimental approaches should be used in association.

Murphy’s group [12,8] has developed an elegant bypass for the concerns listed above: a mitochondrially-accumulated probe, MitoB, that reacts with H$_2$O$_2$ to form a stable phenol, MitoP. The two forms can be extracted from the tissue or cells and quantified by liquid chromatography-tandem mass spectrometry, providing a snapshot of in vivo mitochondrial H$_2$O$_2$ [28]. While this method is technically far superior to the others discussed, it does not allow for continuous time-scans, which are often desired by researchers.

7. Is measuring mitochondrial oxidants necessary?

While many studies follow fluorescent markers in order to estimate mitochondrial oxidant production or release over time, we saw above that there are several technical limitations that make real-time oxidant measurements using fluorescent indicators untrustworthy, especially when dealing with whole cell, tissue, or animal measurements. Indeed, we would like to urge researchers to consider the actual necessity of this form of measurement in their experimental setup.

First, consider instead the use of a probe isolated from the cells or tissue and quantified at specific time-points such as the western blot or mass spectrometry experimental setups described above. These provide far more dependable data, since they isolate the indicator from the biological setup that generated the oxidants, avoiding artifacts generated by the large complexity inherent to biological systems.

Second, consider if you need a direct measurement of mitochondrial oxidants altogether. You do not always need to measure an oxidant to understand biological phenomena that involve this chemical species. Molecules modified by oxidants are good indicators of their presence, including oxidatively-modified lipids, proteins and nucleic acids in mitochondria, which can undergo specific modifications with specific oxidants. In fact, aconitase, a citric acid cycle enzyme, is sensitive to inactivation by superoxide radicals as well as some reactive nitrogen species [49]. Other small molecule markers in mitochondria are good indicators of redox state and redox balance, such as oxidized versus reduced glutathione levels [13,30] or NADP$^+$ /NADPH [3,42]. All of these measurements are quantitative and provide solid information regarding the redox state of mitochondria.

In addition to measuring molecules that are modified by the action of oxidants, and effective manner to link mitochondrial oxidants to a biological process is by using mitochondrially-targeted antioxidants to remove these oxidants (reviewed by [57]). Two groups of mitochondrial antioxidants have been extensively tested in this sense: MitoQ, which contains ubiquinone as the antioxidant (reviewed by [33,35]) and SkQ molecules, which contain plastoquinone (reviewed by [46]). Loss of a biological effect when using these antioxidants is a strong indication that mitochondrial oxidants are involved, although controls should include the mitochondrial target (usually triphenylphosphonium cations) in the absence of the antioxidant and the antioxidant in the absence of the mitochondrial target [1].

Conversely, specifically generating O$_2$ within mitochondria using
mitochondrially-targeted parquat [40] can identify mitochondrial oxidant-triggered effects. Mitochondrial oxidant production can also be modulated by stimulating or inhibiting the electron transport chain at specific points [48]. Beware that inhibition of electron transport chain activity does not usually inhibit mitochondrial oxidant production. In fact, respiratory inhibition usually enhances oxidant generation by causing accumulation of reduced intermediates capable of promoting mono-electronic oxygen reduction to produce O₂⁻ [51]. Overall, measuring oxidant biomarkers and following the effects of pro- or anti-oxidant molecules can be a solid and less artifact-prone manner to uncover biological effects of mitochondrial oxidants. These strategies should be considered when developing experimental designs.

8. Strategic design in studies involving mitochondrial oxidants

As we saw, most techniques to measure mitochondrial oxidants have significant caveats and are prone to many artifacts, most notably when studies involve cultured cells or tissues. As a result, care must be taken when designing experimental strategies in mitochondrial redox biology.

While fluorescent probes are often used, we stress the importance of the following precautions: (i) Keep in mind that these probes are very rarely specifically oxidized by a single reactive oxygen species. (ii) Do not equate fluorescence increases to a proportional increase in oxidant content – the response curves of these probes are not usually linear. Instead, indicate the data as relative fluorescence. (iii) Use mitochondrially-loaded probes and whole cell fluorescence measurements (cytometry, suspension fluorimetry or whole cell fluorescence microscopy) in the presence of a loading normalizer such as a mitochondrial fluorescent probe which does not require oxidation to be detected. (iv) Consider that changes in fluorescence may be caused by changes in pH, inner membrane potentials, and/or mitochondrial morphology, independently of changes in oxidant levels. (v) Finally, and most importantly, do not use fluorescent probes to measure oxidants as the only experimental evidence for the presence of these species in the process you are studying.

Indeed, the ideal experimental strategy to study mitochondrial oxidants includes a combination of techniques and methodological approaches, which together bring strength and reliability to the findings:

- Measure oxidants using extracellular probes (for diffusible oxidants) or, ideally, probes that involve quantification after removal from the biological system.
- In addition to (or maybe even instead of) oxidant measurements in the system, use measurements of oxidized products, including biological macromolecules and/or small molecule redox markers such as glutathione.
- Change mitochondrial oxidant levels using mitochondrially-targeted oxidants, antioxidants and/or electron transport modulators and measure the biological outcome of these modifications.

Through the combination of these different approaches, more trustworthy mitochondrial redox results can be obtained in the future, bringing substantial new mechanistic insights into the roles of mitochondrial oxidants.

Acknowledgements

Prof. Kowaltowski is supported by the Centro de Pesquisa, Inovação e Difusão de Processos Redox em Biomedicina (Redoxoma) Grant 13/07937-8, from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional de Pesquisa (CNPq), and Coordenação de Aperfeiçoamento do Pessoal do Ensino Superior (CAPES) Finance Code 001 and PROEX 1888/2016. The author wishes to thank Prof. Ohara Augusto, Dr. Phablo Abreu and Pâmela Kakimoto for critical reading of the manuscript.

References

[1] V.J. Adlam, J.C. Harrison, C.M. Porteous, A.M. James, R.A. Smith, M.P. Murphy, I.A. Sammut, Targeting an antioxidant to mitochondria decreases cardiac ischemia-reperfusion injury, FASEB J. 19 (2005) 1088–1095.
[2] R.S. Balaban, S. Nemoto, T. Finke, Mitochondria, oxidants, and aging, Cell 120 (2005) 483–495.
[3] T.S. Blacker, M.R. Duchen, Investigating mitochondrial redox state using NADH and NADPH autofluorescence, Free Radic. Biol. Med. 100 (2016) 53–65.
[4] M.G. Bonini, G. Rota, A. Tomasi, R.P. Mason, The oxidation of 2’7’-dichlorofluorescin to reactive oxygen species: a self-filling prophesy? Free Radic. Biol. Med. 40 (2006) 968–975.
[5] M. Bourdi, J.S. Davies, L.R. Pohl, Mispairing C57BL/6 substrains of genetically engineered mice and wild-type controls can lead to confounding results as it did in studies of JNK2 in acetaminophen and concanavalin A liver injury, Chem. Res. Toxicol. 24 (2011) 794–796.
[6] M.D. Brand, D.G. Nicholls, Assessing mitochondrial dysfunction in cells, Biochem. J. 435 (2011) 297–312.
[7] R.L. Budd, R.F. Castillo, D.G. Nicholls, Mitochondrial membrane potential and hydroethidine-monitored superoxide generation in cultured cerebellar granule cells, FEBS Lett. 415 (1997) 21–24.
[8] A.G. Caires, S.J. McQuaker, M.P. Murphy, R.C. Hartley, Targeting mitochondria with small molecules: the preparation of Mitofl and Mitof as exomarkers of mitochondrial hydrogen peroxide, Methods Mol. Biol. 1265 (2015) 25–50.
[9] A.R. Cardoso, B. Chausse, F.M. da Cunha, L.A. Luévano-Martínez, T.B. Marazzi, P.S. Pessoa, B.B. Queiroz, A.J. Kowaltowski, Mitochondrial compartmentalization of reductases, Free Radic. Biol. Med. 52 (2012) 2201–2208.
[10] F.M. Cerqueira, A.J. Kowaltowski, Commonly adopted caloric restriction protocols often involve malnutrition, Aging Res. Rev. 9 (2010) 424–430.
[11] F.M. Cerqueira, A.J. Kowaltowski, Mitochondrial oxidation in aging: effect of dietary interventions, Aging Res. Rev. 12 (2013) 22–28.
[12] H.M. Cochemé, C. Quin, S.J. McQuaker, F. Cabreiro, A. Logan, T.A. Prime, I. Abokumova, J.V. Patel, I.M. Foaunari, A.M. James, C.M. Porteous, R.A. Smith, S. Saeed, J.E. Carré, M. Singer, D. Gems, R.C. Hartley, L. Partridge, M.P. Murphy, Measurement of H₂O₂ within living Drosophila during aging using a ratiometric mass spectrometry probe targeted to the mitochondrial matrix, Cell Metab. 13 (2011) 340–350.
[13] J.G. de la Asuncion, A. Millan, R. Pla, L. Bruseghini, A. Esters, F.V. Pallardo, J. Sastre, J. Viña, Mitochondrial glutathione oxidation correlates with age-associated oxidative damage to mitochondrial DNA, FASEB J. 10 (1996) 333–338.
[14] J. Debcki, R. Smulik, J. Želoskova, B. Michalowski, M. Jakubowska, K. Dobosz, J. Adamus, A. Marciek, B. Kalyanaraman, A. Sikora, Mechanism of oxidative conversion of Amplex® Red to resorufin: pulse radiolysis and enzymatic studies, Free Radic. Biol. Med. 95 (2016) 323–332.
[15] B.C. Dickinson, C.J. Chang, A targetable fluorescent probe for imaging hydrogen peroxide in the mitochondria of living cells, J. Am. Chem. Soc. 2008 130 (2008) 9638–9639.
[16] B.C. Dickinson, D. Srikun, C.J. Chang, Mitochondrial-targeted fluorescent probes for reactive oxygen species, Curr. Opin. Chem. Biol. 14 (2010) 50–56.
[17] B.C. Dickinson, V.S. Lin, C.J. Chang, Preparation and use of MitofP1 for imaging hydrogen peroxide in mitochondria of live cells, Nat. Protoc. 8 (2013) 1249–1259.
[18] R.K. Emaus, R. Grunwald, J.J. Lemasters, Rhodamine 123 as a probe of transmembrane potential in isolated rat-liver mitochondria: spectroscopic and metabolic properties, Biochem. Biophys. Acta 850 (1986) 436–448.
[19] D.C. Fernandes, R.C. Gonçalves, F.R. Lurarding, Measurement of superoxide production and NADPH oxidative activity by HPLC analysis of dihydroethidium oxidation, Methods Mol. Biol. 1527 (2017) 233–249.
[20] T.R. Figueira, M.H. Barros, A.A. Camargo, R.F. Castillo, J.C. Ferreira, A.J. Kowaltowski, F.E. Sluse, N.C. Souza-Pinto, A.E. Vercesi, Mitochondria as a source of reactive oxygen and nitrogen species: from molecular mechanisms to human health, Antioxid. Redox Signal. 18 (2013) 2029–2074.
[21] C. Fleury, B. Mignotte, J.L. Vaysseire, Mitochondrial reactive oxygen species in cell death signaling, Biochimie 84 (2002) 131–141.
[22] D.L. Hoffman, P.S. Brookes, Oxygen sensitivity of mitochondrial reactive oxygen species generation depends on metabolic conditions, J. Biol. Chem. 284 (2009) 16236–16245.
[23] J. Hu, L. Dong, C.E. Outten, The redox environment in the mitochondrial intermembrane space is maintained separately from the cytosol and matrix, J. Biol. Chem. 283 (2008) 29126–29134.
[24] P.A. Kakimoto, A.J. Kowaltowski, Effects of high fat diets on rodent liver bioenergetics and oxidative imbalance, Redox Biol. 8 (2016) 216–225.
[25] B. Kalyanaraman, V. Darley-Usmar, K.J. Davies, P.A. Denness, H.J. Forman, M.B. Grisham, G.E. Mann, K. Moore, L.J. Roberts 2nd, H. Ischiropoulos, Measuring reactive oxygen and nitrogen species with fluorescent probes: challenges and limitations, Free Radic. Biol. Med. 52 (2012) 1–6.
[26] A.J. Kowaltowski, R.G. Casas, C.B. Campos, G. Fiskum, Effect of Bel-2 overexpression on mitochondrial structure and function, J. Biol. Chem. 277 (2002) 42802–42807.
[27] M. Liesa, O.S. Shiraih, Mitochondrial dynamics in the regulation of nutrient utilization and energy expenditure, Cell Metab. 17 (2013) 491–506.
[28] A. Logan, I.G. Shabalina, T.A. Prime, S. Rogatti, A.V. Kalinovich, R.C. Hartley, R.C. Budd, B. Cannon, M.P. Murphy, In vivo levels of mitochondrial hydrogen peroxide increase with age in mtDNA mutant mice, Aging Cell 13 (2014) 765–768.
[29] P. Lipert, M. Patel, Nicotinamide nucleotide transhydrogenase (Nt) links the substrate requirement in brain mitochondria for hydrogen peroxide removal to the thioredoxin/peroxiredoxin (Tx/Px) system, J. Biol. Chem. 289 (2014) 15611–15620.

[30] M. Mari, A. Morales, A. Collell, C. García-Ruiz, J.C. Fernández-Checa, Mitochondrial glutathione, a key survival antioxidant, Antioxid. Redox Signal. 11 (2009) 2685–2700.

[31] A.J. Meyer, T.P. Dick, Fluorescent protein-based redox probes, Antioxid. Redox Signal. 13 (2010) 621–650.

[32] E.W. Miller, A.E. Albers, A. Pralle, E.Y. Isaco, M.P. Murphy, R.A. Smith, Targeting antioxidants to mitochondria by conjugation to lipophilic cations, Ann. Rev. Pharmacol. Toxicol. 47 (2007) 629–656.

[33] L.P. Roma, J. Duprez, H.K. Takahashi, P. Gilon, A. Wiederkehr, J.C. Jonas, Dynamic redox state in rat pancreatic β-cells using ratiometric fluorescent probes: confounding effects of pH with HyPer but not roGFP1, Biochem. J. 441 (2012) 894–899.

[34] A.A. Toye, J.D. Lippiat, P. Proks, K. Shimomura, L. Bentley, A. Hugill, V. Mijat, M. Goldsworthy, L. Moir, A. Haynes, J. Quarteman, H.C. Freeman, F.M. Ashcroft, R.D. Cox, A genetic and physiological study of impaired glucose homeostasis control in C57BL/6J mice, Diabetologia 48 (2005) 675–686.

[35] A.J. Kowaltowski, Redox Biology 21 (2019) 101065.

[36] D.G. Nicholls, M.W. Ward, Mitochondrial membrane potential and neuronal glutamate excitotoxicity: mortality and millivolts, Trends Neurosci. 23 (2000) 166–174.

[37] A. Okado-Matsumoto, I. Fridovich, Subcellular distribution of superoxide dismutase (SOD) in rat liver: Cu, Zn-SOD in mitochondria, J. Biol. Chem. 276 (2001) 38388–38393.

[38] S.J. Remington, Fluorescent proteins: maturation, photochemistry and photophysics, Curr. Opin. Struct. Biol. 16 (2006) 714–721.

[39] J.L. Robbins, J.M. Gawel, D. Aksentijevich, H.M. Cochemé, T.P. Dick, Utilizing natural and engineered peroxiredoxins as intracellular peroxide reporters, Mol. Cells 39 (2016) 46–52.

[40] M. Schwartzländter, S. Wagner, Y.G. Ermakova, V.V. Belousov, R. Radio, J.S. Beckman, G.R. Bertrand, N. Demaurex, M.R. Duchen, H.J. Forman, M.D. Fricker, D. Gems, A.P. Halestrap, B. Halliwell, I.G. Johnston, N.S. Jones, D.C. Logan, B. Morgan, F.L. Müller, D.G. Nicholls, S.J. Remington, P.T. Schumacker, C.C. Winterbourn, J.J. Sweetlove, A.J. Meyer, T.P. Dick, M.P. Murphy, The ‘mionflash’ probe cpYFP does not respond to superoxide, Nature 514 (2014) E12–E14.

[41] L.A. Sena, N.S. Chandel, Physiological roles of mitochondrial reactive oxygen species, Mol. Cell 48 (2012) 158–167.

[42] E.L. Robb, J.M. Gawel, D. Aksentijevich, H.M. Cochemé, T.P. Dick, Properties of the radical intermediate obtained on oxidation of 2,7'-dichlorodihydrofluorescein, a probe for oxidative stress, Free Radic. Biol. Med. 41 (2006) 657–667.

[43] B. Zhao, K. Rangelova, J. Jiang, R.P. Mason, Studies on the photosensitized re-duction of resoru-bin, Ann. Rev. Pharmacol. Toxicol. 47 (2007) 971–978.

[44] J.A. Ronchi, T.R. Figueira, F.G. Ravagnani, H.C. Oliveira, A.E. Vercesi, R.F. Castilho, A spontaneous mutation in the nicotinamide nucleotide transhydrogenase gene of C57BL/6J mice results in mitochondrial redox abnormalities, Free Radic. Biol. Med. 63 (2013) 446–456.

[45] X. Ronot, L. Benel, M. Adolphe, J.C. Mounolou, Mitochondrial analysis in living cells: the use of rhodamine 123 and flow cytometry, Biol. Cell 57 (1986) 1–7.