

## TOPICAL REVIEW

# Mitochondria: isolation, structure and function

Martin Picard<sup>1</sup>, Tanja Taivassalo<sup>1</sup>, Gilles Gouspillou<sup>1,2</sup> and Russell T. Hepple<sup>1,2</sup><sup>1</sup>Department of Kinesiology and Physical Education, McGill University, Montreal, Qc, Canada<sup>2</sup>Critical Care Division, Royal Victoria Hospital and Department of Medicine, McGill University, Montreal, Qc, Canada

**Abstract** Mitochondria are complex organelles constantly undergoing processes of fusion and fission, processes that not only modulate their morphology, but also their function. Yet the assessment of mitochondrial function in skeletal muscle often involves mechanical isolation of the mitochondria, a process which disrupts their normally heterogeneous branching structure and yields relatively homogeneous spherical organelles. Alternatively, methods have been used where the sarcolemma is permeabilized and mitochondrial morphology is preserved, but both methods face the downside that they remove potential influences of the intracellular milieu on mitochondrial function. Importantly, recent evidence shows that the fragmented mitochondrial morphology resulting from routine mitochondrial isolation procedures used with skeletal muscle alters key indices of function in a manner qualitatively similar to mitochondria undergoing fission *in vivo*. Although these results warrant caution when interpreting data obtained with mitochondria isolated from skeletal muscle, they also suggest that isolated mitochondrial preparations might present a useful way of interrogating the stress resistance of mitochondria. More importantly, these new findings underscore the empirical value of studying mitochondrial function in minimally disruptive experimental preparations. In this review, we briefly discuss several considerations and hypotheses emerging from this work.

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**Corresponding author** R. T. Hepple: Critical Care Division, Royal Victoria Hospital, 687 Pine Ave W, Room L3.01, Montreal, Qc, Canada H3A 1A1. Email: russell.hepple@mcgill.ca**Abbreviations** IMM, inner mitochondrial membrane; mPTP, mitochondrial permeability transition pore; ROS, reactive oxygen species.

Mitochondria regulate critical cellular processes, from energy production to apoptosis, and measuring their function is an imperative for scientists whose research focuses on different aspects of cellular metabolism. The in-depth study of mitochondrial function in muscle

tissue is most often achieved using one of two types of preparations: isolated mitochondria, where mitochondria are extracted and purified by mechanical homogenization and differential centrifugation (Frezza *et al.* 2007b); or permeabilized myofibres, where the plasma membrane

**Tanja Taivassalo** (far left) is in the Department of Kinesiology and Montreal Neurological Institute at McGill University, with a background in Neuroscience and Exercise Physiology. **Russ Hepple** (second from left) is in the Department of Kinesiology and Department of Medicine at McGill University, with a background in Physiology. **Martin Picard** (second from right) and **Gilles Gouspillou** (far right) are a PhD candidate and Postdoctoral Fellow, respectively, in the Hepple and Taivassalo laboratories. Together, we are working on animal model and clinical projects aiming to better understand the role of mitochondria in skeletal muscle structure and function in health, ageing, and disease. Our recent work reveals that routine mitochondrial isolation procedures from skeletal muscle have a striking impact on mitochondrial function. This and other work is driving the continued evolution of how we study and interpret mitochondrial function.



of myofibres is selectively permeabilized leaving the mitochondria intact within their native cytoarchitectural environment and in direct contact with the incubation medium (Saks *et al.* 1991; Kuznetsov *et al.* 2008). Both methods permit the direct manipulation of mitochondrial function through the addition of specific substrates and inhibitors to the incubation medium, permitting insights into a wide range of mitochondrial functions, as detailed below.

Historically, isolated mitochondrial preparations were introduced more than half a century ago by the pioneering work of Chance & Williams (1956) and led to seminal discoveries about mitochondrial biology, including the chemiosmotic theory of oxidative phosphorylation (Mitchell, 1961) and elucidation of the Krebs cycle (Williams, 1965). To overcome important yield and selective isolation bias issues inherent to traditional isolated mitochondrial preparations, the permeabilized myofibres method was developed by Valdur Saks and colleagues several decades later (Veksler *et al.* 1987; Saks *et al.* 1998). Experimentally, mitochondria in both preparations are ultimately fed substrates and inhibitors in a carefully ordered manner allowing the experimenter to measure, among other parameters, mitochondrial respiration, reactive oxygen species (ROS) production and scavenging, and susceptibility to apoptotic events such as opening of the mitochondrial permeability transition pore (mPTP) (Zoll *et al.* 2003; Anderson & Neuffer, 2006; Picard *et al.* 2008; Ljubicic *et al.* 2010).

Despite the development of the permeabilized myofibre method, isolated mitochondria continue to be the more widely used method of investigating mitochondrial function in skeletal muscle. It seems likely that the assumption that isolated organelles preserve the function of mitochondria *in vivo* stems from the old 'textbook' view of mitochondrial structure, where these organelles were considered as bean-shaped spheroids that became liberated from their intracellular tethers upon mechanical isolation. However, we have known for more than a quarter of a century that skeletal muscle mitochondria exhibit a markedly diverse architecture (Bakeeva *et al.* 1978; Kirkwood *et al.* 1986; Kayar *et al.* 1988; Ogata & Yamasaki, 1997), with some mitochondria exhibiting elongated tubular branched structures (Ogata & Yamasaki, 1997) and extensive functional connections (Fang *et al.* 2011) between what would appear to be individual mitochondria in tissue cross-sections. With this view in mind, it is clear that mitochondrial structure after isolation procedures would be radically altered, owing to the ripping apart of the more elaborate tubular structures to yield relatively homogeneously sized spheroid organelles during mechanical homogenization. Based on the growing appreciation for the fact that mitochondrial structure is intricately linked to their function, alteration of mitochondrial function should

be an expected outcome following routine isolation procedures. An overview of our current understanding of the events occurring during routine mitochondrial isolation procedures and their impact on mitochondrial morphology is depicted in Fig. 1.

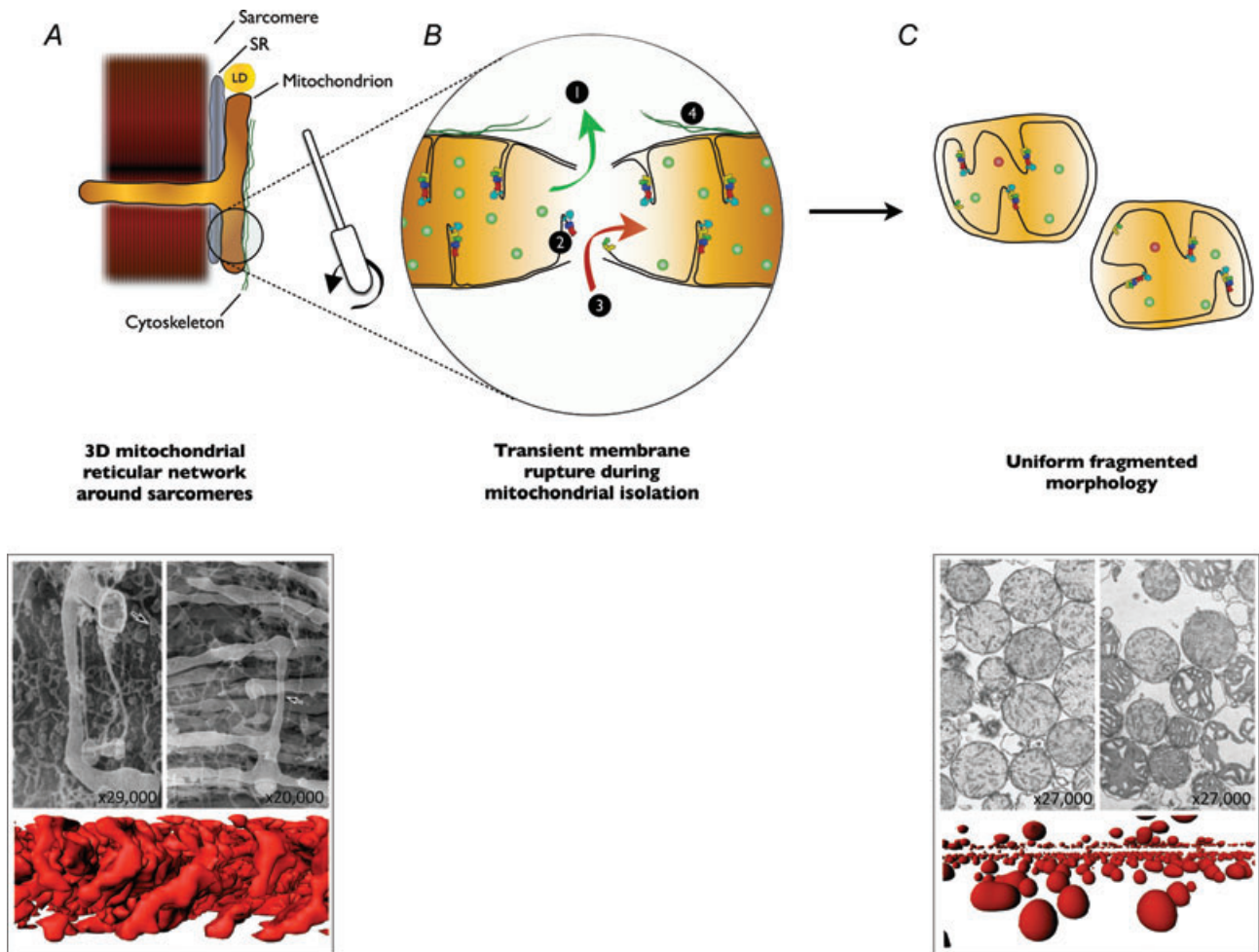
### Morphology–function relationship

As evidence of the impact of mitochondrial structure on function (Heath-Engel & Shore, 2006), some have shown that enhanced network branching induced by upregulating mitochondrial fusion (Sugioka *et al.* 2004; Ong *et al.* 2010) or downregulating fission (Ong *et al.* 2010) can reduce or prevent apoptotic signalling. Blocking mitochondrial fission also prevents fission-induced ROS release in hyperglycaemic conditions (Yu *et al.* 2006). The opposite also appears to occur: enhanced network fragmentation by upregulating mitochondrial fission (Frank *et al.* 2001; Ong *et al.* 2010) or downregulating mitochondrial fusion (Lee *et al.* 2004; Sugioka *et al.* 2004) can promote pro-apoptotic signalling in live cells, although this causal link has not always been observed (Youle & Karbowski, 2005). Further to this, promoting mitochondrial fission and network fragmentation has been associated with reduced respiratory capacity and increased ROS production (Koopman *et al.* 2005; Benard *et al.* 2007; Frezza *et al.* 2007a; Yu *et al.* 2008). In addition, the major protein involved in mitochondrial fusion – mitofusin 2 – influences expression of oxidative phosphorylation genes (Pich *et al.* 2005), indicating overlap at the genetic level between regulatory pathways for mitochondrial morphology and metabolism (Zorzano *et al.* 2010). Mitochondrial membrane potential is also closely associated with reversible changes in mitochondrial morphology (Guillery *et al.* 2008), and additional findings demonstrate an intricate relationship between mitochondrial dynamics, structure and function (Chen & Chan, 2010; McBride & Soubannier, 2010). Clear demonstration that mitochondrial fusion plays a major physiological role in skeletal muscle function comes from skeletal muscle-specific Mfn2<sup>-/-</sup> mice, which accumulate mutations in mitochondrial DNA, exhibit severe deficits in growth (body weight 30–50% of Mfn2<sup>+/+</sup>) and muscle oxidative capacity, and have impaired thermogenesis (Chen *et al.* 2010). This profound interdependence among mitochondrial morphology and function, emerging decades following the establishment of isolation methods as the gold standard to study mitochondrial function, obligates re-consideration of the functional characteristics of isolated organelles.

If we compare the morphology of mitochondria in intact skeletal muscle fibres – branched elongated structures, of heterogeneous size and shape (Bakeeva *et al.* 1978; Kirkwood *et al.* 1986; Kayar *et al.* 1988; Ogata &

Yamasaki, 1997) – to that of isolated mitochondria – quasi-spherical units, of relatively homogeneous size and shape (Figueiredo *et al.* 2008; Picard *et al.* 2010) – we must logically conclude that membranes of the mitochondrial network are ruptured during muscle homogenization and must rapidly re-seal to yield apparently ‘intact’

organelles. Consistent with this, our results (Picard *et al.* 2011) and those of others (Schwerzmann *et al.* 1989) suggest that soluble matrix enzymes are lost from the mitochondrial matrix during isolation, probably during the transient rupturing–resealing of mitochondrial membranes that occurs during isolation procedures. Until



**Figure 1. Mitochondrial isolation fragments mitochondria and alters mitochondrial function**

*A*, *in vivo* and *in situ*, mitochondria exhibit a complex three-dimensional network arrangement around sarcomeres (Kirkwood *et al.* 1986; Ogata & Yamasaki, 1997). In addition, mitochondria maintain functional interactions with the sarcoplasmic reticulum (SR), lipid droplets (LD) (Goodman, 2008) and cytoskeleton (Saks *et al.* 2010). *B*, during mitochondrial isolation, muscle is homogenized using scissors and a Teflon pestle, which separates mitochondria and ruptures mitochondrial membranes. This transient rupture of mitochondrial membranes has four potential direct effects: allowing soluble mitochondrial molecules to escape the mitochondria, thereby diluting matrix components necessary for oxidative phosphorylation or reactive oxygen species detoxification (1); causing disruption of the inner mitochondrial membrane and the structural integrity of its residing supra-molecular complexes (2); allowing soluble molecules from the homogenization medium to enter the mitochondria to alter aspects of mitochondrial function, or allowing the medium itself to enter the matrix causing swelling and dilution of matrix constituents (3); and disrupting functional interactions between mitochondria, SR, LDs and cytoskeleton (4). *C*, the end result of mitochondrial isolation is a relatively homogeneous population of spherical organelles with swollen morphology, disorganized cristae and diluted matrix content. Together, these effects result in altered functional characteristics of isolated mitochondria when compared to intact mitochondria from permeabilized myofibres. EM images reproduced from Ogata & Yamasaki (1997) and Hackenbrock (1968), with permission from Wiley and Rockefeller University Press, respectively. Three dimensional reconstructions are from MitoTracker-labelled confocal imaging of mitochondria in permeabilized myofibres or isolated mitochondria, as described in Picard *et al.* (2011).

recently, the functional consequences of this loss of matrix constituents and altered organelle morphology resulting from mitochondrial isolation have been largely unknown.

### Mitochondrial isolation: functional consequences

Saks and colleagues first reported that the  $K_m$  for ADP during mitochondrial respiration is considerably lower in isolated organelles versus those left *in situ* within permeabilized myofibre bundles (Saks *et al.* 1991). Lack of interactions between the mitochondrial voltage-dependent anion channel (VDAC) and cytoskeletal components such as tubulin, as well as an absence of ADP diffusion limitations appear to cause this effect (Saks *et al.* 2010). However, until recently the full magnitude of differences in respiratory capacity under different states, ROS production, and susceptibility to mPTP opening between these two preparations was unknown.

In an attempt to better understand the impact of isolation on mitochondrial function, we recently conducted a series of experiments directly comparing isolated mitochondria and permeabilized myofibres from the rat gastrocnemius muscle. Strikingly, isolated mitochondria exhibited a 47-fold sensitization of the mPTP to  $Ca^{2+}$  challenge (shorter time to mPTP opening), altered respiratory responses to stepwise substrate addition (lower state 2 respiration, higher  $N,N,N',N'$ -tetramethyl-*p*-phenylenediamine (TMPD)-driven complex IV respiration in isolated mitochondria), and increased ROS production by 9- to 23-fold under different energized states (Picard *et al.* 2011). One could argue that these considerable differences in key indices of mitochondrial function may arise from a selection of a specific mitochondrial population yielded by the isolation procedure. Indeed, muscle cells contain two different mitochondrial populations – subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria – that not only differ in their intracellular localization but also in their functional properties (Adhietty *et al.* 2005) and in their relative ease of extraction. However, the magnitude of difference between SS and IMF mitochondria in mPTP sensitivity to  $Ca^{2+}$  is only around 30–40% (IMF > SS) (Adhietty *et al.* 2005); and the magnitude of difference for ROS production is only around 2- to -3-fold (IMF < SS) (Adhietty *et al.* 2005), suggesting that selective isolation of subpopulations of mitochondria is unlikely to account for the observed differences between mitochondria from isolated and permeabilized preparations. Finally, in the experiments that we employed with isolated mitochondria we used the protease nagarse to facilitate harvest of both SS and IMF mitochondrial populations (Picard *et al.* 2011). Thus, the differences in function between isolated mitochondria and permeabilized myofibre bundles are most likely a

consequence of the isolation procedures on mitochondrial physiology.

To highlight differences between the isolated mitochondria and permeabilized myofibre preparations, here we analyse all of these experiments collectively by using a hierarchical clustering analysis (Fig. 2). This unsupervised analysis – describing similarities between samples and functional parameters – clearly shows that isolated mitochondria and permeabilized myofibres segregate into two distinct clusters. These two clusters reveal that isolated mitochondria and permeabilized myofibres possess strikingly distinct functional signatures across a broad range of mitochondrial functions. It is, however, important to note that state 3 respiratory capacity ( $V_{max}$ ), the most commonly reported index of mitochondrial function from isolated organelles, was not different between preparations. This is likely to explain, at least partially, why the magnitude of altered function in isolated mitochondria has not been appreciated heretofore.

On the one hand, because these findings demonstrate that isolation profoundly impacts several aspects of mitochondrial function, this raises concerns regarding the interpretation of results obtained in studies using isolated mitochondria. For example, age-related changes in mitochondrial function are exaggerated when assessed in isolated mitochondria compared to permeabilized myofibres (Picard *et al.* 2010). On the other hand, because isolated mitochondria exhibit qualitatively similar characteristics of mitochondria undergoing fission during apoptosis *in vivo* (Youle & Karbowski, 2005; Frezza *et al.* 2007a), including marked fragmented morphology, elevated ROS production and sensitization of the mPTP (Picard *et al.* 2011), isolated mitochondria may better represent stressed organelles than mitochondria functioning under normal circumstances *in vivo*, suggesting a novel application of mitochondrial isolation to interrogate the stress resistance of the organelle.

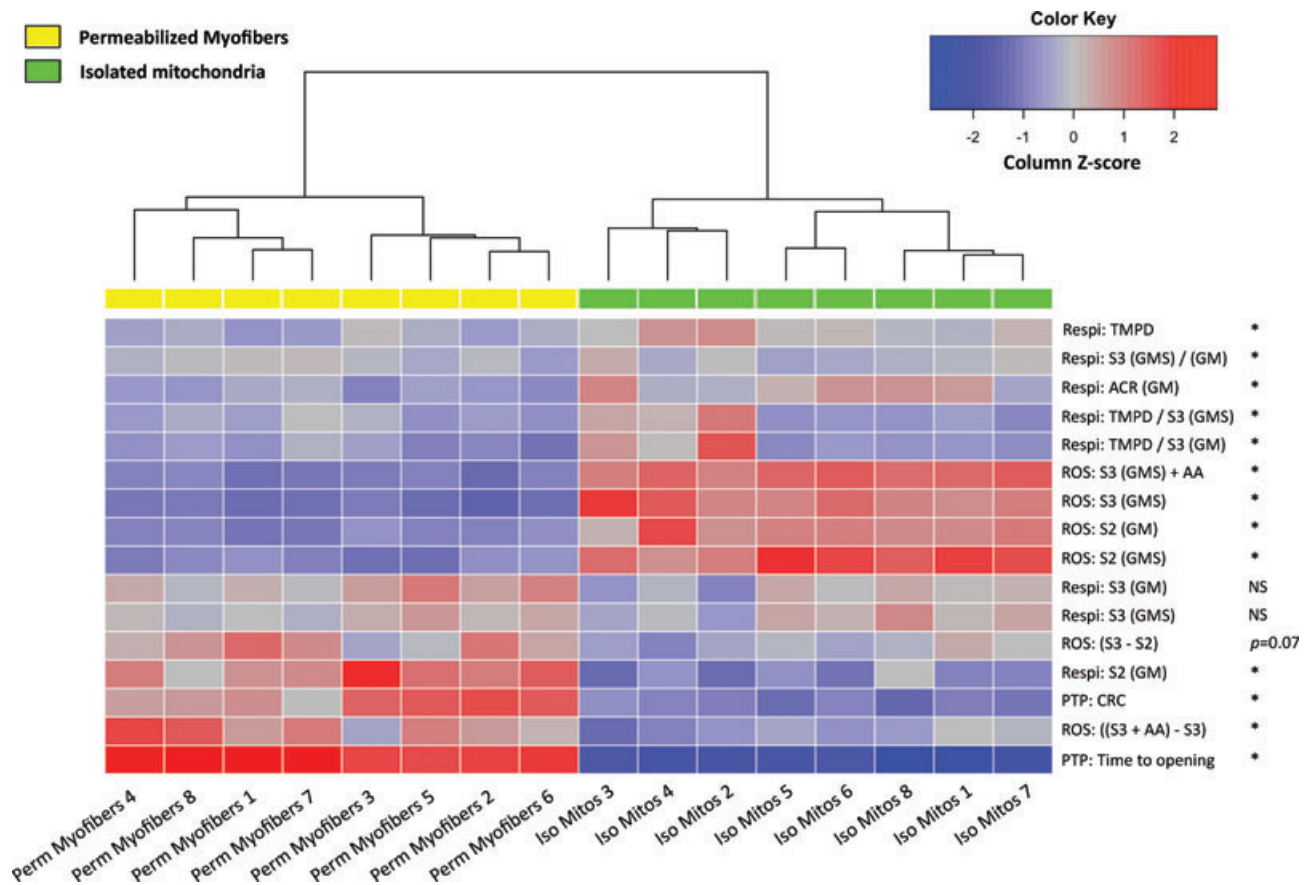
Interestingly, mitochondrial respiration was selectively impaired in isolated mitochondria when complex I (NADH:ubiquinone oxidoreductase) substrates were used, compared to conditions where substrates acting more directly on a given complex were used (succinate to activate complex II; the artificial electron donor, TMPD, used to directly activate complex IV). To this end, we note that complex I-driven respiration with malate and glutamate as substrates depends on the presence and function of soluble components of the Krebs cycle to produce the reducing equivalent NADH, and consequently also depends on the presence of soluble precursor molecules such as the coenzyme  $NAD^+$ . In addition, complex I activity is potentiated by its assembly with complex III and IV within the inner mitochondrial membrane into functional supercomplexes (Schafer *et al.* 2006; Vonck & Schafer, 2009). Based on this and on



the model illustrated in Fig. 1, we speculate that this preferential loss of Complex I activity could be explained by a combination of the following factors: (i) partial loss of matrix constituents like  $\text{NAD}^+$  and Krebs cycle components during isolation (as originally suggested by Schwerzmann *et al.* (1989)); (ii) proteolytic damage to ETC proteins by nagarse, which may enter the mitochondria during isolation, resulting in protein degradation (Wilson, 1987; Patel *et al.* 2009); and/or (iii) cristae remodelling and disruption of supramolecular structures induced by isolation, secondary to either swelling or mitochondrial inner membrane (IMM) rupture. The first two possibilities are

discussed in detail elsewhere (Picard *et al.* 2011). The third is supported by electron microscopy (EM) imaging of isolated mitochondria showing disrupted architecture of the IMM cristae in isolated organelles compared to *in vivo* (Fig. 3).

Whether the processes involved in the mitochondrial membrane resealing that must occur during mitochondrial isolation are passive, involving spontaneous resealing of phospholipid bilayer, or active, involving the mitochondrial fusion proteins mitofusin 1 and 2 (Mfn1 and Mfn2) and optic atrophy 1 (OPA1), is currently unknown. In our recent experiments,



**Figure 2. Isolated mitochondria and permeabilized myofibres have different functional signatures**

Heat map depicting results from an unsupervised hierarchical clustering analysis, whereby samples with the most similar functional signatures are grouped together. Functional signatures are defined here as the integration of functional measures of respiratory capacity (Respi) and  $\text{H}_2\text{O}_2$  production (ROS) under various stimulatory conditions, as well as mitochondrial permeability transition pore (mPTP) responses to a  $\text{Ca}^{2+}$  challenge. The dendrogram above the heat map shows two primary clusters of similar compactness and exhibiting marked distinctiveness, corresponding to permeabilized myofibre and isolated mitochondrial preparations. The heat map analysis was performed with data obtained from mixed gastrocnemius muscles of Fisher 344/Brown Norway  $\text{F}_1$ -hybrid rats (data reported in: Picard *et al.* 2011). To allow direct comparison of both preparations, all data are presented and normalized per international unit of citrate synthase activity, except for time to PTP opening expressed in seconds. The z-score for each value was computed from the mean for each measure. Measures which significantly differ ( $P < 0.05$ ) between preparations are marked with an asterisk. Statistical analysis was performed using 'R' (R Foundation for Statistical Computing, version 2.12.1). List of abbreviations: TMPD, TMPD-driven complex IV respiration; S2, State 2 – Substrates-driven respiration; S3, State 3 – ADP-driven respiration with glutamate + malate (GM) or GM + succinate (GMS); ACR, acceptor control ratio; AA, antimycin A; CRC, calcium retention capacity; NS, not significant. Details of experimental procedures are reported in Picard *et al.* (2011).

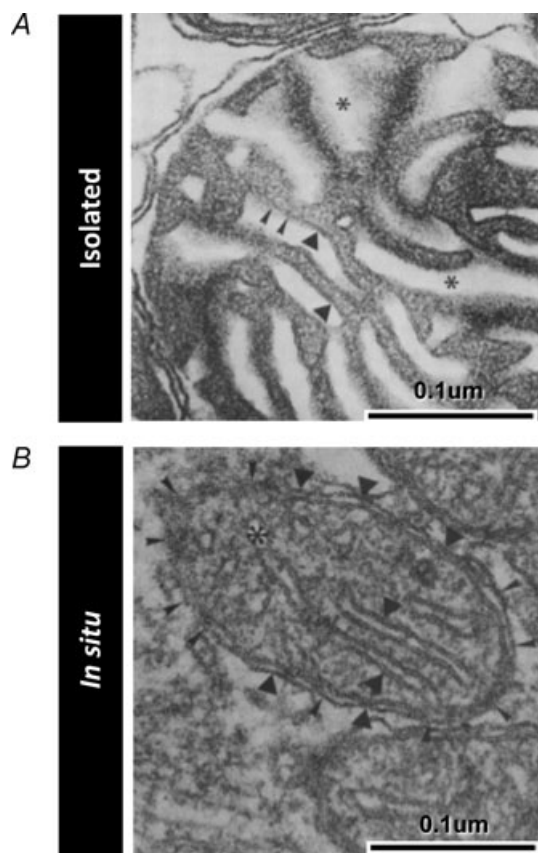
we also observed that the functional alterations induced by the isolation of mitochondria from skeletal muscles were greater in aged muscles (Picard *et al.* 2010). If the successful resealing of disrupted mitochondria during isolation depends upon fusion proteins, these results could in part be explained by dysfunction in the fusion aspect of mitochondrial dynamics that may occur with ageing (Seo *et al.* 2010), handicapping the ability of mitochondria to reconstitute during isolation and rendering them more susceptible to isolation damage. This would also exacerbate loss of matrix constituents during isolation procedures in aged mitochondria, a point consistent with the elevation in ratio of the biochemical activities of the mitochondrial inner membrane enzyme, complex IV, and the mitochondrial matrix enzyme, citrate synthase, that we observed in isolated mitochondria from

aged muscle (Picard *et al.* 2010). Further work will be necessary to more completely address these issues.

### Live cell and *in vivo* methods to assess mitochondrial function

While we favour the use of the permeabilized myofibre over the isolated mitochondrial technique based on its preservation of mitochondrial morphology and its flexibility of use in assessing a wide range of mitochondrial functional outputs, there are methods which permit measurement of certain parameters of mitochondrial function from live cells where the sarcolemma is left intact. For instance, modern microscopy-based imaging methods such as confocal (for cultured cells or *in vivo* imaging) (Duchen, 2004; Frezza *et al.* 2007a; Fang *et al.* 2011) and two-photon fluorescence microscopy (for *in vivo* imaging in tissues) (Rudolf *et al.* 2004; Pozzan & Rudolf, 2009; Romanello *et al.* 2010) have been used to monitor mitochondrial function in different types of muscle cells (Duchen, 2004; Saks *et al.* 2010; Fang *et al.* 2011). Microscopic imaging is used in conjunction with specific fluorescent probes sensitive to mitochondrial membrane potential,  $[Ca^{2+}]$ , or ROS (e.g.  $O_2^{\cdot-}$ ); by exploiting the autofluorescent properties of intracellular molecules such as NAD(P)H and flavoproteins (i.e. FADH) at specific excitation wavelengths (Duchen, 2004), the assessment of intracellular fluxes and relative levels of biochemical parameters linked to mitochondrial function is made possible.

A definite advantage of imaging techniques over *in vitro* preparations (i.e. isolated mitochondria and permeabilized myofibres) is that mitochondrial morphology and certain aspects of function can be assessed simultaneously (Frezza *et al.* 2007a; Fang *et al.* 2011). Additionally, in the *in vivo* situation, mitochondria are not only morphologically intact but also in their native intracellular and systemic environment, which allows one to evaluate certain mitochondrial function parameters in the context of putative soluble cytoplasmic factors that may impact how mitochondria function within the intact muscle. However, imaging techniques do not provide information on one of the most important mitochondrial functional parameters: the oxygen consumption rate. Oxygen consumption can nevertheless be measured in intact cultured cells using high sensitivity systems (such as the Seahorse (Gerencser *et al.* 2009), or Oroboros systems (Gnaiger, 2009)), with the compromise being that these systems do not permit simultaneous microscopic measurements. Alternatively, oxygen consumption has also been monitored using a standard  $P_{O_2}$  electrode in single intact myocytes (Elzinga & van der Laarse, 1988), and this method can probably be adapted to permit monitoring of other aspects of cellular function



**Figure 3. Muscle mitochondrial ultrastructure is disrupted in isolated mitochondria (*in vitro*) compared to normal mitochondria *in situ***

Electron micrographs of mitochondria from skeletal muscle depicting the cristae morphology in isolated mitochondria (A) compared to mitochondria fixed *in situ* (B). Several differences are noted in isolated mitochondria, including: lower electron density of the matrix space, dysmorphic and irregular inner mitochondrial membrane cristae structure, absence of consistent contact sites near cristae junctions, and swelling of inter-membrane spaces. Images are from Schwerzmann *et al.* (1989) and are reproduced here with permission from the National Academy of Sciences of the USA.

simultaneously using confocal imaging methods (Stary & Hogan, 2000, 2005).

A significant drawback of using imaging methods on cells with an intact plasma membrane lies in the fact that the micro-environment surrounding mitochondria cannot be readily and precisely controlled. Consequently, these methods preclude the accurate determination of the effect of certain factors of interest on mitochondrial function. For example, live cell experiments using microscopy prevents one testing the direct effect of specific substrates (e.g. glutamate, malate, succinate, long-chain fatty acids) or non-freely diffusible molecules (ADP) on mitochondrial function. Determining the effect of these different molecules on mitochondrial function is an important step in the characterization of mitochondrial alterations/adaptations induced by physiological or pathophysiological events, but such level of experimental control is currently only possible with isolated mitochondria and permeabilized myofibre methods. Confocal microscopy techniques can nevertheless be applied to permeabilized myofibres with the added benefit of spatial resolution, allowing investigation of subcellular and organelle-level dynamics in  $[Ca^{2+}]$  and redox state (Isaeva *et al.* 2005). Given the breadth of techniques preserving mitochondrial morphology that are available, researchers must determine how much experimental control is necessary to answer physiological questions of interest. Furthermore, one may wish to use intact cells in tandem with more invasive approaches, with the latter being used to provide greater detail of the nature of specific mitochondrial defects identified in intact cells.

### Summary

Over the last several decades, preparations of 'intact' isolated mitochondria from skeletal muscle have been widely employed to study the active function of these organelles. However, we now have convincing evidence that mitochondrial isolation severely disturbs mitochondrial morphology, and not unexpectedly, that this is associated with marked impairments of mitochondrial function. Although these differences urge caution in the interpretation of data collected from isolated mitochondria, a more thorough understanding of the basis for these differences in function could lead to novel applications of this technique. Indeed, developing assays allowing one to experimentally enhance or impair reconstitution of mitochondria during isolation from tissues may provide new insights into the rapidly evolving understanding of structure–function relationships in mitochondria.

In this era where neurodegenerative, muscular, cardiovascular, malignant and metabolic diseases are frequently proposed to involve altered mitochondria, and where several versions of a mitochondrial theory of

ageing dominates the current thinking in gerontology, there is a pressing need to test these theories with the most appropriate and relevant experimental methods. Characterizing the current methods – isolated mitochondria, permeabilized cells and intact cell microscopy-based methods – to permit a clearer understanding of how function is influenced by the preparation used, is a necessary step in this process. This will allow researchers to better exploit the current methods and hopefully pave the way for the development of new ways of investigating mitochondrial function and their impact on physiological/pathophysiological processes, with the ultimate goal of identifying molecular targets for interventions. In this latter respect, while the current review underscores the effect of isolation on mitochondrial function, we must also consider how mitochondrial function may be altered from the *in vivo* condition even in the permeabilized myofibre technique, where the intracellular environment is altered from the native state by the use of standard incubation buffers. This alteration could mask changes in mitochondrial function that occur *in vivo* secondary to disease- and/or ageing-induced alterations in the intracellular milieu in which the mitochondria reside. This critical evaluation of the methods for interrogating mitochondrial function is fundamental to advancing our understanding of how mitochondria may be involved in health, ageing and disease.

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