Mitochondrial morphology and function: two for the price of one!

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Summary
Mitochondrial shape and function are known to be linked; therefore, there is a need to combine three-dimensional EM structural analysis with functional analysis. Cytochrome c oxidase labelling is one approach to examine mitochondrial function at the EM level. However, previous efforts to apply this method have had several issues including inconsistent results, disruption to mitochondrial ultrastructure, and a lack of optimisation for volume EM methods. We have used short fixation and microwave processing to address these issues. We show that our method gives consistent cytochrome c oxidase labelling and improves labelling penetration across tissue volume. We also quantify mitochondrial morphology metrics, including in volume EM, to show that ultrastructure is unaltered by the processing. This work represents a technical advance that allows the correlation of mitochondrial function and morphology with greater resolution and volume than has previously been feasible.

Introduction
Mitochondria were first observed by electron microscopy in 1952 (Palade, 1952). Since then the importance of mitochondria as a central hub in cellular metabolism has been established, along with their many cellular roles such as calcium buffering and iron sulphur cluster formation (Baughman et al., 2011; Zhu et al., 2016). Furthermore it is now well-established that changes in mitochondrial morphology affect the mitochondrial function and vice versa (Chen et al., 2005; Yu et al., 2006; Jahani-Asl et al., 2007; Chen & Chan, 2009; Romanello et al., 2010; Picard et al., 2013).

Skeletal muscle is highly metabolically active, and as such has a high mitochondrial mass. The mitochondria of the skeletal muscle fall into structural and functionally different populations: the intermyofibrillar mitochondria, which are highly branched and the subsarcolemmal mitochondria, which are more spherical (Hood & Iqbal, 2013; Picard et al., 2013; Faitg et al., 2019; Vincent et al., 2019). Light microscopy methods have advanced rapidly allowing the correlation of mitochondrial structure and function (Wolf et al., 2019). Whilst the advent of super resolution techniques has revolutionised what is possible to visualise in cells, the resolving power in tissues is not as good. As such the tightly packed mitochondria of the skeletal muscle, particularly in the subsarcolemmal region, present a resolution problem even for super resolution microscopy techniques. Therefore, electron microscopy is often the only option for accurate quantification of mitochondrial morphology in tissues but is limited by lack of functional information. Transmission electron microscopy can be combined with labelling methods to pinpoint the positions or activity of specific molecules, thus linking morphology and function. Graham & Karnovsky (1966) were the first to introduce 3,3′-diaminobenzidine tetrahydrochloride (DAB) for the electron microscopic demonstration of peroxidase activity in the proximal tubules of the mouse kidney in TEM.

Cytochrome c oxidase (COX) histochemistry is a routinely used histological assay for the detection of COX (complex IV of the mitochondrial respiratory chain) activity. Seligman et al. (1968) proposed the use of COX histochemistry for the ultrastructural localisation and visualisation of cytochrome c (COX) in rat liver. Using DAB as an electron acceptor, the visualisation of COX activity can be demonstrated by the electron-dense deposits forming with osmium on the inner mitochondrial membrane and in the intracristae space with EM (Seligman et al., 1968; Beard & Novikoff, 1969; Novikoff & Goldfischer, 1969; Seligman et al., 1970; Keith & Schulier, 1972; Roels, 1974). Since then, many reports of EM studies of COX localisation have appeared with several changes to the original protocol. The cytochemical visualisation depends not only on the oxidative activity of the mitochondrial membrane but is also influenced by the heterogeneity of the specimen itself, by the denaturing effect of the fixative, size of the sample, the solubility of the substrate and possibly other factors (Seligman et al., 1968; Anderson et al., 1975). However, this cytochemical technique provides 2D information only, it is often associated with ultrastructural changes, suffers from poor penetration.
Fig. 1. Schematic view of conventional bench protocol and short fixation microwave processing (SFMP) protocol for TEM of skeletal muscle from start to finish. The conventional protocol bench processing (overnight at 4 °C) is shown with a grey background. The SFMP (10 min at 4 °C), steps highlighted in green show the steps where processing was completed in the microwave.

Table 1. TEM sample preparation for electron microscopy using microwave processing.

| Process               | Reagent                                         | Power (W) | Time (sec) | Temperature Range (°C) | Vacuum (20 Hg) |
|-----------------------|-------------------------------------------------|-----------|------------|------------------------|----------------|
| 1. Buffer rinse       | 0.1 M Sorenson’s buffer (pH 7.3) – 3 times       | 150       | 40         | 23–27                 | Cycle          |
| 2. Post fixation      | 1% osmium tetroxide (v/v) in water              | 100       | 120 on–120 off–120 on–120 off–120 on | 23–27     | Cycle          |
| 3. Water rinse        | distilled H$_2$O – 3 times                      | 150       | 40         | 23–27                 | Cycle          |
| 4. Dehydration        | Acetone series: 25%, 50%, 75%, 3 × 100%         | 150       | 40         | 23–27                 | Off            |
| 5. Resin infiltration | TAAB medium resin in Acetone series: 25%, 50%, 75%, 3 × 100% | 300       | 180        | 23–27                 | Off            |
| 6. Polymerisation     | Embedded in 100% fresh resin and left to polymerise at 60 °C for a minimum of 24 h. |           |            |                        |                |

Note: Protocol for microwave included washes, dehydration and resin embedding of skeletal muscle.

and is seriously limited in its application to a three-dimensional structure.

Here we optimise sample fixation, processing and COX enzyme histochemistry for TEM in muscle. Mitochondria are known to be anisotropic in skeletal muscle (Vincent et al., 2019), and as such two dimensions provides a quantitative limitation. Therefore, we further develop this method for use with serial block-face scanning electron microscopy (SBF-SEM), to allow correlative analysis of 3D mitochondrial morphology with mitochondrial COX activity in muscle. Such an improvement in sample preparation may be translatable to other tissues allowing advances in characterising mitochondrial pathology and disease mechanisms that were not previously possible.

Materials and methods

Animals and tissue collection

All experiments were performed with wild type (WT) mice under project licence (P76987201) (n = 6). The animals were euthanised by cervical dislocation. The soleus (SOL) muscles from both hind legs were collected, lightly teased apart in 0.1 M Sorenson’s Phosphate buffer to allow penetration of solutions between the muscle fibres, then cut into 1 mm$^3$ pieces, which were divided across the different processing conditions. Samples were immersion fixed in 2% glutaraldehyde with 0.1 M Sorenson’s buffer (pH 7.3) at 4 °C either overnight (standard) or for 10 min [Short Fixation Microwave Processing (SFMP)].

For transmission EM, tissue samples were processed in one of four ways:

1. Conventional overnight fixation and bench processing (to have a TEM ‘gold-standard’ against which to compare the morphology from the other methods).
2. Short fixation, microwave processing (SFMP).
3. Overnight fixation, COX histochemistry and microwave processing.
4. Short fixation, COX histochemistry and microwave processing.

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Fig. 2. Illustration of COX labelling in electron microscopy (EM). (A) Dissection and teasing of muscle fibres. (B) Fixation for 10 min (SFMP) or overnight at 4 °C (conventional protocol). (C) Incubated in COX reaction (2 h at 37 °C) or Sorenson’s buffer (2 h at 4 °C). (D) Following this, samples were processed for TEM or SBFSEM with microwave including postfixation, dehydration, embedding and imaging in electron microscope. *2: Short fixation, microwave processing (SFMP). 3: Standard TEM overnight fixation, COX histochemistry and microwave, 4: short fixation, COX histochemistry and microwave processing, 5: short fixation, heavy metal protocol and microwave-processing (SFMP), 6: short fixation, COX histochemistry, heavy metal protocol and microwave processing.

SFMP Protocol - Microwave processing (5)

SFMP Protocol - COX - Microwave processing (6)

Fig. 3. Schematic view of short fixation microwave processing (SFMP) with or without COX procedures for SBFSEM of skeletal muscle. Standard protocol of SBFSEM is shown with a grey background. For the SFMP protocol the steps where processing was done in the microwave (dehydration, washes and resin embedding) are highlighted in green and the COX incubation in red.

For serial block face scanning EM, tissue samples were processed in one of two ways:

5) Short fixation, heavy metal protocol and microwave-processing (SFMP).

6 Short fixation, COX histochemistry, heavy metal protocol and microwave processing.

Transmission electron microscopy only

For overnight fixation and bench-processing, tissue was postfixed in 1% osmium tetroxide (1 h), dehydrated in graded acetone (25%; 50%; 75%; 2 × 100%; 30 min each, RT) before being impregnated with increasing concentrations of epoxy resin (TAAB medium resin) in acetone (25%, 50%, 75%, 3 × 100%, all for 1 h each, RT). The samples were then embedded in 100% fresh resin and left to polymerise at 60 °C for a minimum of 24 h (Fig. 1).

For SFMP, the Pelco Biowave® Pro+ incorporating the Pelco Coldspot® Pro microwave was used for the following steps of the processing (Fig. 1). The ColdSpot® system improves inconsistent wattage supply to the microwave compartment, therefore protecting samples from excess microwave energy. The range temperature was set 23—27 °C. See Table 1 for full microwave program details. Samples were postfixed in 1% osmium tetroxide for 8 min [pulse microwaved (MW), 100 watts (W)] and rinsed in distilled H₂O (3 × 150 W 40 s per step). Samples were dehydrated in a graded series of acetone (25%; 50%; 75%; 3 × 100%; 150 W 40 seconds per step) before being impregnated with increasing
Table 2. Detailed parameters for microwave processing steps for SBFSEM sample preparation. For electron microscopy using microwave processing.

| Process          | Reagent                                      | Power (W) | Time (sec) | Temperature Range (°C) | Vacuum (20 Hg) |
|------------------|----------------------------------------------|-----------|------------|------------------------|----------------|
| 1. Water rinse   | distilled H₂O – 3 times                      | 100       | 40         | 23–27                  | Cycle          |
| 2. Dehydration   | Acetone series: 25%, 50%, 75%, 3×100%        | 150       | 60 on – 60 off – 60 on | 23–27                  | Cycle          |
| 5. Resin infiltration | TAAB hard resin in acetone series: 25%, 50%, 75%, 3×100% | 300       | 40         | 23–27                  | Cycle          |
| 6. Polymerisation | Embedded in 100% fresh resin and left to polymerise at 60 °C for a minimum of 24 h. |           |            |                        |                |

All resin blocks were trimmed using a razor blade to form a trapezoid block face. Sections were cut in a longitudinal or transverse orientation on an ultramicrotome (Wolf et al., 2019) using a diamond knife. Semithin sections (0.5 μm) were stained with toluidine blue and viewed on a light microscope (LM) to verify orientation of tissue. Ultrathin sections (70 nm) were then cut and picked up onto copper grids. Sections from blocks that had not been incubated in COX medium were stained with 1% uranyl acetate (30 min) and 3% lead citrate (7 min); sections from blocks that had been incubated in COX medium were not further stained. All sections were examined using a HT7800 120kV TEM (Hitachi). Digital micrographs were captured using an EMSIS Xarosa CMOS Camera with Radius software.

Fig. 4. Transmission electron microscopy images comparing mitochondrial structure following conventional bench processing and short fixation microwave processing. TEM images of mouse muscle processed with the conventional overnight fixation and bench processing protocol (A), short fixation microwave processing (B). Healthy subsarcolemmal and intermyofibrillar mitochondria with well-defined uniform cristae following staining with lead citrate and uranyl acetate stain (scale bar = 500 nm).

Transmission electron microscopy and COX labelling

After fixation (either overnight or 10 min), samples were washed in three changes of Sorenson’s buffer in microwave (3 × 150 W 40 sec per step). Samples were then incubated either with COX reaction (5 mM 3′,3 diaminobenzidine tetrahydrochloride (DAB), 1000 μM cytochrome c and 0.2 μg catalase in 0.1 M phosphate buffer, pH 7.0) for 2 h at 37 °C in the dark, or with Sorenson’s buffer for 2 h at 4 °C (Fig. 2). As cytochemical controls for COX activity, the same solution of COX reaction was made with additional 10 mM of sodium azide, a specific inhibitor of COX activity. After several washes in buffer, the tissue was then processed as for the SFMP protocol above.

Serial block face scanning electron microscopy and COX labelling

After fixation (10 min), samples were washed in three changes of Sorenson’s buffer in microwave (3 × 150 W 40 sec per step) and incubated either with COX reaction (5 mM 3′,3 concentrations of epoxy resin (TAAB medium resin) in acetone (25%; 50%; 75%; 3×100%; 300 W 3 min per step). The samples were then embedded in 100% fresh resin and left to polymerise at 60 °C in a conventional oven for a minimum of 24 h (Table 1).
Fig. 5. Mitochondrial morphology does not differ between conventional overnight fixation and bench processing and SFMP in mouse muscle in TEM. (A–E) Frequency distribution of shape descriptors and morphological parameters for intermyofibrillar mitochondria in transverse orientation. (A) Area, (B) perimeter, (C) circularity, (D) aspect ratio, (E) form factor. Differences in frequency distributions were tested using Mann–Whitney test to compare cumulative distributions (mice; conventional n = 3, SFMP n = 3; mitochondria; standard n = 649; SFMP n = 667).
A Mann–Whitney test was used for ± et al. ± 0.015 0.714 ± 0.006 ± μ ± 0.037 1.848 3 SFMP; mitochondria ± 0.040 ± 0.043 1.739 ± 0.008 0.007 0.784 0.043 1.739 ± 0.052 0.007 0.784 ± 0.192 ± 0.007 0.382 ± 0.007 ± 0.008 0.008 0.192 ± 0.008 0.189 ± 0.008 ± μ 0.189 ± 0.008 ± μ 0.189 ± 0.008 0.007 0.721 ± 0.015 0.721 ± 0.015 ± μ 0.0368 ± 0.007 0.382 ± 0.007 ± μ 0.1841 ± 0.043 1.739 ± 0.052 Form factor 1.273 ± 0.026 1.276 ± 0.031 n 649 667

Note: Data are presented as median ± SEM. Data were obtained from mouse soleus (n = 3 conventional; n = 3 SFMP; mitochondria n = 649, conventional; n = 667, SFMP). A Mann–Whitney test was used for all comparisons.

diaminobenzidine tetrahydrochloride (DAB), 1000 μM cytochrome c and 0.2 μg catalase in 0.1 M phosphate buffer, pH 7.0) for 2 h at 37 °C in the dark, or with Sorenson’s buffer for 2 h at 4 °C (Fig. 2). As a cytochemical controls for COX activity, the same solution of COX reaction was made and 10 mM of sodium azide added.

The tissue was then processed using a heavy metal protocol adapted from Wilke et al. (2013). Tissue was immersed in 3% potassium ferricyanide with 2% osmium tetroxide for 1 h at room temperature. Tissue was placed in filtered 0.1% thiocarbohydrazide (TCH) for 20 min and then 2% osmium tetroxide for 30 min. Samples were left in 1% uranyl acetate overnight at 4 °C. The day after, the samples were washed three times for 5 min each with ddH2O and immersed in lead aspartate solution. 0.12 g of lead nitrate in 20 mL aspartic acid, for 30 min at 60 °C. Finally, samples were dehydrated using the microwave as above, in graded acetone from 25% to 100% and then infiltrated in increasing concentrations (25%–100%) of Taab 812 hard resin in acetone (Fig. 3, Table 2).

The samples were embedded in 100% fresh resin and left to polymerise at 60 °C for 36–48 h. After polymerisation, blocks were trimmed and sectioned for TEM to identify areas of interest for SBFSEM. To verify tissue orientation, penetration and morphology several sections (0.5 μm) were taken and stained with toluidine blue and viewed under a LM. Blocks were then trimmed to approximately 0.75 mm x 0.75 mm and glued onto a pin. In order to reduce sample charging within the SEM, the block was painted with silver glue and sputter-coated with a 5 nm layer of gold. The pin was placed into a Zeiss Sigma SEM incorporating the Gatan 3view system (Gatan inc., Abingdon, UK) for SBFSEM, which allows sectioning of the block and the collection of serial images in the z-plane. For each mouse two regions of interest (ROIs) in the intermyofibrillar (IMF) mitochondria region of the muscle fibre were selected, sectioned and captured in a series of images (50 images per stack) at 70 nm sectioning thickness with dimensions of 2000 × 2000 pixels and a pixel size of 0.07 μm x 0.07 μm (Cocks et al., 2018).

3D-reconstructions and quantitative analysis

COX-SBFSEM z-stacks were analysed and mitochondria were followed in all three dimensions for reconstructions. Image stacks from the SBFSEM were converted to Tiff files and greater than 100 mitochondria reconstructed for each fibre using 3D-MIB (Microscopy Image Browser, Helsinki (Bleievich et al., 2016)) analysis software for the segmentation and AMIRA (AMIRA image analysis software) for the 3D reconstruction. Mitochondria were manually traced in MIB using the ‘brush’ drawing tool on each section and were excluded if they were not completely within the ROI. Mitochondria were segmented separately, and only IMF mitochondria were analysed. For each completely reconstructed mitochondrion, the total volume and surface area were extracted from AMIRA and used in analyses. Mitochondrial Complexity Index was calculated using the formula (Vincent et al., 2019):

\[
MCI = \left( \frac{SA^{1.5}}{4\pi V^2} \right)^2 = \frac{SA^3}{16\pi^2 V^2}.
\]

This equation is a three-dimensional equivalent to form factor, to assess mitochondrial morphological complexity.

All statistics of shape descriptors used to assess mitochondrial morphology were statistically analysed using non-parametric Mann–Whitney test, because normalisation was not effective. Volume and MCI were fitted to a linear mixed model and were log-transformed before analysis. All statistical analyses were performed using the Prism 8 software (GraphPad Software, San Diego, CA, USA).

Morphological and statistical analyses from TEM images

Mitochondrial shape descriptors and size measurements were obtained using Image J (version 1.52i, National Institutes of Health, Bethesda, MD, USA) by manually tracing mitochondria from TEM images. Surface area (mitochondrial size) is reported in squared micrometres; perimeter in micrometres; aspect ratio (AR) is computed as [(major axis)/(minor axis)] and reflects the ‘length-to-width ratio’; form factor (FF) [(perimeter2)/(4π · surface area)] reflects the complexity and branching aspect of mitochondria; circularity [4π · (surface area/perimeter2)] is an index of sphericity with values of 1 indicating perfect spheroids; Feret’s diameter represents the longest distance (μm) between any two points within a given mitochondrion; Minimum Feret’s represents the closest distance between two parallel tangents of a mitochondrion.
Fig. 6. Comparing COX labelling penetration in COX-TEM using conventional overnight fixation and bench processing and short fixation microwave processing (SFMP). (A, C) Conventional overnight fixation COX histochemistry and microwave processing demonstrates weak reaction visible in the subsarcolemmal (SS, blue) mitochondria on the edges but none of the intermyofibrillar (IMF, red) mitochondria centrally located show activity. (A) Low magnification of muscle fibre (scale bar = 5 µm). (C) High magnification of subsarcolemmal (SS, blue) and intermyofibrillar (IMF, red) (scale bar = 500 µm). (B, D) Short fixation microwave processing (SFMP) protocol demonstrates clear reaction visible in the SS (blue) mitochondria and in the IMF (red) mitochondria. (B) Low magnification of muscle fibre (scale bar = 5 µm). (D) High magnification of subsarcolemmal (SS, blue) and intermyofibrillar (IMF, red) (scale bar = 500 µm). Arrows show clear COX activity with a dark deposit in the cristae.
Fig. 7. Clear labelling of mitochondrial cristae after COX-TEM reaction in oxidative muscle fibres from soleus mouse muscle. (A) Low magnification of muscle fibres (scale bar = 5 μm). (B) High magnification of the same muscle fibres (scale bar = 1 μm). (C–F) High magnification images of mitochondria from other muscle fibres showing COX-TEM reaction (scale bar = 1 μm). Arrows show clear COX activity with a dark deposit in the cristae. All mitochondria are stained dark by the cytochrome c oxidase medium.
Fig. 8. Cytochemical controls for enzyme activity in TEM. (A) Skeletal muscle mitochondria control stained with lead citrate and uranyl acetate. (B) Skeletal muscle mitochondria control without staining. (C) Cytochrome c oxidase activity in skeletal muscle mitochondria. (D) Skeletal muscle fibres incubated with COX plus sodium azide (10 mM) showed no electron-dense deposits. Arrow showing unstained mitochondria, with no COX activity. (Scale bar = 1 μm).

Values were imported into Microsoft Excel and Prism 8 software (GraphPad Software, San Diego, CA, USA) for data analysis. Statistical significance was evaluated based on 99% confidence interval (CI) of the median.

Analysis of COX staining intensity

Images to be analysed were normalised to ensure comparable contrast between all conditions (Non-COX, COX and COX azide) using MIB. Using Image J (version 1.52i, National Institutes of Health, Bethesda, MD), the optical density threshold was manually set (threshold 50) for measurements of COX activity per mitochondrial area. Mean intensity and percent area are measured per total image area (n = 3 fibres per conditions). When comparing between methods all mitochondria were included irrespective of COX labelling since, in order to assess differences caused by the method. The samples compared for each method were from the same skeletal muscle.

Results

Many enzymes are very sensitive to fixation and the duration of fixation is critical in the preservation of enzyme
Fig. 9. Mitochondrial morphology does not differ between non-COX and COX incubations in mouse muscle in TEM. (A–E) Frequency distribution of shape descriptors and morphological parameters for intermyofibrillar mitochondria in transverse orientation. (A) Area, (B) perimeter, (C) circularity, (D) aspect ratio, (E) form factor. Differences in frequency distributions were tested using Mann–Whitney test to compare cumulative distributions (mice non-COX n = 3; COX n = 3; mitochondria non-COX n = 667; COX n = 582).
Table 4. Comparison of mitochondrial morphological parameters and shape descriptors of intermyofibrillar mitochondria following non-COX and COX protocols in TEM.

| Parameter                  | non-COX     | COX         |
|----------------------------|-------------|-------------|
| Area (μm²)                 | 0.192 ± 0.006 | 0.230 ± 0.007 |
| Perimeter (μm)             | 1.848 ± 0.040 | 1.981 ± 0.036 |
| Circularity                | 0.784 ± 0.008 | 0.789 ± 0.008 |
| Feret diameter (μm)        | 0.714 ± 0.016 | 0.751 ± 0.014 |
| Minimum feret (μm)         | 0.382 ± 0.007 | 0.442 ± 0.007 |
| Aspect ratio               | 1.739 ± 0.052 | 1.639 ± 0.046 |
| Form factor                | 1.276 ± 0.031 | 1.270 ± 0.025 |
| n                          | 667         | 582         |

Note: Data are presented as median ± SEM. Data were obtained from mouse soleus (mice n = 3 non-COX, n = 3 COX; Mitochondria n = 667, non-COX; n = 582, COX). A Mann–Whitney test was used for all comparisons.

activity. Furthermore, overfixation of a sample may hinder the penetration of solutions such as COX medium. We hypothesised that a short fixation (10 min at 4°C) microwave protocol (Fig. 1), could address previous penetration issues for the use of cytochrome c oxidase cytochemistry with EM.

We and other laboratories have used the conventional bench processing TEM protocol (Fig. 1). More recently, microwave technology has been used, to assist in getting comparable, and in some tissue, improved ultrastructural morphology, whilst decreasing the time to process. The speed of processing following COX incubation is likely to be particularly important if we use a short fixation protocol, since it decreases time taken to get to the secondary fixation steps to preserve morphology.

**Short fixation microwave processing does not impact mitochondrial morphology in transmission electron microscopy**

To test if the short fixation microwave processing (SFMP) protocol would compromise the mitochondrial morphology, we compared it to the overnight fixation and bench processing (Figs. 1 and 4). The mitochondria do not show any marked alteration in their characteristic ultrastructure, which was very well-preserved following both conditions. The outer membrane is well-defined, and the cristae are clearly visible in both samples. All the images were normalised based on the myofibrill grey scale for analysis with MIB software.

To determine the impact of SFMP on the morphology of intermyofibrillar mitochondria in transversely orientated muscle, mitochondria were analysed with Fiji software by manual tracing and standard shape descriptors were extracted. There were no significant differences observed in the distribution of mitochondrial shape descriptors (area, perimeter, circularity, Feret’s diameter, minimum Feret’s diameter, aspect ratio, form factor) between the conventional overnight fixation and bench processing protocol and the SFMP (Fig. 5, Table 3), based on a nonparametric Mann–Whitney test, with significance set at p < 0.05 (mice: conventional n = 3, SFMP n = 3; mitochondria: conventional n = 649, SFMP n = 667). These data show that processing with SFMP preserves the structure similar to the conventional overnight fixation and bench processing protocol (Figs. 4, and 5, Table 3). These results demonstrated preservation of cell structure with the microwave in addition to faster processing. Therefore, we next wanted to test if it improves COX labelling penetration for TEM.

**Demonstration of cytochrome c oxidase activity in transmission electron microscopy**

We next introduced a COX reaction to our protocols. We have previously found that a 1 h fixation at 4°C and bench processing yielded poor COX penetration and poor sample morphology (data not shown). The poor penetration is likely due to length of fixation, therefore we reduced the fixation time used to 10 min at 4 °C, as suggested by previous work on muscle (Hirai, 1971; Nonaka et al., 1989). The poor morphology was likely due to the sample only being lightly fixed and the time taken for bench processing. Therefore, in order to compare overnight fixation protocol and the short 10 min fixation protocol, we opted to use microwave processing to speed up the time taken for sample processing after the COX incubation.

Overnight fixation with the COX incubation results in inadequate penetration with the subsarcolemmal mitochondria exhibiting more electron dense deposits from the COX reaction than the intermyofibrillar mitochondria (Figs. 6A and C). In comparison, fixing for 10 min at 4 °C, resulted in homogenous labelling throughout the cell (Figs. 6B and D).

There are no general criteria for good fixation, but the ultrastructure of mitochondria is often considered to be a sensitive indicator of good or bad fixation. Figure 7 shows the electron microscopic pattern of mitochondria following COX cytochemistry. The electron dense deposits from the COX reaction can be observed in the mitochondrial membranes but especially on the cristae membrane (Figs. 7A and B). Higher magnification images demonstrate clear cristae morphology and electron dense deposits as indicated by arrows (Figs. 7B–F). In order to check that we were seeing detectable COX labelling, we also included several controls for comparison. Figure 8(A) is a conventional reference for the appearance of normal TEM images with lead citrate and uranyl acetate staining. Figure 8(B) shows a lack dark deposits in mitochondria when COX medium is replaced with Sorenson’s only. Most mitochondria contain dark deposits following treatment with COX medium (Fig. 8C). Mitochondria also lack dark deposits when azide, a specific inhibitor of COX activity, is added (10 mM) to the COX medium (Fig. 8D). These results indicate that formation of the dark deposits occurs specifically due to incubation with the COX medium and is dependent on COX enzyme activity. On-section staining with lead citrate and uranyl acetate is not completed.
Fig. 10. Intensity quantification measurement of 2D SBFSEM images of mouse muscle. SBFSEM images low and high magnification from muscle blocks prepared using short fixation and (A) heavy metal protocol and microwave-processing, (B) COX histochemistry, heavy metal protocol and microwave processing or (C) COX azide histochemistry 10mM incubation, heavy metal protocol and microwave processing (scale bar = 2 μm). The contrast background of A, B and C images were normalised and a threshold of 50 set in image J (A1, B1 and C1). Mean intensity of the mitochondria and the percentage of the image area that is mitochondrial were quantified (D). The COX activity in image B is clearly demonstrated compared to a non-COX and COX-azide.

for Figures 8(C), (D) to enhance the contrast between the dark deposits and the surrounding tissue.

Mitochondrial function is known to be heterogeneous in skeletal muscle fibres both intercellular and intracellularly (Vincent et al., 2018). Even in COX-EM on control mouse muscle we detected heterogeneity of mitochondrial COX labelling (arrow, Fig. 8C). This appears to be due to the heterogeneity of the mitochondrial population and not artefact due to solution penetration based on the pattern of labelling in the surrounding mitochondria.
Fig. 11. Quantitative analysis of mitochondrial morphology and volume in IMF mitochondria in mouse skeletal muscle from serial block face—scanning electron microscopy. IMF mitochondrial surface area (A) volume (B) and MCI (C) in mouse muscle non-COX (blue) and COX incubation (red). IMF mitochondrial volume (D) and MCI (E) shown as cumulative frequency distributions for non-COX (blue) and COX incubation (red). (F) Bivariate plot of volume and MCI for non-COX (blue) and COX incubations (red). Each point represents a single mitochondrion. A Mann—Whitney’s test was used for all comparisons (mice \( n = 3 \) non-COX, \( n = 3 \) COX; 3 fibres per mouse; mitochondria non-COX; \( n = 283 \) COX).

Selective staining does not impact mitochondrial morphology in transmission electron microscopy

After showing the positive activity with the COX incubation, we sought to determine whether the addition of the COX medium affects mitochondrial morphology. As above, intermyofibrillar mitochondria were manually traced and mitochondrial shape descriptors calculated. No significant differences appear in the shape descriptors (area, perimeter, circularity, aspect ratio, form factor) between the non-COX and COX conditions (Fig. 9, Table 4). Therefore, incubation at 37 °C for 2 h does not result in loss of morphological complexity.
Table 5. Effect of COX incubation on morphological parameters and shape descriptors of intermyofibrillar mitochondria in three-dimensions from SBFSEM.

|                    | non-COX       | COX            |
|--------------------|---------------|----------------|
| Surface (μm²)      | 4.013 ± 0.266 | 3.073 ± 0.204  |
| Volume (μm³)       | 0.223 ± 0.021 | 0.240 ± 0.024  |
| MCI                | 5.634 ± 0.321 | 5.333 ± 0.199  |
| n                  | 283           | 415            |

Note: Data are presented as median ± SEM. Data were obtained from mouse soleus (mouse non-COX n = 3, COX n = 3; 3 fibres per mice; mitochondria non-COX n = 283, COX n = 415). A Mann–Whitney test was used for all comparisons.

These results demonstrate that the mitochondrial network organisation can be studied in samples prepared for COX-TEM.

Short fixation microwave processing and COX-EM allows detection of COX reaction product in samples prepared for serial block face – scanning electron microscopy, and does not impact three-dimensional mitochondrial morphology

Whilst TEM can be used to study mitochondrial morphology, it is often affected by the orientation of the tissue and as such SBFSEM is considered superior for morphological quantification. Since we have established that COX labelling penetrates through the tissue samples using the SFMP, we next sought to adapt the COX-TEM protocol for SBFSEM and Figure 10 shows that the COX labelling can be easily detected when compared to non-COX and COX azide. The COX activity in Figure 10(B) is clearly demonstrated compared to non-COX (Fig. 10A) and COX-azide (Fig. 10C) images.

We further quantified COX activity using Image J and demonstrate that mean intensity of the total mitochondrial area is higher with COX labelling than non-COX or COX azide (Fig. 10D). COX azide mitochondria results in the lowest mean mitochondrial intensity, likely due to both inhibiting COX activity and to disrupting heavy metal labelling reacting with lead compared to the non-COX sample.

Three-dimensional reconstructions from a stack with 50 sections showed no visible difference between ‘non-COX’ and ‘COX’ samples (Fig. 11, Videos S1 and S2). Complex shaped mitochondria with branches > 1 μm in length could be observed in equal abundance with both non-COX and COX incubation. We then determined their dimensions with measurements performed in Amira Software from the 3D reconstructions (Fig. 11, Videos S1 and S2). We calculated Mitochondrial Complexity Index (MCI), a 3D equivalent to form factor which quantifies mitochondrial branching, as described previously (Vincent et al., 2019). Neither mitochondrial volume, surface area nor MCI values differed significantly between non-COX and COX incubations (Fig. 11, Table 5), demonstrating that COX incubation for 2 h does not result in loss of morphological complexity. These results demonstrate that the 3D mitochondrial network organisation can be studied in fixed skeletal muscle from samples that have been processed for COX-EM.

Discussion

We have successfully developed a COX-EM approach to assess mitochondrial morphology in two- and three-dimensions. This technique will give new insights into how mitochondrial function is linked to mitochondrial morphology and will be used to understand the mechanisms underpinning COX deficiency. The histological detection of COX activity has been used routinely since it was established by Seligman et al. (1968) but without any morphological analysis. Initial studies combining COX histochemistry with TEM were inconsistent with varying degrees of staining success (Seligman et al., 1968; Angermüller & Fahimi, 1981; Nonaka et al., 1989; Haginoya et al., 1990; Oguro et al., 1992). Possible explanations for the discrepancies likely include problems with tissue penetration, fixative solution, artefacts or overfixation (Anderson, 1970; Seligman et al., 1970; Rupec & Brühl, 1970; Ekes, 1971; Reith & Schuler, 1972; Divac et al., 1995).

Short fixation microwave processing: fixation

Here, we used a short fixation time of 10 min at lower temperature (4 °C) with 2% glutaraldehyde to have adequate preservation of both the mitochondrial morphology and the enzyme activity, and staining penetration. We have shown that the procedure preserves the tissue without any damage and is comparable to a conventional protocol with overnight fixation.

It is known that mitochondrial ultrastructural preservation is a common problem in cytochemical studies, since fixation reduces enzymatic function by hindering conformational changes. Furthermore, incubation conditions (Seligman et al., 1967) as well as fixation time, type of fixative (Sabatini et al., 1963) and fixative concentration will also influence the cytochemistry reaction (Sabatini et al., 1963; Seligman et al., 1967; Novikoff & Goldfischer, 1969; Nonaka et al., 1989; Saprunova et al., 2008). Indeed, Hirai and colleagues demonstrated that DAB is inactivated in overfixed tissues and enzyme activity, including COX and peroxidases, is impaired (Hirai, 1971; Roels, 1974; Herzog & Fahimi, 1976). They also showed a similar effect on enzymatic activity when increasing the time and temperature of the fixation (HiraI, 1971; LeHir et al., 1979). Short fixation time and cold temperature (Seligman et al., 1968; Nir & Seligman, 1971) is required for COX and other oxidative enzymes, since fixation with aldehydes at room temperature even for short times resulted in a substantial loss of enzyme activity (Yoo et al., 1974). Further, Wakabayashi and colleagues demonstrate that glutaraldehyde has a severe effect on COX enzyme activity even with a shortened fixation time (45 s) and
low concentration of glutaraldehyde (0.01%, 0.05%, 0.1% and 0.1%); however, they did use a higher temperature of fixation, 25 °C (Wakabayashi et al., 1975). These combined benefits of reducing fixation time likely explain the improvements in results observed here.

**Short fixation microwave processing: microwave processing**

In this study, the Pelco Biowave® Pro+ incorporating the Pelco Coldspot® Pro microwave was used in TEM processing for postfixation, washing, dehydration and embedding, and in SBFSEM for washing, dehydration and embedding. It is well established in the literature that using a microwave for processing and embedding in EM can shorten sample preparation and improve penetration of buffers as reviewed in several papers (Login & Dvorak, 1985; Login et al., 1986; Login et al., 1990). Thus, it is possible to process samples in under a day (Mayers, 1970; Giberson et al., 2003) without any loss of fine structure in tissue samples. This increase in processing speed is particularly important if a shorter fixation is required for cytochemical labelling, since it reduces the time that the sample is only lightly fixed and liable to structural changes. Therefore, the microwave processing used here has likely allowed us to reduce the initial fixation time and improve COX labelling without a noticeable change in sample morphology. A recent study evaluated the comparison between conventional processing techniques versus microwave processing and showed indistinguishable results or even superior in section and image quality (Gerrity & Forbes, 2003).

Microwaves induce heat inside materials by exciting molecules and rotating them (Kok & Boon, 1990; Login & Dvorak, 1994). This ‘thermal effect’ (Leonard & Shepardson, 1994; Giberson & Demaree, 1995) occurs uniformly and simultaneously in all the tissue subjected to the action of microwaves, unlike traditional heating, where the action of heat starts from the external parts (Hayat, 2002). The controlled heat increases the stabilisation, the diffusibility of these fluids and the action of the microwaves increases this effect further (Boon et al., 1986; Leong et al., 1988). As such using the microwave for washes after fixation may be beneficial by removing excess fixative solution from the tissue.

The advanced 3D-COXEM technique: morphology

At the present time, it is thus possible to study morphology and mitochondrial function in two dimensions. However, two dimensions provides limited information about mitochondrial morphology, which tends to be anisotropic in skeletal muscle fibres (Glancy et al., 2015). SBFSEM provides us with three-dimensional information and allows us to track the morphology of mitochondria through the complex architecture of the skeletal muscle in the z plane as well as the x and y planes (Vincent et al., 2019). Previous work has performed three-
Conclusion

Here we present the development of a three-dimensional EM assay that allows simultaneous detection of mitochondrial function. This technique allows the correlation of mitochondrial COX function and mitochondrial morphology at a subcellular level in three dimensions, therefore providing a future opportunity to solve questions around mitochondrial distribution, morphology and mitochondrial disease pathology.

Author contributions

JF, DMT, KW and AEV contributed to the conception and design of the study. JF, TSD and KW developed the protocol. JF performed all lab work and data analysis. JF wrote the first draft of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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### Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Video S1.** Serial block-face scanning electron microscopy images stack from mouse non-COX muscle fibre and three-dimensional reconstruction. Reconstruction of mitochondria from the Internyofibrillar region of soleus muscle fibre. Each mitochondrion is shown in a different colour. The mitochondrial network is shown in transverse orientation. Each image (serial section) is separated by 70 nm, and the total volume includes 50 images. Dimensions are $0.07 \times 0.07 \mu m (x/y)$ and $14 \mu m (z)$.

**Video S2.** Serial block-face scanning electron microscopy images stack from mouse COX muscle fibre and three-dimensional reconstruction. Reconstruction of mitochondria from the Internyofibrillar region of soleus muscle fibre. Each mitochondrion is shown in a different colour. The mitochondrial network is shown in transverse orientation. Each image (serial section) is separated by 70 nm, and the total volume includes 50 images. Dimensions are $0.07 \times 0.07 \mu m (x/y)$ and $14 \mu m$. 