Video Article

Immunodetection of Outer Membrane Proteins by Flow Cytometry of Isolated Mitochondria

Sarah Pickles[1], Nathalie Arbour[2], Christine Vande Velde[2]

[1]Department of Biochemistry, Université de Montréal, CRCHUM

[2]Department of Neurosciences, Université de Montréal, CRCHUM

Correspondence to: Christine Vande Velde at c.vande.velde@umontreal.ca

URL: http://www.jove.com/video/51887
DOI: doi:10.3791/51887

Keywords: Cellular Biology, Issue 91, Mitochondria, flow cytometry, organelle isolation, immunolabeling, spinal cord, TMRM

Date Published: 9/18/2014

Citation: Pickles, S., Arbour, N., Vande Velde, C. Immunodetection of Outer Membrane Proteins by Flow Cytometry of Isolated Mitochondria. *J. Vis. Exp.* (91), e51887, doi:10.3791/51887 (2014).

Abstract

Methods to detect and monitor mitochondrial outer membrane protein components in animal tissues are vital to study mitochondrial physiology and pathophysiology. This protocol describes a technique where mitochondria isolated from rodent tissue are immunolabeled and analyzed by flow cytometry. Mitochondria are isolated from rodent spinal cords and subjected to a rapid enrichment step so as to remove myelin, a major contaminant of mitochondrial fractions prepared from nervous tissue. Isolated mitochondria are then labeled with an antibody of choice and a fluorescently conjugated secondary antibody. Analysis by flow cytometry verifies the relative purity of mitochondrial preparations by staining with a mitochondrial specific dye, followed by detection and quantification of immunolabeled protein. This technique is rapid, quantifiable and high-throughput, allowing for the analysis of hundreds of thousands of mitochondria per sample. It is applicable to assess novel proteins at the mitochondrial surface under normal physiological conditions as well as the proteins that may become mislocalized to this organelle during pathology. Importantly, this method can be coupled to fluorescent indicator dyes to report on certain activities of mitochondrial subpopulations and is feasible for mitochondria from the central nervous system (brain and spinal cord) as well as liver.

Video Link

The video component of this article can be found at http://www.jove.com/video/51887/

Introduction

Mitochondria are highly dynamic organelles that undergo multiple rounds of fission and fusion, are transported to sites of high energy demand and respond rapidly to physiological stimuli[1]. Since it is increasingly recognized that mitochondria within different tissues, even different cellular compartments, have distinct functional profiles, new methods are needed to identify these distinct mitochondrial subsets.

Microscopy provides a means whereby individual mitochondria can be visualized and the presence of a protein at or in mitochondria can be determined by immunofluorescence[2]. However, quantitative analysis by this method is labor intensive and is more suitable for experiments using immortalized or primary cell lines. The study of individual mitochondria derived from tissue is significantly more difficult and most methods do not allow for easy identification of mitochondrial subsets concurrently with the evaluation of mitochondrial function[3].

In order to address this hurdle, a novel method to immunolabel mitochondria isolated from rodent tissues and subsequently analyzed by flow cytometry has been developed. This allows for the rapid detection and quantification of proteins localized to the mitochondrial outer membrane, which compared to analysis by microscopy, is much less labor intensive and permits the analysis of thousands of mitochondria in a single sample. This assay can be applied to monitor the fate and relative amount of mitochondrial outer membrane proteins that are thought to be constitutively present at the mitochondria, the recruitment of proteins to the mitochondrial surface, or the detection of proteins mislocalized to the mitochondria in pathological conditions. Moreover, the incorporation of conventional fluorescent indicator dyes permits the simultaneous evaluation of certain aspects of mitochondrial function in distinct mitochondrial subpopulations.

Protocol

Animals used in this study were treated in strict accordance to a protocol (N08001CVsr) approved by the Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM) Institutional Committee for the Protection of Animals which follows national standards as outlined by the Canadian Council on Animal Care (CCAC).

Prepare all reagents required to perform this protocol (**Table 1**). All other details regarding equipment, supplies and suppliers can be found in List of Materials.

Copyright © 2014 Journal of Visualized Experiments

Buffer	Composition	Step used	Comments
Homogenization Buffer (HB)	210 mM Mannitol	2.1-2.8	Protease inhibitors can be added
	70 mM Sucrose		
	10 mM Tris pH 7.5		
	1 mM EDTA		
HB+ KCL	HB, as above with the addition of 50 mM KCl	2.3	Protease inhibitors can be added
M-Buffer	220 mM Sucrose	3.3-3.9	Protease inhibitors can be added. This buffer is supplemented with the complex II substrate succinate and complex I inhibitor rotenone so as to prevent reverse electron flow.
	68 mM Mannitol		
	10 mM KCl		
	5 mM KH_2PO_4		
	2 mM $MgCl_2$		
	500 µM EGTA		
	5 mM Succinate		
	5 µM Rotenone		
	10 mM HEPES pH 7.2		
	0.1 % fatty-acid free Bovine Serum Albumin		

Table 1. Buffer Compositions.

1. Collection of Rat Spinal Cord

1. Deeply anaesthetize the rat (Sprague Dawley) with 4% isoflorane. Verify anaesthetization by a lack of reflex upon pinching of the forepaw. Euthanize the rat by decapitation via guillotine. This method of euthanasia is preferred over others, which might distort the spinal cord.
2. Cut the skin of the back to expose the spine. Cut the spinal column with bone scissors just above the pelvic bone. Visualize the opening of the vertebral column.
3. Insert a 10 ml syringe, with a 200 µl pipette tip (attached via melting slightly over flame), filled with Phosphate Buffered Saline (PBS), into the vertebral column.
4. Flush out the spinal cord by applying a medium amount of pressure to the plunger.
5. If any blood is present on the spinal cord, rinse with PBS before proceeding to the next step.
   NOTE: This method has also been validated for brain and liver. If including these tissues, collect half the brain from the euthanized rat and a piece of liver equal in weight to the spinal cord. All other steps remain identical.

2. Isolation of Spinal Cord Mitochondria (Adapted from Vande Velde *et al.* [4])

1. Collect the whole intact spinal cord and place in 5 ml glass homogenizer with 5 volumes (~3.25 ml) Homogenization Buffer (HB). For optimal recovery of isolated mitochondria, perform all steps on ice or in cold room. Homogenize tissue by hand until no large pieces of tissue remain, approximately eight strokes. Place homogenate in two (2 ml) or three (1.7 ml) microcentrifuge tubes. Centrifuge 1,300 x g for 10 min at 4 °C in a benchtop microcentrifuge.
2. Recover supernatant and place in a 5 ml ultracentrifuge tube. Add 750 µl (~0.5 volumes) HB to the pellet-containing microcentrifuge tube and gently resuspend the pellet. Repeat centrifugation and resuspension steps two more times. Pool all supernatants (S1a and S1b) into the same 5 ml ultracentrifuge tube above. This step serves to remove small debris.
3. Centrifuge pooled S1 using an ultracentrifuge equipped with a swinging bucket rotor and centrifuge at 17,000 x g for 15 min at 4 °C.
4. Keep supernatant (S2) for further processing if the cytosolic fraction is of interest (for example for Western blot analysis). Resuspend the pellet (P2), the crude mitochondrial fraction, in 4 ml HB+50 mM KCl. Centrifuge 17,000 x g for 15 min at 4 °C in a swinging bucket rotor. Discard the supernatant and gently resuspend the pellet (P3) in 800 µl HB.
   NOTE: Mitochondria are washed with HB+50 mM KCl to remove any non-specific mitochondrially-associated contaminants.
5. In a new 5 ml ultracentrifuge tube, add exactly 800 µl of resuspended pellet (P3).
6. To this tube add, 200 µl of Iodixanol (density gradient medium), thereby creating a final concentration of 12% Iodixanol. Mix the contents of the tube gently, but thoroughly via pipetting with a P1000. Addition of Iodixanol first, and then resuspended pellet (P3) may be preferable to facilitate thorough mixing. Centrifuge in an ultracentrifuge equipped with a swinging bucket rotor at 17,000 x g for 15 min at 4 °C.
7. NOTE: Liver does not contain myelin and therefore this step is not necessary if only the liver is being processed. However, if liver is being processed concurrently with CNS mitochondria, it is recommended to treat all tissues equally.
8. Aspirate the layer of myelin at the top of the tube and carefully remove and discard the supernatant. Pellet may be loose. Resuspend the pellet in 4 ml HB. Centrifuge again at 17,000 x g for 10 min at 4 °C. Discard the supernatant and resuspend the pellet in 4 ml HB. Repeat the centrifugation and remove the supernatant.
9. Resuspend the final pellet (P7) in 100-200 µl HB and transfer to a 1.7 ml microcentrifuge tube. This sample contains isolated mitochondria.
10. Proceed to protein quantification. Dilute samples and the standard curve in 2% sodium dodecyl sulfate (SDS) to ensure adequate solubilization of mitochondria during protein quantification.

3. Immunolabeling of Isolated Mitochondria for Flow Cytometry

1. For each staining mix to be tested, pipette 25 µg of isolated mitochondria into a 1.7 ml microcentrifuge tube. Include an unstained sample in each experiment; and for each antibody, be sure to include a sample for the appropriate isotype control.
2. Centrifuge at 17,000 x g for 2 min at 4 °C in a bench-top microcentrifuge.
3. Remove supernatant and resuspend isolated mitochondria in 50 µl Mitochondria Buffer (M Buffer) supplemented with 10% fatty-acid free BSA for 15 min at 4 °C (blocking step).
NOTE: During labeling, perform incubations in a refrigerator at 4 °C.
4. Add primary antibody (rabbit anti-Mfn2, 20 µg per ml) to tube and incubate for 30 min at 4 °C.
NOTE: Determine optimal concentration of each antibody empirically by titration. Due to variability in concentration and/or purity, different lots of the same antibody from the same manufacturer may lead to different results; therefore titration is needed for each new lot of antibody.
5. Wash out unbound primary antibody: Centrifuge at 17,000 x g for 2 min at 4 °C. Remove the supernatant and gently resuspend the pellet in 200 µl M Buffer. Centrifuge at 17,000 x g for 2 min at 4 °C. Remove supernatant and resuspend the pellet in 50 µl M Buffer.
6. Add secondary antibody (Donkey anti-rabbit IgG Phycoerythrin (PE), 0.5 µg per ml) to tube and incubate samples for 30 min at 4 °C, protected from light.
7. Wash out unbound secondary antibody: Centrifuge at 17,000 x g for 2 min at 4 °C. Remove the supernatant and resuspend the pellet in 200 µl M Buffer. Centrifuge at 17,000 x g for 2 min at 4 °C. Remove supernatant and resuspend the pellet in 500 µl M Buffer.
8. To ensure events are in fact mitochondria, stain isolated mitochondria with a mitochondria specific fluorescent dye for 15 min at RT, protected from light. If staining of other functional parameters (mitochondrial transmembrane potential or superoxide production) is desired, proceed to step 4. If not, proceed to step 5 for acquisition.
NOTE: It is important to verify that the emission spectrum of the secondary antibody is compatible with that of the functional dyes. For example, if verifying mitochondrial purity with a commercial dye with spectral properties similar to FITC and transmembrane potential with Tetramethylrhodamine methyl ester (TMRM), a viable secondary antibody would be allophycocyanin (APC: Ex 650 nm/Em 660 nm). Add compensations controls, *i.e.,* a sample immunolabeled or stained with a single fluorophore, when applicable.
9. Transfer to a tube suitable for loading flow cytometer. (To facilitate the small sample size, a microtiter tube is placed inside 3 ml flow cytometer tube.) Keep samples on ice and proceed immediately to flow cytometer for acquisition.

4. Assaying Mitochondrial Transmembrane Potential and Mitochondrial Superoxide Production by Flow Cytometry

1. Verify that isolated mitochondria have an intact transmembrane potential by staining with 100 nM TMRM (Ex 548 nm/Em 574 nm)[5], at step 3.8, for 15 min at RT, protected from light. For comparison of transmembrane potential between samples and populations, use of lower/non-quenching concentrations of TMRM (1 to 30 nM), may be more appropriate[6].
    1. As a control for TMRM staining, stain isolated mitochondria with 100 nM TMRM in the presence of 100 µM carbonyl cyanide m-chloro phenyl hydrazine (CCCP), a mitochondrial uncoupler that will depolarize mitochondria. The concentration of CCCP required to depolarize the mitochondria may be less if lower concentrations of dye are used.
2. Verify that isolated mitochondria produce mitochondrial superoxide by staining with an appropriate mitochondrial superoxide indicator[7], also at step 3.8, for 15 min at RT, protected from light.
    1. As a control for mitochondrial superoxide production, stain isolated mitochondria with dye in the presence of 10 µM Antimycin A, an inhibitor of complex III of the respiratory chain that will augment mitochondrial superoxide production.

5. Acquisition and Analysis of Immunolabeled Isolated Mitochondria by Flow Cytometry

1. Instrument set up: Switch voltages from linear to log mode to facilitate analysis of isolated mitochondria and set voltages (FSC: 450; SSC: 250). Ensure that events are collected in FSC-A (area) mode as well as FSC-W (width) and FSC-H (height), to be able to exclude doublets (*i.e.,* two events, passing through the detector at the same time) in analysis post-data collection. Set the number of events to be collected to 100,000. Acquire compensation controls, if applicable.
2. Data acquisition: Before data acquisition, avoid vortexing samples. Instead mix by gently tapping tube. Initially collect events at a low pressure, during gating. Gate on total population. Adjust voltages of histograms accordingly, usually the peak of the unstained sample will correspond to the second decade (10^2). Once gates are established, and samples are being processed, the pressure can be switched to high.
3. Analysis: Visualize doublets by plotting FSC-W versus FSC. Identify singlets and doublets. Gate on singlets. Select the mitochondrial population by gating on events that are stained positively with a mitochondrial-selective dye.
4. Determine background labeling from isotype control. Using the isotype control sample, determine the percentage of the mitochondrial population labeling positive for Mfn2 antibody.

Representative Results

Mitochondria derived from rat spinal cords can be immunolabeled with an antibody targeted to Mitofusin2 (Mfn2), a protein implicated in the fusion of the outer membrane of mitochondria[8]. Following isolation and labeling with a Mfn2 specific antibody and a fluorescently conjugated secondary antibody, mitochondria are processed by flow cytometry (**Figure 1**). Following data acquisition, samples are analyzed using flow cytometry analysis software, by first visualizing all collected events on a dot plot (**Figure 2A**). Doublets and singlets are differentiated when the events are plotted in FSC, width (W) versus area (A) (**Figure 2B**). Once singlets are selected, gate the mitochondrial population via FSC/SSC (**Figure 2C**), and verify the number of events staining positive for mitochondria-specific dye by co-plotting a histogram of the unstained sample with a sample stained with the mitochondria-specific dye. To determine the mitochondrial events, place a gate at the intersection of the two peaks

(**Figure 2D**). For spinal cord preparations, typically >90% of the events are positive for the mitochondria-specific dye. For other tissues such as liver, ~98% of the events will label with the dye (data not shown). After selecting only events that label positively for the mitochondria-specific dye, determine background labeling with the isotype control by selecting a gate ($Mfn2^+$) that includes 1% or less isotype labeling (**Figure 2E**, *left*). Apply this gate uniformly to all samples labeled with antibody Mfn2 to determine the percentage of mitochondria with Mfn2 present on the outer mitochondrial membrane (**Figure 2E**, *right*). In this experiment, 30% of mitochondria derived from spinal cord label positive for Mfn2. It is important to note here, that although Mfn2 is considered to be a ubiquitously expressed mitochondrial outer membrane protein, it has not been previously quantified. Moreover, an immunocytochemical analysis of Mfn2 in cultured cells shows a non-homogenous labeling of individual mitochondria[9]. It is also possible that the Mfn2 epitope was unavailable due to post translational modifications or due to interactions with itself or other binding partners. Of note, the antibody in this study was generated using a synthetic peptide to the N-terminus (amino acids 38-55). There is a predicted splice variant of Mfn2 lacking the first 302 amino acids, although this variant has yet to be confirmed experimentally (UniProt database). Thus, this assay is unable to detect alternatively spliced Mfn2 lacking the N-terminal sequence, given the antibody used.

Mitochondrial transmembrane potential ($\Delta\Psi_m$) and superoxide production can be assessed in this assay. Across the inner mitochondrial membrane, there is a separation of charge which drives ATP production via oxidative phosphorylation. TMRM is a cationic dye that accumulates within the mitochondria in a membrane potential dependent manner[5], and therefore can be used as a reporter of mitochondrial transmembrane potential. The majority of mitochondria (95%) are TMRM positive after staining, compared to the unstained control (**Figure 3A**). However, when the uncoupler CCCP is added there is a significant decrease in the number of mitochondria able to retain TMRM (**Figure 3A**). CCCP allows the free passage of ions across the inner mitochondrial membrane, essentially destroying the separation of charge and depolarizing the membrane.

Mitochondria release superoxide as a normal byproduct of oxidative phosphorylation from complex I and III of the electron transport chain. Mitochondrial superoxide can be measured via a membrane permeable dye that is targeted to mitochondria and becomes fluorescent following a reaction with superoxide[7]. Functional mitochondria produce a basal amount of superoxide compared to unstained samples (**Figure 3B**). Addition of the complex III inhibitor Antimycin A yields an increase in superoxide, as seen by a rightward shift in florescence and a higher number of mitochondria (typically ~10%) that are fluorescent with this mitochondrial superoxide indicator dye (**Figure 3B**).

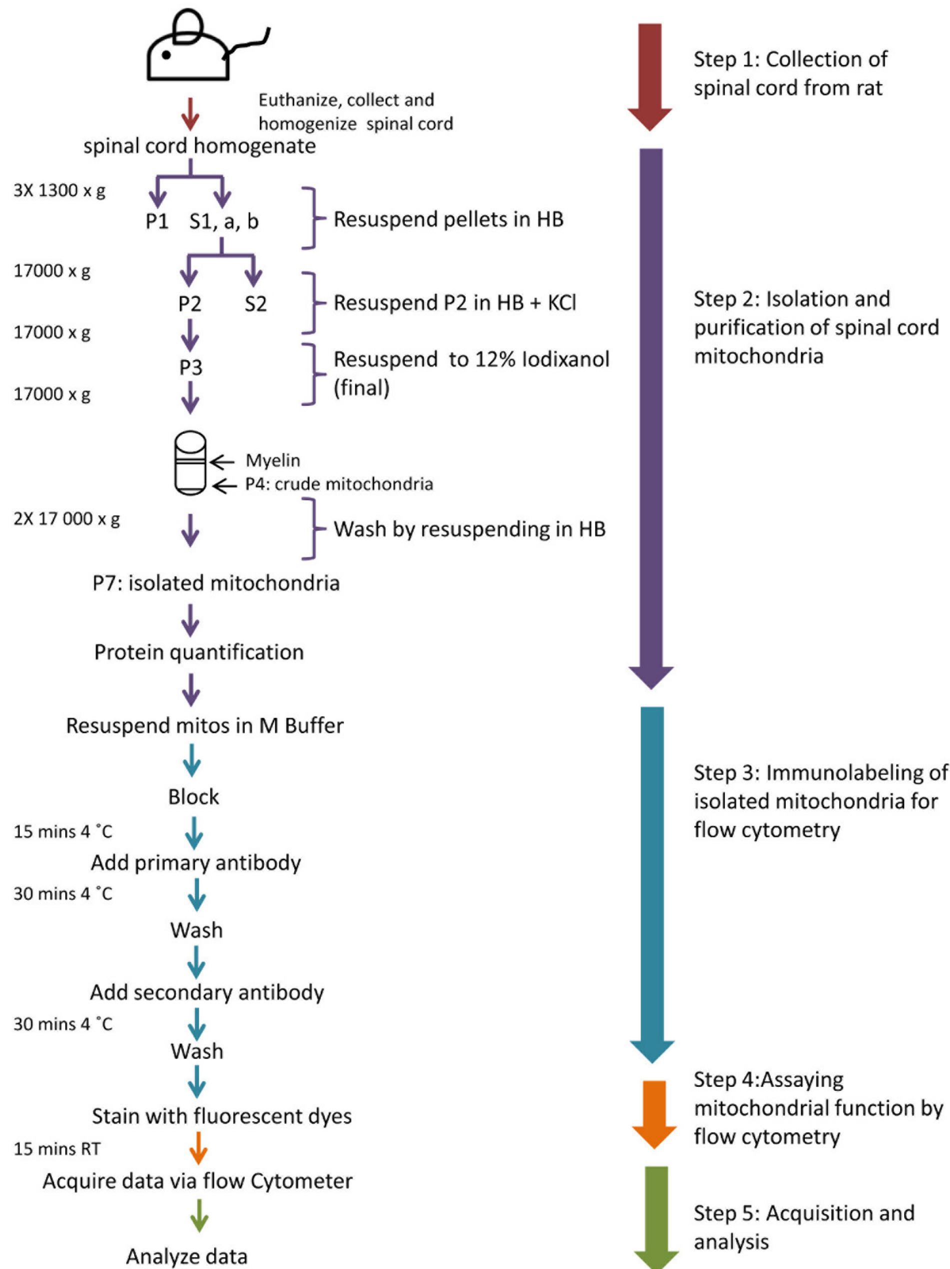

Figure 1. Schematic of isolation, immunolabeling and analysis of mitochondria. Step 1: Collect tissue and homogenize. **Step 2:** The isolation procedure contains eight centrifugation steps. Myelin, a major containment of central nervous system tissue is removed by diluting mitochondria in Iodixanol (density gradient medium) and centrifuging, resulting in the myelin floating to the top of the tube, while the mitochondria are pelleted. **Step 3**: Following isolation and quantification, mitochondria are blocked, labeled with primary antibody and washed. A secondary antibody conjugated to a fluorophore is then added. Unbound antibody is washed out. **Step 4**: At this point fluorescent dyes that report on mitochondrial purity, or mitochondrial function can be added. **Step 5**: Mitochondria are now ready to be analyzed by flow cytometry.

Copyright © 2014 Journal of Visualized Experiments

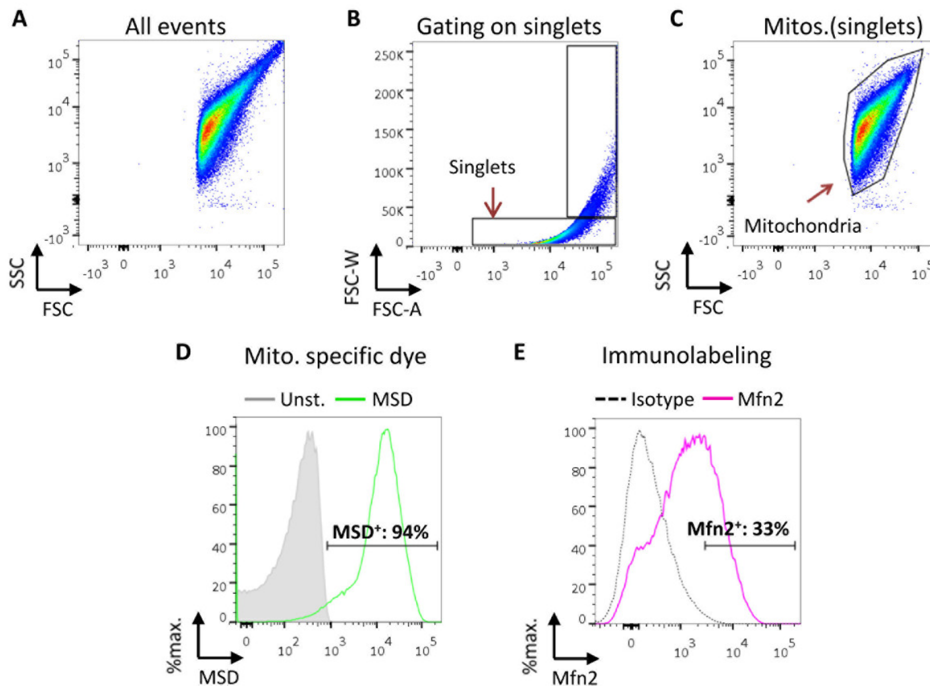

Figure 2. Strategy for the analysis of isolated mitochondria by flow cytometry. The Forward Scatter (FSC) and Side Scatter (SSC) voltages must be adjusted for small events, using both parameters in logarithmic mode. FSC width (FSC-W) data must be collected to exclude doublets. Note, on most flow cytometers, the default setting for FSC and SSC is linear mode. **(A)** Visualize all collected events on a dot plot. **(B)** Doublets, two mitochondria passing by the laser at the same time, can be distinguished from singlets by plotting FSC versus FSC-W (linear mode). Events are excluded if the FSC-W value is more than twice the mean FSC-W value of the majority of events, *i.e.,* those that are part of the dense cloud. Events under this threshold are gated as singlets. **(C)** Again, visualize the events in a dot plot, and gate on the remaining events. **(D)** Plot a histogram of the unstained sample (solid, grey, filled) and sample stained with a mitochondrial specific dye (MSD: solid, green). Gate the events staining positive for the MSD (MSD^+). **(E)** Histogram of the isotype control, rabbit IgG (dashed, black) and Mfn2 labeled sample (solid, pink). Set the gate so as to yield ≤ 1% $Mfn2^+$ on the isotype control peak and apply this same gate to experimental sample to determine the percentage of events labeling positive for Mfn2 ($Mfn2^+$). Please click here to view a larger version of this figure.

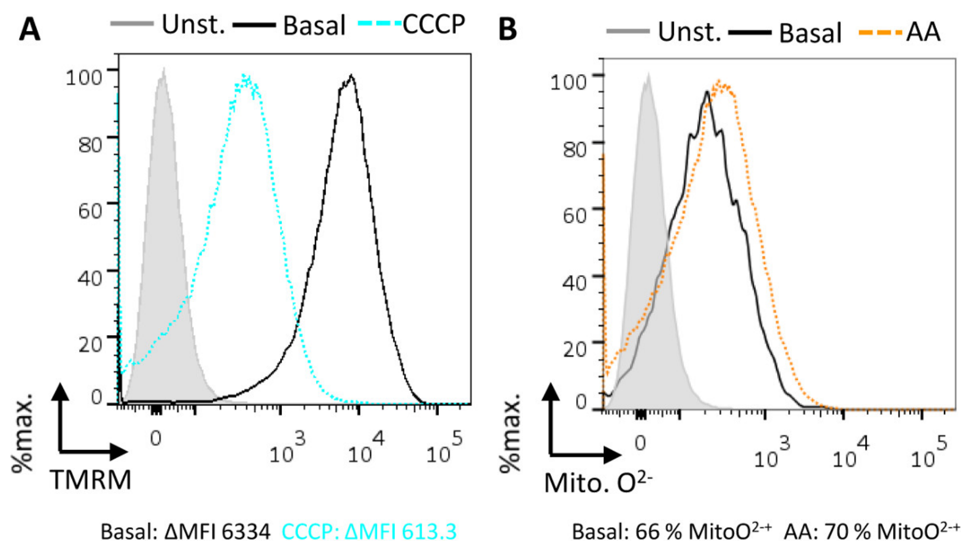

Figure 3. Assaying mitochondrial transmembrane potential ($\Delta\Psi_m$) and superoxide production in isolated mitochondria by flow cytometry. (A) Under basal conditions all active mitochondria will label with TMRM (solid, black), compared to unstained control (solid, grey, filled) because the dye accumulates in mitochondria with a transmembrane potential. Addition of the protonophore CCCP (dashed, blue) dissipates the transmembrane potential causing the mitochondria to depolarize and retain less dye compared to basal conditions. Data is reported as the delta mean fluorescent intensity (ΔMFI), which is the mean fluorescent intensity of the unstained control subtracted from the mean fluorescent intensity of the sample. **(B)** Under basal conditions, mitochondria produce superoxide as a by-product of oxidative phosphorylation. This mitochondrial source of superoxide can be assayed with a mitochondrial superoxide indicator, ($MitoO^{2-}$: solid, black) compared to unstained control (solid, grey, filled). Addition of the complex III inhibitor, Antimycin A (dashed, orange) results in increased superoxide production, compared with basal levels. Data is reported as percent of cells staining positive for the $MitoO^{2-}$ indicator. Please click here to view a larger version of this figure.

Discussion

It is increasingly evident that mitochondria are key players in both normal physiology and disease. While immunoblotting can determine which proteins are found within mitochondria or at the mitochondrial surface in a certain condition, this method reports on the average of the entire population. This method cannot yield information about relative abundances of mitochondrial subpopulations or subsets. While it has been previously assumed that all mitochondria are created equally, the field is increasingly recognizing that mitochondria within a cell have extensive variability in terms of morphology and/or function[10].

Fluorescent microscopy approaches do take into account the heterogeneity of mitochondria. However, quantification of this type of data is labor intensive. Furthermore, this approach is better suited to studies using cultured cells, as labeling of mitochondria *in vivo/in situ* is difficult due to the excessive number of mitochondria present, making differentiation of individual organelles inherently difficult. In most immunocytochemistry protocols, it is also not possible to simultaneously label outer mitochondrial membrane proteins and assess mitochondrial function due to the cellular permeabilization step required for antibody labeling. The current method works with isolated mitochondria, and thus does not require a permeabilization step. In addition, the visualization of individual mitochondria is only possible via electron microscopy, which is not amenable to the analysis of mitochondrial function. That being said, we recognize that isolation of mitochondria from tissue leads to disruption of the mitochondrial network and this could affect some elements of mitochondrial function. However, comparisons of functional aspects of isolated mitochondria from tissues that are processed similarly remain valid.

This method of immunolabeling of tissue-derived isolated mitochondria and subsequent analysis by flow cytometry allows for a rapid and quantifiable method to detect and monitor the presence of a protein located on the outer membrane. Detection of a highly abundant mitochondrial protein (like Mfn2) is possible with this technique. Similarly, this method can detect low abundance proteins that are only deposited on the mitochondrial membrane in disease, like misfolded SOD1 in the context of Amyotrophic Lateral Sclerosis (ALS)[11]. In addition, this method could be useful to monitor the proteins that transiently associate with the mitochondrial surface as part of their normal function. Examples include Dynamin-Related protein-1 (Drp1), a cytosolic protein that is recruited to the mitochondria to promote mitochondrial fission[12] and tumor necrosis factor receptor-associated factor 6 (TRAF6), which translocates to mitochondria to augment mitochondrial reactive oxygen species as a part of an innate immune response[13].

At present this technique is amenable only to proteins located at the mitochondrial surface, as standard permeabilization protocols for intracellular labeling require a detergent that disrupts the structural integrity of mitochondria. While a number of possible reagents have been tried (unpublished), further optimization of the immunolabeling protocol is still needed to make this protocol amenable to detecting intra-mitochondrial components.

This technique has broad applications and can be used to detect the presence or recruitment of one or more proteins to the mitochondria under different experimental paradigms. Furthermore, mitochondria can be co-labeled with two different antibodies, as well as with fluorescent indicators[11]. Other fluorescent probes could also be incorporated to characterize additional aspects of mitochondrial function. For example, commercially available dyes to monitor mitochondrial pH[14], calcium uptake with Calcium green-5N[15], and ATP levels[16] are possible.

Copyright © 2014 Journal of Visualized Experiments

Disclosures

The authors have nothing to disclose.

Acknowledgements

We thank Laurie Destroismaisons and Sarah Peyrard for outstanding technical support and Dr.Alexandre Prat for access to the flow cytometer. We would also like to acknowledge Dr. Timothy Miller for his contribution regarding the removal of myelin from the preparations. This work was supported by the Canadian Institutes of Health Research (CIHR) Neuromuscular Research Partnership, Canadian Foundation for Innovation, ALS Society of Canada, the Frick Foundation for ALS Research, CHUM Foundation and Fonds de la Recherche en Santé du Québec (C.V.V.). Both C.V.V. and N.A. are Research Scholars of the Fonds de la Recherche en Santé du Québec and CIHR New Investigators. S.P. is supported by the Tim Noël Studentship from the ALS Society of Canada.

References

1. Itoh, K., Nakamura, K., Iijima, M, Sesaki, H. Mitochondrial dynamics in neurodegeneration. *Trends Cell Biol.* **23**, 64-71. doi: 10.1016/j.tcb.2012.1010.1006. Epub 2012 Nov 1016. (2013).
2. Narendra, D., Tanaka, A., Suen, D.F., Youle, R.J. Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. *J Cell Biol.* **183**, 795-803. doi: 710.1083/jcb.200809125. Epub 200802008 Nov 200809124. (2008).
3. Zorov, D.B., Kobrinsky, E., Juhaszova, M., Sollott, S.J. Examining intracellular organelle function using fluorescent probes: from animalcules to quantum dots. *Circ Res.* **95**, 239-252. (2004).
4. Vande Velde, C., Miller, T.M., Cashman, N.R., Cleveland, D.W. Selective association of misfolded ALS-linked mutant SOD1 with the cytoplasmic face of mitochondria. *Proc Natl Acad Sci U S A.* **105**, 4022-4027. doi: 4010.1073/pnas.0712209105. Epub 0712202008 Feb 0712209122. (2008).
5. Loew, L.M., Tuft, R.A., Carrington, W., Fay, F.S. Imaging in five dimensions: time-dependent membrane potentials in individual mitochondria. *Biophys J.* **65**, 2396-2407. (1993).
6. Perry, S.W., Norman, J.P., Barbieri, J., Brown, E.B., & Gelbard, H.A. Mitochondrial membrane potential probes and the proton gradient: a practical usage guide. *Biotechniques.* **50**, 98-115. doi: 110.2144/000113610. (2011).
7. Xu, X., Arriaga, E. A. Qualitative determination of superoxide release at both sides of the mitochondrial inner membrane by capillary electrophoretic analysis of the oxidation products of triphenylphosphonium hydroethidine. *Free Radic Biol Med.* **46**, 905-913. doi: 910.1016/j.freeradbiomed.2008.1012.1019. Epub 2009 Jan 1017. (2009).
8. Otera H, Ishihara, N., Mihara, K. New insights into the function and regulation of mitochondrial fission. *Biochim Biophys Acta.* **1833**, 1256-1268. doi: 1210.1016/j.bbamcr.2013.1202.1002. Epub 2013 Feb 1220. (2013).
9. de Brito, O. M., Scorrano, L. Mitofusin 2 tethers endoplasmic reticulum to mitochondria. *Nature.* **456**, 605-610. doi: 610.1038/nature07534. (2008).
10. Wikstrom, J.D., Twig, G., Shirihai, O.S. What can mitochondrial heterogeneity tell us about mitochondrial dynamics and autophagy? *Int J Biochem Cell Biol.* **41**, 1914-1927. doi: 1910.1016/j.biocel.2009.1906.1006. Epub 2009 Jun 1921. (2009).
11. Pickles, S., Destroismaisons, L., Peyrard, S.L., Cadot, S., Rouleau, G.A., Brown, R.H. Jr., Julien, J.P., Arbour, N., Vande Velde, C. Mitochondrial damage revealed by immunoselection for ALS-linked misfolded SOD1. *Hum Mol Genet.* **22**, 3947-3959. doi: 3910.1093/hmg/ddt3249. Epub 2013 Jun 3944. (2013).
12. Frank, S., Gaume, B., Bergmann-Leitner, E.S., Leitner, W.W., Robert, E.G., Catez, F., Smith, C.L., Youle, R.J. The role of dynamin-related protein 1, a mediator of mitochondrial fission, in apoptosis. *Dev Cell.* **1**, 515-525. (2001).
13. West, A.P., Brodsky, I.E., Rahner, C., Woo, D.K., Erdjument-Bromage, H., Tempst, P., Walsh, M.C., Choi, Y., Shadel, G.S., Ghosh, S. TLR signalling augments macrophage bactericidal activity through mitochondrial ROS. *Nature.* **472**, 476-480. doi: 410.1038/nature09973. (2011).
14. Abad, M.F., Di Benedetto, G., Magalhães, P.J., Filippin, L., Pozzan, T. Mitochondrial pH monitored by a new engineered green fluorescent protein mutant. *J Biol Chem.* **279**, 11521-11529. Epub 12003 Dec 11530. (2004).
15. Murphy, A.N., Bredesen, D., Cortopassi, G., Wang, E., Fiskum, G. Bcl-2 potentiates the maximal calcium uptake capacity of neural cell mitochondria. *Proc Natl Acad Sci U S A.* **93**, 9893-9898. (1996).
16. Tantama, M., Martinez-François, J.R., Mongeon, R., Yellen, G. Imaging energy status in live cells with a fluorescent biosensor of the intracellular ATP-to-ADP ratio. *Nat Commun.* **4:2550.**, 10.1038/ncomms3550. (2013).

Copyright © 2014 Journal of Visualized Experiments