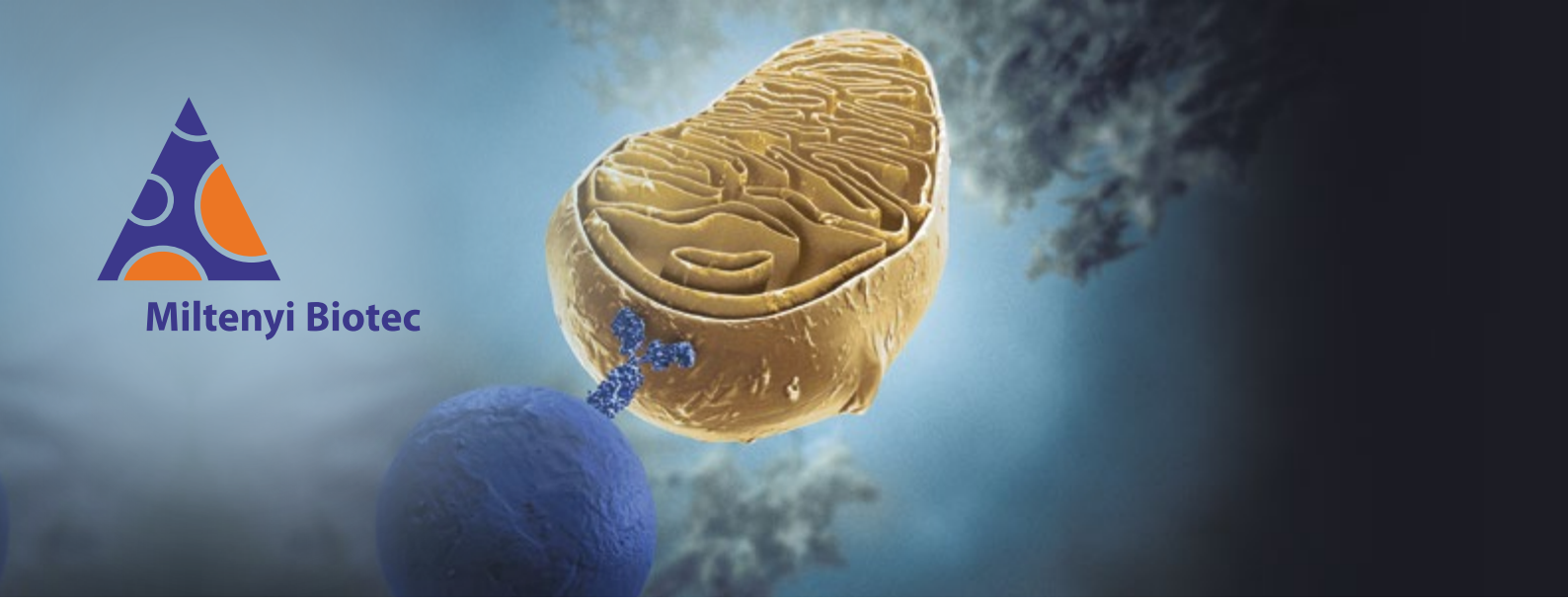


Synaptic mitochondria isolation

Novel use of MACS® Technology to isolate, purify, and separate brain-derived non-synaptic and synaptic mitochondria

W. Brad Hubbard [1,2,3,4], Christopher L. Harwood [1,2], Paresh Prajapati[1,2], Joe E. Springer[1,2], Kathryn E. Saatman[1,3], and Patrick G. Sullivan[1,2,4]

[1]Spinal Cord and Brain Injury Research Center, University of Kentucky, Lexington, KY, USA.
[2]Dept. of Neuroscience, University of Kentucky, Lexington, USA.
[3]Dept. of Physiology, University of Kentucky, Lexington, USA.
[4]Lexington VA Healthcare System, Lexington, KY, USA.

Introduction

Mitochondria maintain essential cellular functions by providing a majority of cells' energy in the form of adenosine triphosphate (ATP), by regulating calcium homeostasis, and by playing a role in cell signaling and apoptotic pathways. Mitochondrial function is particularly important in the brain where the central nervous system (CNS) consumes around 20% of the body's oxygen[1]. Specifically, mitochondria located in the neural synapse (synaptic mitochondria) may undergo the highest bioenergetic demand in the brain, supplying energy needed during neurotransmission[2].
Other mitochondria in the brain (non-synaptic) can be derived from multiple cell types or locations, such as astrocytes, microglia, endothelial cells, and neuronal soma.

Mitochondrial dysfunction is well established in neurodegenerative disease[3,4]. Because synaptic mitochondria are more vulnerable than non-synaptic mitochondria to calcium overload, aging, and neurotrauma[5–7], functional studies of neurodegenerative disease may necessitate isolating synaptic mitochondria. The isolation of synaptic mitochondria is possible due to the formation of synaptoneurosomes, which are the result of the attachment of presynaptic compartments (synaptosomes) to postsynaptic elements (neurosomes) during brain tissue homogenization. Although ultracentrifugation (UC) with sucrose density gradients can be used to separate non-synaptic and synaptic mitochondria[5,8,9], the method requires access to an expensive ultracentrifuge, necessitates a high level of technical proficiency, can be time consuming, and has limitations in mitochondrial output. Thus, there is a need for a mitochondrial isolation technique, such as affinity purification, that can preserve mitochondria yields and mitochondrial heterogeneity when using lower amounts of brain tissue.

The use of magnetically labeled antibodies for mitochondrial extraction has been shown to yield intact functional mitochondria from cell culture and various tissues[10–13], but a proto-col for isolating brain synaptic and non-synaptic mitochondria using this method has yet to be refined. In this application note, we detail how to apply magnetic immunolabeling using MACS Technology to target the TOM22 protein on the outer membrane of the mitochondria for synaptic and non-synaptic mitochondrial isolation. We call this technique fractionated mitochondrial magnetic separation (FMMS). This novel, optimized technique is widely relevant to any laboratory focused on assessing neurodegenerative disease or CNS injury and eliminates the need for an ultracentrifuge for mitochondrial isolation.

Materials and methods

Non-synaptic mitochondrial isolation

Mouse brain tissues were rapidly dissected from naïve C57BL/6J male mice. Samples were kept at 4 ˚C on ice and all reagents were previously stored at 4 ˚C to prevent degradation of the mitochondria. Mitochondria were isolated from brain tissue samples using a Mitochondria Isolation Kit for mouse tissue (Miltenyi Biotec # 130-096-946), which includes anti-TOM22 microbeads and a MACS Separation LS Column. Anti-TOM22 MicroBeads (Miltenyi Biotec # 130-127-693) were used in combination with the Mitochondria Isolation Kit for mouse tissue, since a higher concentration of beads than those provided in the kit was required for this application. For collection of the paramagnetic anti-TOM22 MicroBeads, we used a magnetic QuadroMACS™ Separator (Miltenyi Biotec, # 130-090-976). A schematic of the procedure, as well as isolation using UC, is shown in figure 1.

Selected brain region(s) were removed from euthanized mice, dissected, and rinsed thoroughly with Isolation Buffer (IB; 215 mM mannitol, 75 mM sucrose, 0.1% BSA, 20 mM HEPES, 1 mM EGTA, adjusted pH 7.2 with KOH) to remove blood and other debris. Tissue was mechanically homogenized with 2 mL of IB at 4 ˚C. The sample was then centrifuged, and the supernatant was transferred to a 15 mL conical tube. The mitochondrial pellet was resuspended in 1 mL of IB, the resuspension was centrifuged again, and the resulting supernatant was added to the 15 mL conical tube.

IB was added at a concentration of 1 mL for every 7.5 mg of tissue weight and anti-TOM22 MicroBeads were added at a concentration of 4 µL for every 1 mg of tissue weight. (Note that this is a larger volume of beads than in the standard protocol and we recommend performing an anti-TOM22 MicroBead titration for optimization). The sample was then incubated at 4 ˚C for 30 minutes while ensuring constant rotation before proceeding to magnetic separation with LS Columns and Isolation Buffer using a MACS Separator. The flow-through, which contains synaptoneurosomes, was collected and set aside for synaptic mitochondrial isolation (described in the next section). Non-synaptic mitochondria were collected from the MACS Separator following column washing and collection of the magnetically labeled mitochondria in IB.

Synaptic mitochondrial isolation

The eluate from the non-synaptic pulldown was centrifuged, the supernatant was discarded, and the pellet was resuspended in 500 µL of IB. The samples were placed in a nitrogen cell disruptor at 1,200 PSI for 10 minutes and this pressure was then quickly released to rupture the synaptoneurosomes. Anti-TOM22 MicroBeads were added at a concentration of 1 µL for every 1 mg of original tissue (we recommend optimizing the amount of anti-TOM22 MicroBeads). Samples were leveled off at 2 mL and incubated at 4 ˚C for 30 minutes while ensuring constant rotation before a second round of magnetic separation with LS Columns and IB using a MACS Separator (as described in the previous section).

Measuring oxygen consumption rates

Oxygen consumption rates (OCR) were measured using a Seahorse XFe24 Flux Analyzer (Agilent Technologies). The non-synaptic and synaptic mitochondria (purified using MACS Technology) were diluted to 5 µg and 10 µg, respectively, before loading onto standard Seahorse XFe24 assay plates.

Repeated mild traumatic brain injury model

Experimental murine closed head injury was induced following previously established methods[14]. After euthanasia at 48 hours post-injury, mitochondria were extracted by UC or FMMS. For UC methods, brain regions were pooled from three mice for one data point to achieve a measurable synaptic mitochondria fraction.

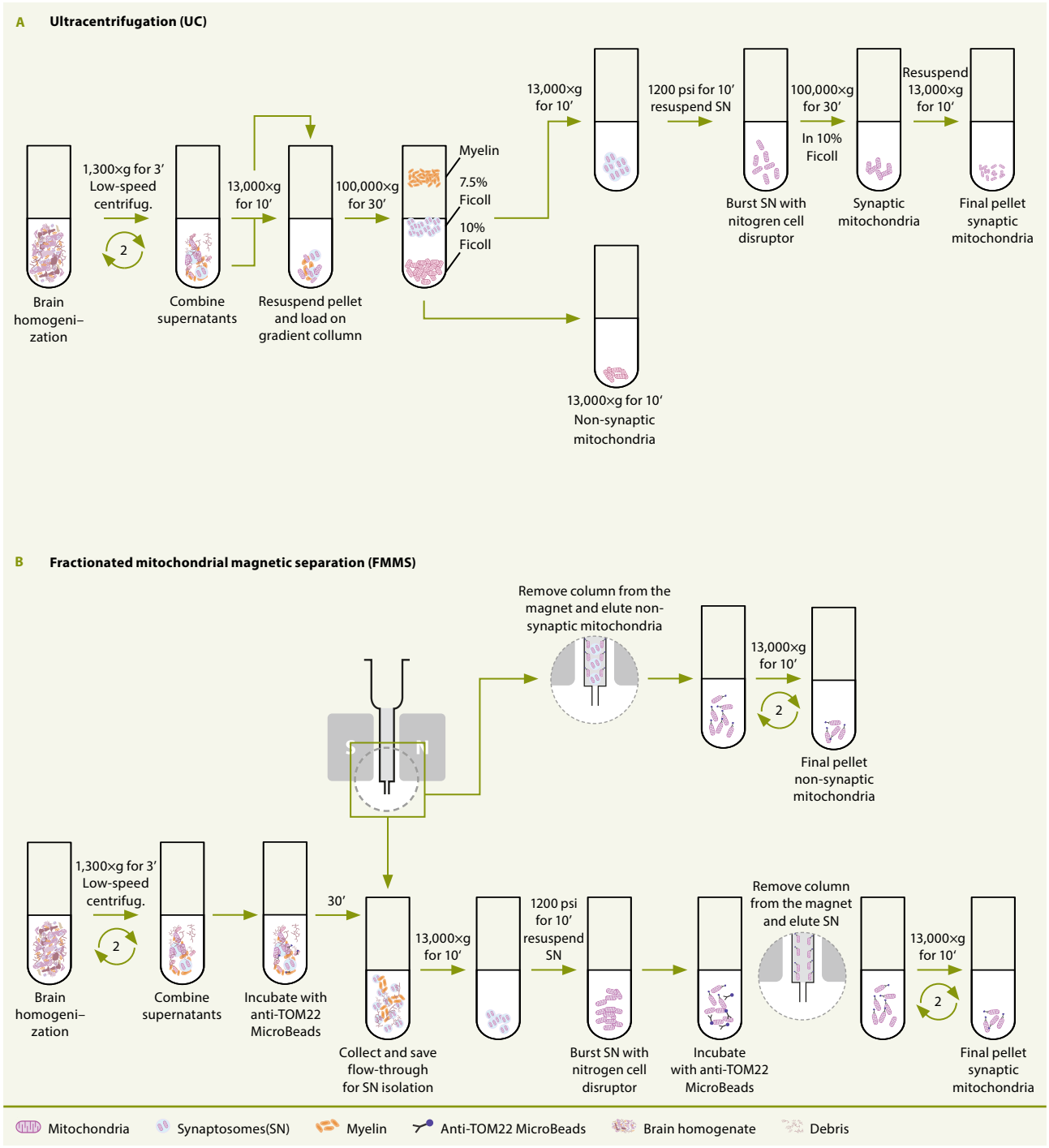


Figure 1: Schematic of mitochondrial isolation techniques (adapted from Hubbard, *et al.*[15]). (A) Workflow of density gradient ultracentrifugation (UC) to isolate synaptic and non-synaptic mitochondrial fractions from mouse brain samples. (B) Workflow of FMMS technique to isolate synaptic and non-synaptic mitochondrial fractions from mouse brain samples.

Results

Protocol optimization – non-synaptic fraction

To ensure that all free mitochondria were labeled, we ran assays of differing anti-TOM22 magnetic beads to tissue concentrations and measured protein output. The range of magnetic bead concentrations (1 to 6 µL of beads per 1 mg of tissue) showed major differences in protein yield, suggesting that mouse brain mitochondria require higher antibody concentrations for saturation. In the cortex and hippocampus, we found that the antibody titration for non-synaptic mitochondria demonstrated a plateau effect, indicating saturation (data not shown[15]). Saturating the non-synaptic mitochondria with antibody is imperative for preventing free, unbound non-synaptic mitochondria from passing into the eluate during the first pass through the magnet (fig. 1B). If saturation is not reached, non-synaptic mitochondria would be present in the synaptosomal fraction, biasing the mitochondrial population and leading to potentially inaccurate results. To ensure complete saturation, we increased the non-synaptic antibody concentration in our final protocol to 4 µL of magnetic antibody solution per mg of initial brain tissue. Increasing the antibody concentration for the non-synaptic pulldown also ultimately led to a ~1.5-fold increase in mitochondrial yield compared to UC methods (fig. 2A). Additionally, respiratory control ratios (RCRs) were comparable, even slightly higher, in mitochondria obtained from FMMS compared to those obtained by UC (fig. 2B), indicating that both methods yield intact and functional mitochondria.

Protocol optimization – synaptic fraction

After determining the appropriate anti-TOM22 magnetic bead amounts for non-synaptic mitochondria, we optimized amounts for the synaptic fraction. Due to the relatively smaller amount of synaptic mitochondria, a lower antibody concentration range was tested (0.2 to 1 µL of antibody per mg of tissue). As with the non-synaptic fraction, yield was dependent on antibody concentration. A change in synaptic antibody concentration from 0.2 to 1 µL per mg of tissue resulted in a 5-fold increase in yield in the hippocampus and an even greater increase in yield in the cortex. Furthermore, FMMS yielded 3× more synaptic mitochondria compared to UC (fig. 2C). RCR values of synaptic mitochondrial preparations were similar between FMMS and UC, although FMMS-derived samples demonstrated higher values (fig. 2D). These data indicate that the FMMS protocol isolates intact and functional synaptic mitochondria from the mouse brain.

Mitochondrial purity

We compared UC- and FMMS-derived synaptic fractions using three different markers, NDUAF9 (mitochondria), tubulin (cytosol), and calnexin (mitochondria-associated membrane). We found that, as expected, both NDUAF9 and calnexin levels were similar for the two isolation methods. However, tubulin levels were significantly lower in the FMMS-derived sample, indicating that mitochondria isolated using FMMS exhibit higher purity compared to UC-isolated mitochondria (fig. 3).

Detecting mitochondrial dysfunction after injury

Using a model of repeated mild traumatic brain injury that had previously shown mitochondrial deficits[14] we evaluated UC and FMMS protocols to assess synaptic and non-synaptic mitochondrial dysfunction after injury. A disadvantage of the UC method is the requirement of pooling samples from different mice in order to attain sufficient sample amounts. In bilateral hippocampus (3×40 mg wet weight), we did not observe any significant changes in state III respiration adenosine disphosphate (ADP-stimulated respiration to generate ATP) from either synaptic or non-synaptic mitochondrial fractions using the UC isolation procedure. In contrast, FMMS could be performed on hippocampal tissue (40 mg wet weight) from individual mice exposed to the same injury paradigm. Analysis of the FMMS-derived non-synaptic mitochondrial fraction revealed a statistically significant decrease in state III function compared to the sham group (fig. 4). This suggests that, in addition to increasing mitochondrial yield, FMMS produces a higher level of resolution, potentially by capturing a higher percentage of damaged mitochondria that would be lost using the UC isolation technique.

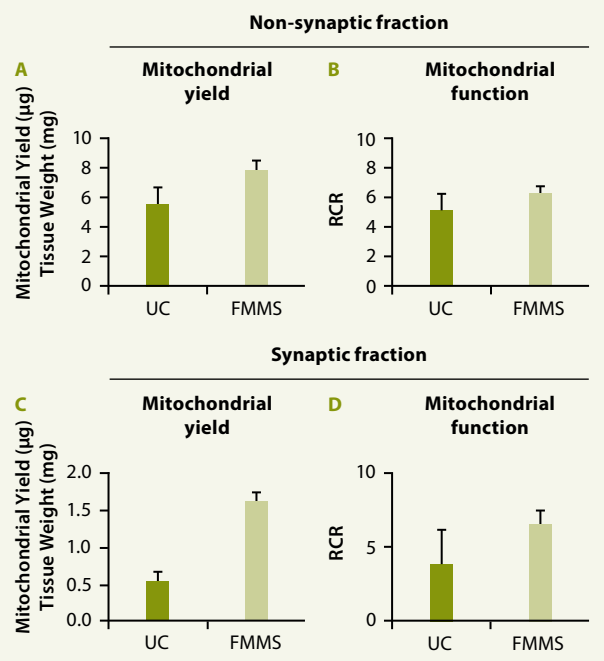


Figure 2: Non-synaptic fraction optimization. Non-synaptic mitochondrial fractions were obtained from mouse hippocampus using the UC and FMMS protocols. (A) Mitochondrial yield (µg) was normalized to initial brain tissue amount (mg). (B) RCR was calculated by dividing state III oxygen OCR respiration values by state IV OCR respiration values. No significant difference was observed. **Synaptic fraction optimization** Synaptic mitochondrial fractions were obtained from mouse hippocampus using the UC and FMMS protocols. (C) Mitochondrial yield (µg) was normalized to initial brain tissue amount (mg). The FMMS technique produced significantly higher ($p < 0.01$) levels of mitochondrial protein compared to UC methods. (D) Oxygen consumption rates were measured in the synaptic mitochondrial fractions. No significant difference was observed (adapted from Hubbard, *et al.*[15]).

	UC	FMMS
Yield	–	++
Purity	+	++
Function	+	+
Sub-population separation	+	+
Sensitivity (mitochondrial function)	–	+

Table 1: Advantages and disadvantages of the described techniques: density gradient ultracentrifugation (UC) and fractionated mitochondrial magnetic separation (FMMS). (–) technique does not have the capability or is a disadvantage. (+) technique has the capability, can perform well, or is an advantage. (++) technique has superior capability or has greater advantage compared to other methods (adapted from Hubbard, *et al.*[15]).

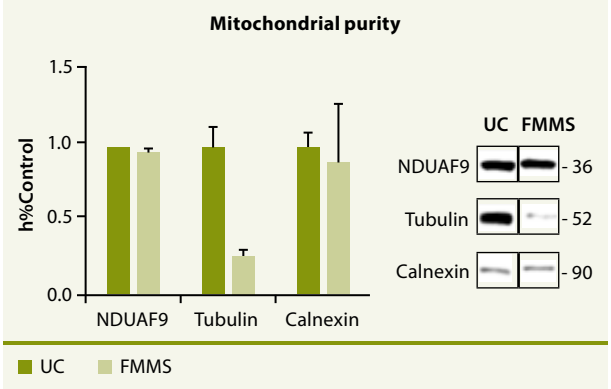


Figure 3: Markers of mitochondrial fraction purity after UC or FMMS procedures. (Left) Protein levels, obtained by western blot, were normalized to UC values. NDUAF9 and calnexin levels were not significantly different between the groups. Tubulin levels were significantly lower after FMMS methods compared to UC methods. (Right) Representative western blot for all markers. These blots were cropped from different parts of the same gel for clarity (adapted from Hubbard, *et al.*[15]).

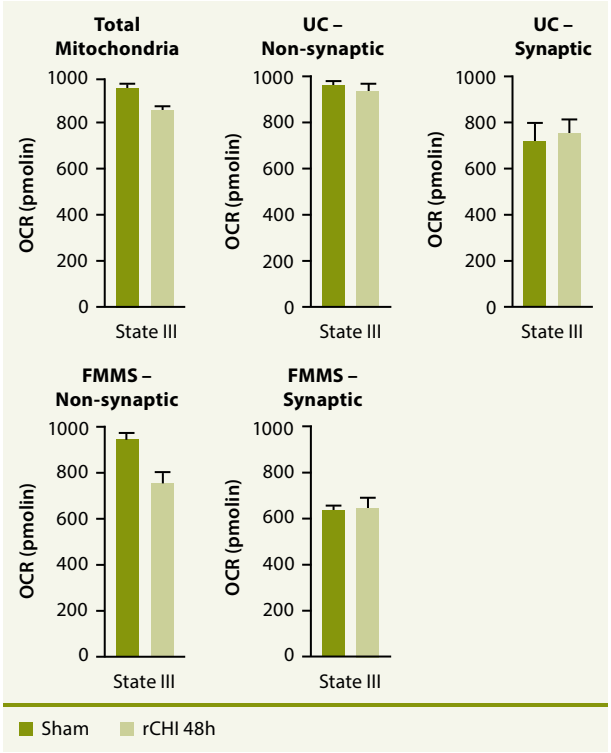


Figure 4: Application of FMMS in a model of repeated closed head injury. Mice were given either a repeated closed head injury (rCHI) at a 48 h interval or sham procedure. At 48 h after the final CHI, bilateral hippocampus was extracted and homogenized for mitochondrial respiration assessment. (Left) Total mitochondria obtained through UC methods demonstrated a state III OCR decrease in the repeated CHI group compared to sham. This data was modified from previously published work[14]. (Middle) Non-synaptic and synaptic fractions were obtained by UC procedures. Neither fraction showed any significant differences between repeated CHI and sham groups. (Right) Non-synaptic and synaptic fractions were obtained by FMMS. While the synaptic fraction did not show any significant differences between repeated CHI and sham groups, state III OCR was lower in the non-synaptic fraction of the repeated CHI group compared to sham (adapted from Hubbard, *et al.*[15]).

Conclusion

In this study, we developed and optimized FMMS to isolate synaptic and non-synaptic mitochondria from the hippocampus and cortex. The MACS Technology–based technique, FMMS, yields significant increases in mitochondrial protein compared to UC techniques. Table 1 lists the advantages of FMMS, including increased yield, sensitivity, and purity. Additionally, because of the increased yield, studies employing FMMS may avoid the limitations of pooled samples in neurodegenerative models. The use of anti-TOM22 MicroBeads confers additional advantages to the FMMS method. Due to the small size of the MicroBeads (50 nm), their biodegradability, and non-toxic nature, a bead removal step is eliminated. This renders isolated mitochondria ready for downstream applications without further processing. FMMS has wide-ranging implications in the field of brain metabolism and neurodegenerative disease. This novel technique may enable a greater understanding of how mitochondria within specific brain subregions are altered after pathological insult.

For the full protocol and additional details about this study, see: Hubbard, W. B. *et al.* (2019) Fractionated mitochondrial magnetic separation for isolation of synaptic mitochondria from brain tissue. Sci. Rep. 9: 9656.

References

1. Raichle, M.E. and Gusnard, D.A. (2002). Appraising the brain's energy budget. Proceedings of the National Academy of Sciences 99, 10237-10239.
2. Harris, Julia J., Jolivet, R. and Attwell, D. (2012). Synaptic energy use and supply. Neuron 75, 762-777.
3. Lin, M. T. & Beal, M. F. Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. Nature 443, 787–795, https://doi.org/10.1038/nature05292 (2006).
4. Johri, A. & Beal, M. F. Mitochondrial dysfunction in neurodegenerative diseases. The Journal of pharmacology and experimental therapeutics 342, 619–630, https://doi.org/10.1124/jpet.112.192138 (2012).
5. Brown, M.R., Sullivan, P.G. and Geddes, J.W. (2006). Synaptic mitochondria are more susceptible to ca2+overload than non-synaptic mitochondria. J Biol Chem 281, 11658-11668.
6. Kulbe, J.R., Hill, R.L., Singh, I.N., Wang, J.A. and Hall, E.D. (2017). Synaptic mitochondria sustain more damage than non-synaptic mitochondria after traumatic brain injury and are protected by cyclosporine a. J Neurotrauma 34, 1291-1301.
7. Du, H., Guo, L., Yan, S., Sosunov, A.A., McKhann, G.M. and Yan, S.S. (2010). Early deficits in synaptic mitochondria in an alzheimer's disease mouse model. Proceedings of the National Academy of Sciences of the United States of America 107, 18670-18675.
8. Kristian, T. (2010). Isolation of mitochondria from the cns. Curr Protoc Neurosci Chapter 7, Unit 7.22.
9. Rendon, A. and Masmoudi, A. (1985). Purification of non-synaptic and synaptic mitochondria and plasma membranes from rat brain by a rapid percoll gradient procedure. J Neurosci Methods 14, 41-51.
10. Franko, A., Baris, O.R., Bergschneider, E., von Toerne, C., Hauck, S.M., Aichler, M., Walch, A.K., Wurst, W., Wiesner, R.J., Johnston, I.C.D. and de Angelis, M.H. (2013). Efficient isolation of pure and functional mitochondria from mouse tissues using automated tissue disruption and enrichment with anti-tom22 magnetic beads. PLoS ONE 8, e82392.
11. Macheiner, T., Fengler, V.H.I., Agreiter, M., Eisenberg, T., Madeo, F., Kolb, D., Huppertz, B., Ackbar, R. and Sargsyan, K. (2016). Magnetomitotransfer: An efficient way for direct mitochondria transfer into cultured human cells. Scientific Reports 6, 35571.
12. Banik, B. and Dhar, S. (2017). Centrifugation-free magnetic isolation of functional mitochondria using paramagnetic iron oxide nanoparticles. Curr Protoc Cell Biol 76, 25.24.21-25.24.20.
13. Ahier, A., Dai, C.Y., Tweedie, A., Bezawork-Geleta, A., Kirmes, I. and Zuryn, S. (2018). Affinity purification of cell-specific mitochondria from whole animals resolves patterns of genetic mosaicism. Nat Cell Biol 20, 352-360.
14. Hubbard, W. B. *et al.* (2019) Acute mitochondrial impairment underlies prolonged cellular dysfunction after repeated mild traumatic brain injuries. J Neurotrauma 36, 1252-1263.
15. Hubbard, W. B. *et al.* (2019) Fractionated mitochondrial magnetic separation for isolation of synaptic mitochondria from brain tissue. Sci. Rep. 9: 9656.



Miltenyi Biotec

Miltenyi Biotec B.V. & Co. KG | Friedrich-Ebert-Straße 68 | 51429 Bergisch Gladbach | Germany | Phone +49 2204 8306-0 | Fax +49 2204 85197
macsde@miltenyi.com | **www.miltenyibiotec.com**

Miltenyi Biotec provides products and services worldwide. Visit **www.miltenyibiotec.com/local** to find your nearest Miltenyi Biotec contact.

Unless otherwise specifically indicated, Miltenyi Biotec products and services are for research use only and not for therapeutic or diagnostic use.
MACS and the Miltenyi Biotec logo are registered trademarks or trademarks of Miltenyi Biotec and/or its affiliates in various countries worldwide.
Copyright © 2022 Miltenyi Biotec and/or its affiliates. All rights reserved.

130-130-785