Improved protocols for isolation of intact mitochondria from tissue samples

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Introduction

Defects in mitochondrial function have been linked to many of the diseases associated with aging, including cancer, Type II diabetes mellitus, Parkinson's disease, atherosclerotic heart disease, stroke, and Alzheimer's dementia. To understand these diseases, as well as fundamentals of basic cell biology, studies often need to be done at the subcellular level. Additionally, there are other applications for which isolated organelles may be important, e.g. cytotoxicity assays in drug discovery, search for potential therapeutic agents that target mitochondria, diagnosis of certain pathological disorders, and the elucidation of various signaling pathways. Isolated mitochondria are also required for proteomic profiling studies that are likely to provide insights into mitochondrial function and dysfunction associated with disease.

While many common cell disruption techniques, e.g. mortar-pestle, bead beater, sonicator or French press, are highly efficient at disintegration of cellular material, they are much less suitable for extraction of intact subcellular components from cells or tissues, due to excessive damage to the desired organelles. Organelle isolation from whole tissue is typically done using a Potter-Elvehjem homogenizer or similar manual disruption methods. Reproducibility of such homogenization techniques usually depend to a great extent on the level of user experience.

The Pressure Cycling Technology Sample Preparation System (PCT SPS, Figure 1) subjects the sample to alternating cycles of high hydrostatic and ambient pressures. When pressure levels above 25,000 psi are employed, pressure cycling is a powerful tool for extracting nucleic acids, proteins and small molecules from cells and tissues. We have explored pressure cycling at lower levels of pressure (between 5,000 and 20,000 psi) for selective disruption of pressure-sensitive cellular structures such as plasma and nuclear membranes, while keeping the smaller organelles intact. Gentle automated PCT-based homogenization is conducted under controlled thermodynamic parameters (temperature, pressure) and, therefore, is expected to be more selective and reproducible than conventional manual homogenization techniques.

In this study we show that the PCT SPS can be used to isolate mitochondria from various types of tissue. Our results demonstrate that mitochondria extracted by pressure cycling are intact and exhibit a protein profile comparable to samples isolated using a conventional Potter-Elvehjem homogenizer technique.

Materials and Methods.

1. Kidneys, gastrocnemius muscles and lungs were harvested from 4 month old male Fisher 344 *x* Brown Norway F_1 rats. (All steps of homogenization and centrifugation were carried out on ice or at 4 ^{0}C).

2a. Freshly harvested **kidney** tissue was chopped with scissors in N1 to wash away blood. Tissue pieces were transferred into 3 FT-500 PULSE^{TM} Tubes and brought up to 1.4 ml with N1 buffer (Figure 2A).

CONTINUED AT STEP 3.

2b. Freshly harvested **muscle** tissue was minced with scissors for 5 minutes in 0.25 mg/ml Nagarse (bacterial proteinase type XXIV from Sigma). The enzyme was removed by 2 washes in MMIB. The minced tissue was split into 8 FT 500-SR Shredder PULSE Tubes and shredded for 10 seconds with a pre-cooled PCT Shredder (Pressure BioSciences, Inc.) in 0.5 ml MMIB buffer (Figure 2B).

CONTINUED AT STEP 3.

2c. Flash frozen rat **lungs** were thawed in cold N2 or MMIB buffer (as shown). The tissue was roughly minced to wash away blood, transferred to 3 FT 500-SR Shredder PULSE Tubes, shredded as described above and brought up to 1.4 ml with N2 or MMIB buffers.

CONTINUED AT STEP 3.

3. Pressure cycling was performed at 10,000 or 20,000 psi at 4˚C in an NEP2320 Barocycler^{TM} (Pressure BioSciences, Inc.). Each cycle consisted of 20 seconds at high pressure and 5 seconds at atmospheric pressure.

4. The homogenates were **centrifuged** at 1000 x g for 8 minutes and the supernatants were centrifuged at 14,000 x g for 8 minutes to pellet the mitochondria-enriched fraction. In lung and kidney preparations, this pellet was washed and stored as the mitochondria-enriched fraction. In muscle preparations, the mitochondrial pellet consisted of two layers. The darker bottom layer was comprised of intact mitochondria and the pale top layer contained damaged mitochondria and myofibrils. To separate the layers, the bottom of the tube was gently tapped on the bench several times, causing the top layer to slide down the side of the tube while the lower layer remained attached. The pale material was then gently aspirated and discarded.

Figure 1. PCT-based mitochondrial isolation workflow

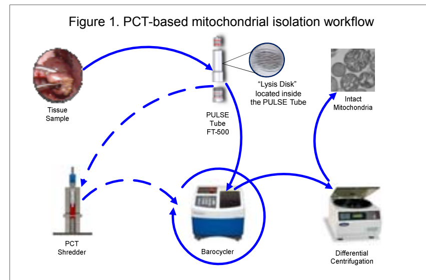


Figure 2. SDS-PAGE and Western Blotting of Mitochondria prepared by PCT

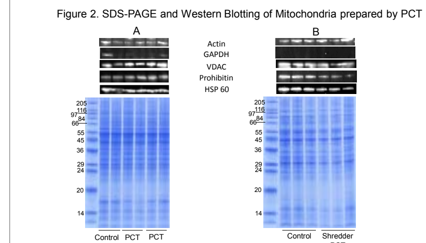


Figure 2 A. Kidney mitochondria SDS PAGE and Western blots.

Kidney mitochondria were isolated in N1 buffer (250 mM sucrose, 10 mM HEPES, 1 mM EGTA, pH 7.4 supplemented with 0.5% BSA) [3]. The mitochondria pellets were washed with N2 (250 mM sucrose, 10 mM HEPES, pH 7.4) to wash away BSA. Control kidney mitochondria were isolated by homogenization in 3ml of N1 buffer in a glass homogenizer with a Teflon pestle using 8-10 strokes. All subsequent steps were performed as for PCT samples. 10 mg of protein was loaded per lane.

Figure 2 B. Muscle mitochondria SDS PAGE and Western blots.

Muscle mitochondria were isolated in Muscle Mitochondria Isolation Buffer (MMIB; 120mM KCl, 20mM HEPES, 5mM MgCl2, 1mM EGTA, pH adjusted to 7.4 with KOH, [2]. Control skeletal muscle mitochondria were isolated by homogenization after incubation in Nagarse. Tissue was manually homogenized in ~10 ml ice-cold MMIB in a glass homogenizer with a Teflon pestle. All subsequent steps were performed as for PCT samples.

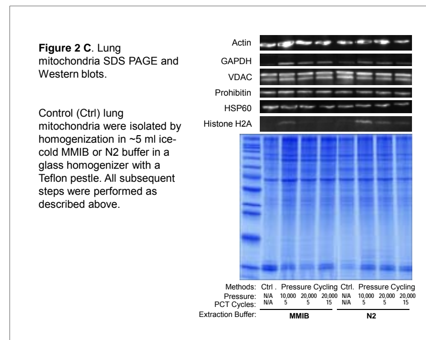


Figure 2 C. Lung mitochondria SDS PAGE and Western blots.

Control (Ctrl) lung mitochondria were isolated by homogenization in ~5 ml ice-cold MMIB or N2 buffer in a glass homogenizer with a Teflon pestle. All subsequent steps were performed as described above.

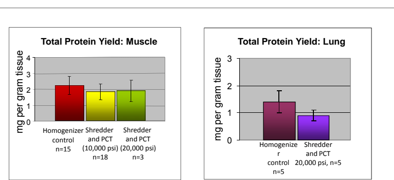
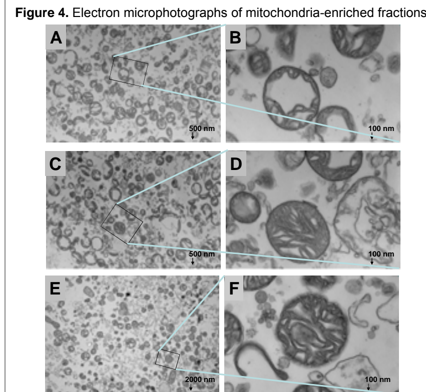


Figure 3. Comparable protein yields are obtained by the new PCT-mediated method and conventional manual methods.

Figure 4. Electron microphotographs of mitochondria-enriched fractions



A, B – Muscle mitochondria, homogenizer control;
C, D - Muscle mitochondria, Shredder + PCT at 10,000 psi, 5 cycles;
E, F - Kidney mitochondria, PCT (10,000 psi, 5 cycles).

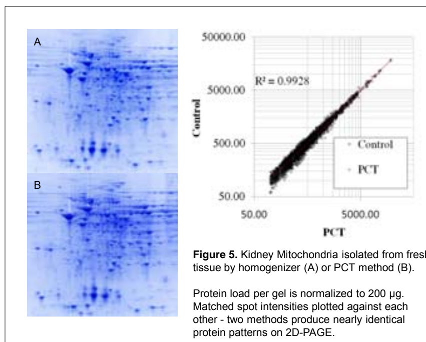
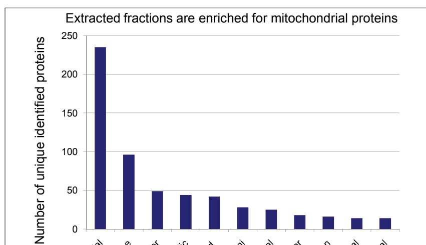


Figure 5. Kidney Mitochondria isolated from fresh tissue by homogenizer (A) or PCT method (B).

Protein load per gel is normalized to 200 µg. Matched spot intensities plotted against each other - two methods produce nearly identical protein patterns on 2D-PAGE.

Extracted fractions are enriched for mitochondrial proteins



Three samples of PCT-based mitochondrial extracts form rat kidney were subjected to proteomic analysis. Sample digestion was done in a barocycler NEP2320 (Pressure BioSciences, MA), conditions are reported in the poster authored by E. Freeman and A. Ivanov at this conference. Each sample was analyzed three times using NanoLC-2D HPLC system (Eksigent, CA) and LTQ Orbitrap (ThermoElectron, CA). MS data was analyzed with the SEQUEST-Sorcerer algorithm on the Sorcerer IDA2 (SageN Research, CA). Protein Prophet output was searched against UniProt database for available data on subcellular locatlization of unique proteins identified in the PCT-based fractions enriched for kidney mitochondria.

Results and Discussion

Pressure-mediated cell disruption has previously been shown to be an effective method for the extraction of intact mitochondria from cultured mammalian cells [2]. Here we expand the utility of this method by demonstrating that intact mitochondria can be efficiently isolated from a variety of fresh or frozen tissue samples. We have developed tissue-specific protocols for the isolation of mitochondria from 3 distinct mammalian tissues (kidney, skeletal muscle, and lung). PCT-mediated tissue disruption is a rapid and automated method that is independent of the operator's skill and experience. We confirm that the quality and purity of the mitochondria recovered using PCT is comparable to samples processed using the conventional method of tissue disruption with a Potter-Elvehjem homogenizer. PCT-mediated tissue disruption allows for up to three samples to be processed concurrently in a hands-off fashion. Electron microscopy and Western blotting have confirmed that the mitochondria are intact, and that they exhibit markers of outer (VDAC) and inner (prohibitin) mitochondrial membranes, as well as soluble inner matrix protein (HSP 60). The mitochondrial samples also contained little or no GAPDH or histones, indicating little carry-over of nuclei or soluble cytosolic protein (note: the small amount of histone detected in some mitochondrial samples isolated from frozen-thawed lung tissue may be an artifact due to freeze-thaw-induced lysis of nuclei. No histone was detected in either kidney or muscle samples, which were processed from fresh tissue). Additionally, preliminary mass spectrometry results indicate that mitochondria isolated by PCT-mediated tissue disruption are suitable for proteomic analysis. Resulting mitochondrial fractions are highly enriched in mitochondrial proteins. 235 unique proteins identified with sequence coverage over 25% were found to be highly abundant in these samples. Several other proteins reportedly associated with membranes or cytoskeleton (96 and 16, respectively) have been found in these samples. Some of the proteins in this group are expected to be associated with mitochondria as well.

The PCT-mediated tissue disruption workflow is straightforward and can be readily adjusted to optimize extraction of mitochondria from most types of tissue samples. Mitochondria can be easily extracted from relatively soft tissue (such as kidney) by PCT without the need for extensive pre-processing or grinding of the tissue. The tissue pieces are placed into the PULSE Tube, and gently forced through the holes in the lysis disk (Figure 1, inset). This simple step greatly increases the sample surface area and improves subsequent pressure-mediated tissue disruption. For tougher tissues, such as lung and muscle, a gentle pre-PCT grinding step can be performed using the PCT-Shredder. This device uses a serrated surface on the PULSE Tube Ram to grind the tough tissue and push it through the holes in the lysis disk. The shredding step takes just 10 seconds, generates minimal heat, and is performed directly in the PULSE Tube prior to PCT. In addition, PCT-based extraction can be readily combined with an enzymatic digestion step for certain tissues, such as skeletal muscle, that contain subpopulations of mitochondria that can not be readily extracted using mechanical means alone. The PCT profile can also be optimized depending on the needs of the researcher. Higher pressures or number of pressure cycles may increase total yield, but are likely to result in more damaged mitochondria. Therefore, depending on whether yield or function of mitochondria is more important for downstream analyses, the PCT parameters can be adjusted by the user.

Advantages of PCT include automation-friendly reproducible mitochondrial extraction that is independent of operator skill level and experience. The PCT-mediated tissue disruption method yields mitochondria that are intact, functional, and compatible with common downstream applications, such as Western blotting and 2D gel electrophoresis or LC-MS-based proteomic analysis. The PCT-mediated tissue disruption method is also suitable for applications that require functional mitochondria, such as respiration and swelling assays.

References

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