Isolation of Functional Mitochondria from Whole Rat Heart Using a PBI Shredder and Pressure Cycling Technology (PCT)

Introduction

Isolation of intact mitochondria from human and animal tissue is crucial for studies that focus on the elucidation of their function and dysfunction in conditions such as aging, diabetes and cancer. As potential drug targets, high quality functional mitochondrial isolates are important for drug screening studies [1]. Mitochondria isolation from solid tissue is usually carried out using labor-intensive homogenizer-based methods [2] that require extensive operator experience. Here we describe a semi-automated method to release mitochondria from whole rat heart tissue, using a PBI Shredder and PCT in place of traditional manual homogenization.

The PBI Shredder

The PBI Shredder comes in two configurations; *The PCT Shredder* and *The* Shredder SG3 are both designed to quickly and gently grind solid tissues such as brain and kidney, or even fibrous samples such as plant leaves and muscle tissue. Since shredding is performed in a disposable sample container (Figure 1), the likelihood of cross contamination with other samples is very low. In addition, shredding for 10-20 seconds generates relatively little heat and thus does not increase protein denaturation or damage delicate organelles.

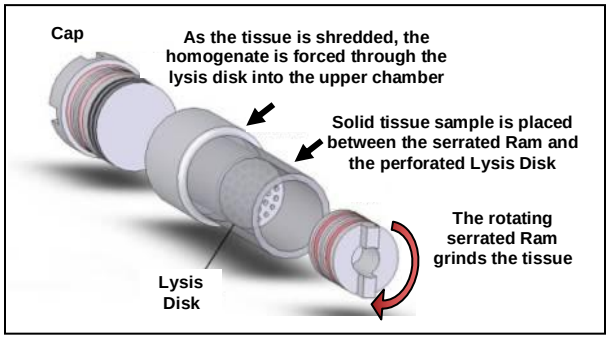


Figure 1. The FT500-S Shredder PULSE Tube. As the tissue is disrupted, the Shredder Ram of the PULSE Tube forces the homogenate through the holes of the Lysis Disk into the upper compartment, significantly reducing the risk of sample over-homogenization and mitochondrial damage.

Pressure Cycling Technology (PCT)

PCT destabilizes molecular interactions by rapidly and repeatedly raising and lowering pressure in the reaction vessel from ambient to high pressures (up to 45,000 psi [310 MPa, 3000 Atm]). High hydrostatic pressure acts preferentially on the more compressible constituents of the sample, leading to destabilization of molecular interactions, but having little impact on covalent bonds. At lower pressures, e.g. 10,000 - 20,000 psi, PCT can be used to lyse cells and release their intracellular contents, including intact organelles. This method has been shown to be relatively gentle, and has already been used to isolate intact mitochondria from cell culture [3], as well as from fresh skeletal muscle and kidney tissues [4].

Methods

Mitochondria-enriched fractions were prepared from freshly harvested, whole adult rat heart weighing ~1gram each (1 heart per sample, n=4). Tissue was rapidly excised and placed into semi-frozen slush of Heart Mitochondria Isolation Buffer (HMIB) (220mM mannitol, 70mM sucrose, 10mM HEPES (free acid), 1.0 mM EGTA, pH adjusted to 7.4 with KOH, supplemented with 0.05% BSA) [5]. The samples were kept on ice, or at 4˚C, for all subsequent steps.

The tissue was roughly chopped in HMIB to wash away surface blood. The tissue pieces were transferred to BSA-free HMIB supplemented with 0.25 mg/mL bacterial proteinase type XXIV (Sigma). The tissue was further minced while incubating for 5 minutes at 4˚C in the enzyme solution. The minced tissue was washed twice in ice-cold HMIB, and split evenly into three FT500-S PULSE Tubes. Shredding was performed for 10 seconds with 0.5mL HMIB. The metal Shredder stand was pre-chilled to compensate for any heat that might be generated during shredding.

Prior to pressure cycling, the volume of each PULSE Tube was brought up to 1.4mL with additional HMIB. PCT was performed at 10,000psi for 5 cycles at 4˚C. Each cycle consisted of 20 seconds at high pressure and 5 seconds at atmospheric pressure.

Tissue homogenates from the three PULSE Tubes were pooled and centrifuged for 10 minutes at 1000 x g. The supernatant was then centrifuged for 10 minutes at 14,000 x g to pellet the mitochondria-enriched fraction. The pellet was suspended in 1.5ml HMIB, transferred to a microcentrifuge tube and centrifuged at 14,000 x g to wash the mitochondria-enriched fraction. The mitochondria were washed once more in HMIB and then

resuspended in Heart Mitochondria Wash Buffer (HMWB: 220mM mannitol, 70mM sucrose, 10mM HEPES free acid, pH 7.4 with KOH) to help wash away HMIB-derived BSA. After the final wash, the pellet was suspended by gently homogenizing in 50µL of HMWB using a small plastic pestle. The final volume of the mitochondria-enriched suspension was ~80-100µl.

Multiparameter Kinetic Assessment of Isolated Rat Heart Mitochondria

Simultaneous measurements of changes in mitochondrial membrane potential ($\Delta\varphi_m$), Ca^{2+} fluxes, mitochondrial swelling, and changes of pyridine nucleotide oxidation (NAD(P)H), were performed using a multichannel fluorescent setup as previously described ([4] and references therein). Mitochondria (0.25mg/ml) were incubated with 5mM succinate, 150µM ATP, 150µM Mg^{2+} and were challenged with a series of bolus additions of 20µM Ca^{2+}. Alamathicin (Ala), a pore-forming agent, was added at the end of each run to assess complete swelling, Ca^{2+} release, de-energization and oxidation of pyridine nucleotides.

Tetramethylrhodamine methyl ester (TMRM) (Invitrogen) was used in self-quenching mode to detect changes in mitochondrial membrane potential. Ca^{2+} fluxes were detected using Ca-Green 5N (Invitrogen, Carlsbad Ca.), a Ca^{2+}-sensitive, non-permeable fluorescent dye which increases its fluorescence when complexed with free extra-mitochondrial Ca^{2+}. Oxidation of mitochondrial pyridine nucleotides was measured as a decrease in autofluorescence of NADH and NADPH at $\lambda_{ex}/\lambda_{em}$ = 360/440 nm. Mitochondrial swelling was detected as a decrease in light scattering at excitation and emission wavelengths of 587 nm measured at an angle of 90^0.

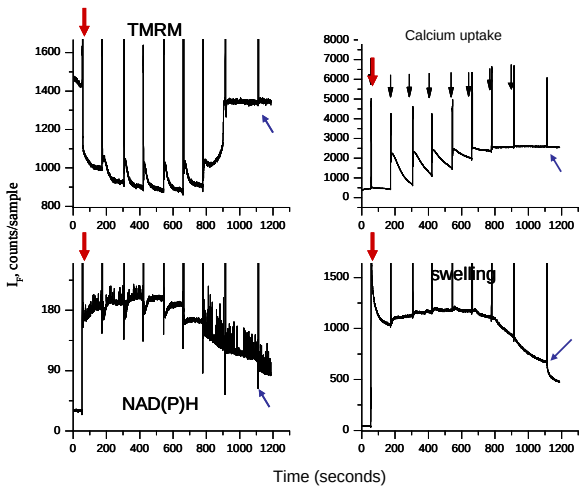
Results and Discussion

Here we demonstrate that functional mitochondria from whole rat heart can be efficiently extracted using a PBI *Shredder* and pressure cycling. This method is based on previous data that support the use of the PBI Shredders and PCT for isolation of intact mitochondria from cell cultures [3], kidney and skeletal muscle [4], and lung tissue [6].

Total protein concentration of the final mitochondria preparations was 39 ± 12.4 mg/ml (n=4). Previous data suggest that no improvement in yield of skeletal muscle mitochondria is achieved when pressure is increased above 10,000 psi [4]; however it is possible that with heart tissue, higher pressures could be beneficial.

The ability of isolated heart mitochondria to respond to Ca^{2+} is shown in Figure 2. These results confirm that mitochondria exposed to hydrostatic pressure cycling at 10,000 psi are intact and exhibit a normal response to Ca^{2+} overload.

Figure 2. Mitochondria Isolated by the Shredder/PCT Method are Intact and Respond to Exogenous Ca^{2+}. The Ca^{2+}-induced response was monitored by simultaneous four channel recording. Addition of mitochondria to the sample chamber is indicated by the red arrow. Each Ca^{2+} addition is indicated by black arrows (since all four traces are recorded simultaneously, the timing of Ca^{2+} addition is identical in all four traces, but is marked with arrows only in the calcium uptake trace). Addition of alamithicin at the end of the run is indicated by blue arrows.



Conclusions

Traditional manual methods for isolation of functional mitochondria from tissues rely heavily on operator experience and skill. Considerable training is required to avoid common mistakes such as tissue over-homogenization, which can result in damaged mitochondria and highly variable results. Here we describe a convenient method for isolation of intact and functional mitochondria from fresh rat heart tissue using a PBI Shredder and PCT. During shredding, the design of the FT 500-S PULSE Tube forces the homogenate through the holes of the Lysis Disk into the upper compartment. This simple but effective design significantly reduces the likelihood of sample over-homogenization. Following the shredding step, brief (total pressure cycling time per sample is <3 mins) PCT treatment at 10,000 psi is used extract the intact mitochondria.

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The PBI Shredders and PCT are physical tissue disruption/lysis methods that are widely applicable to many sample preparation needs. In the case of heart mitochondria, the proteinase enzyme step is optional, and may be omitted in some applications. In addition, the formulations of the isolation and wash buffers described above were based on published protocols for isolation of rat heart mitochondria [5], and can be readily replaced by other mitochondria isolation buffers depending on the needs and preferences of the user. As previously demonstrated with other tissue types, the mitochondria isolated using this convenient method can be used for proteomic as well as functional studies [3, 4]. This method is simple, easy to learn, and eliminates many of the problems of traditional manual mitochondria preparation methods.

References

[1] Armstrong (2007) Brit. J. Pharm. 151:1154-1165
[2] Rasmussen et al. (1997) Anal. Biochem. 252:153-159.
[3] Gross et al. (2008) Biotechniques 45(1):99-100.
[4] Gross et al., (2011) Anal. Biochem. *In Press*
[5] Stavrovskaya et al. (2010) Free Rad. Biol. & Med. 49: 567-579.
[6] Application Note-0021: Isolation of Mitochondria from Rat Lung Using *The PCT Shredder* and Pressure Cycling Technology (PCT) http://www.pressurebiosciences.com/downloads/2010-12-13/AN-00021-Lung-Mito-Final.pdf

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