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Preparation of permeabilized muscle fibres for diagnosis of mitochondrial respiratory function

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Summary: Application of permeabilized muscle fibres and O2k High-Resolution FluoRespirometry (HRFR) offer a sensitive diagnostic test of mitochondrial dysfunction in small biopsy specimens of human muscle. By using these techniques in conjunction with multiple substrate-uncoupler-inhibitor titration (SUIT) protocols, respirometric studies of human and animal tissue biopsies improve our fundamental understanding of mitochondrial respiratory control and the pathophysiology of mitochondrial myopathies.

1 Preparation of Permeabilized Muscle Fibres

1.1 Requirements

Ketamine, xylazine; 2 pairs of sharp scissors (large and small); 2 pairs of forceps with sharp tips (one straight tip, one angular tip; non-magnetic), 2 pairs of forceps with very sharp angular tips (non-magnetic); ice; tissue culture plate with 12 wells (Falcon 35/3043); 5 ml beaker; magnetic stirrer plate; small (3 ml) test tubes, microbalance (5 digits; 0.01 mg).

BIOPS The relaxing and biopsy preservation solution BIOPS contains 10 mM Ca-EGTA buffer, 0.1 µM free calcium, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM DTT, 6.56 mM $MgCl_2$, 5.77 mM ATP, 15 mM phosphocreatine, pH 7.1 [MiPNet03.02]. BIOPS can be stored and shipped frozen at -20 °C.

Animals After full anaesthesia of the the mouse or rat through intraperitoneal injection (e.g. Fentanyl, 1 ml/kg of mouse), the condition of the animal is checked (heart beat is visible, the reflex test on the hindlimb is negative).

1.2 Tissue preparation

The muscle (heart or skeletal muscle) is excised. Skeletal muscle is cut lengthwise into small samples of 10-20 mg W_w, and put into a Falcon tube with 10 ml of ice cold BIOPS. In this preservation solution, the sample can be stored for several hours at 0 °C, depending on the source of muscle tissue (e.g. mouse skeletal muscle for at least six hours; human skeletal muscle for up to 24 h; Skladal et al 1994). This provides the possibility for shipping of biopsies on ice for functional analysis by High-Resolution FluoRespirometry (HRFR).

Place the tissue sample in ice cold preservation solution (BIOPS) into a small petri dish on ice. Remove all connecting tissue using the pair of sharp or very sharp forceps.

1.3 Tissue separation

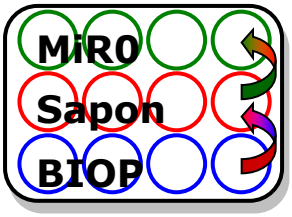
Fibre bundles are separated mechanically with the two pairs of very sharp forceps, in a small petri dish on ice. A period of five minutes is required for preparation of a 2-mg sample of fibres. The degree of separation may be evaluated by observing a change from red to pale colouring of the separated fibre bundles. At least during an initial start-up period, use of a dissecting scope is recommended for observation of the mechanical

separation, during which fibres are partially teased appart and streched out. The tissue should still remain connected in a mesh-like framework.

This preparation leads to partial permeabilization of skeletal muscle, but full permeabilization of cell membranes in heart (Kuznetsov et al. 2004) or liver tissue (Kuznetsov et al. 2002).

After tissue separation, the fibre bundels are placed sequentially into 2 ml ice cold BIOPS into individual wells of a Falcon 12-well tissue culture plate.

1.4 Tissue permeabilization



After completion of separation of fibre bundles for two or more O2k-chambers, the fibre bundles are transferred quickly into 2 ml of icecold BIOPS, containing 20 µl of saponin stock solution (5 mg/ml; final concentration 50 µg/ml).

Shake by gentle agitation in the cold room (on ice) for 30 min. After the 30 min period, all samples are quickly transferred from the saponin solution into 2 ml of MiR06 (see figure), and shaken by gentle agitation for 10 min in the cold room (on ice).

Additional samples may prepared with or without saponin permeabilization for other assays (histochemical/morphological; enzymatic; mtDNA).

2 Wet weight

METTLER TOLEDO microbalance XS205DU

Weight measurements are made after permeabilization, eliminating variations in water contents due to osmotic stress, allowing for elimination of connective tissue during mechanical separation before weight measurement, and for partitioning of subsamples of similar wet weight.

Before adding the tissue into the O2k chamber, take wet weight measurements of several loosly connected fibre bundles of c. 1-3 mg wet weight (skeletal muscle) or 0.5-2 mg wet weight (heart). Blot the bundles carefully on filter paper. Take the sample with the sharp pair of forceps (angular tip) and place it for 5 s onto the filter paper. During this time, wipe off any liquid from the tip of the forceps with another filter paper. Then take the sample from the filter paper and touch it once more shortly onto a dry area of filter paper while holding it with the forceps. Then immediately place the sample onto a small plastic plate on the table of the tared balance.

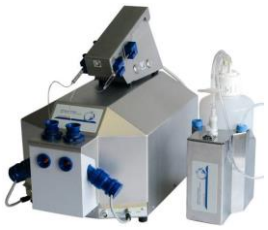
Immediately after reading the wet weight, the sample is transferred into a well with 2 ml ice cold MiR06. Check that the tare balance reading returns to zero. Each well receives a sample for an O2k-chamber.

3 Oroboros O2k

Calibrate the oxygen sensors in MiR06 at experimental temperature in the 'open' chamber (gas phase above the stirred medium, with a partially inserted stopper) of the Oroboros O2k at a Gain of 1, to ensure that the raw signal does not reach the limiting value of 10 V at high experimental oxygen concentrations.

Respiration is measured (at 37 °C for mammalian tissues) in mitochondrial respiration medium (MiR06) designed for optimal protection of mitochondrial function [MiPNet14.13]. From the vial with MiR06, the sample is transferred into the Oroboros O2k chamber containing air saturated MiR06. The sample is immersed with the pair of straight forceps into the stirred medium in the O2k-chamber.

After closing the chamber, the oxygen concentration is increased to 350 µM, adding microliter volumes of H_2O_2 solution, using the TIP2k or by manual titration [MiPNet14.06]. The oxygen signal increases rapidly at each titration step, and the titration is continued until the final oxygen level is reached. 5 to 10 min are required for stabilization of the signal. Oxygen concentration is prevented to drop below 200 µM by intermittent H_2O_2 titrations, preventing diffusion limitation of respiration in permeabilized fibres (Gnaiger 2003).



Respiratory flux of muscle fibre bundles is limited by oxygen diffusion even above 50% air saturation (O_2 concentration of 100 µM). This problem is carefully controlled and avoided by application of a high-oxygen regime. It is essential that instrumental and chemical oxygen background fluxes are routinely determined as a function of oxygen concentration in the experimental range. With appropriately calibrated background parameters inserted into the 'Edit Experiment' window of Datlab 4, background corrections are automatically obtained on-line over the entire experimental oxygen range. This is an indispensable prerequisite for accurate measurements particularly at low fluxes, when the amount of biological material is limited.

4 References

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Protocols

MiPNet03.02	Selected media and chemicals.
MiPNet14.06	Instrumental background correction and accuracy of oxygen flux.
MiPNet14.13	Mitochondrial respiration medium – MiR06.