Measuring mitochondrial respiration in C2C12 myotubes

C2C12 myotube differentiation

1. Grow 1 x 10^5 C2C12 myoblasts per well in 6-well plate in DMED with 20% FBS for 24 hrs. Remove the culture medium and feed the cells with differentiation medium (see below for recipe).
2. Change differentiation medium every two days.
3. Around 4-5days, the myotubes will be ready for test.

Cell permeabilization (a titration is preferred for each experiment)

4. Take differentiated C2C12 myotube from the tissue culture incubator.
5. Remove the culture medium from one well completely and add 500 µl of pre-warmed trypsin (GIBCO, Cat# 25200) to cover the cells.
6. Remove the trypsin immediately and completely and add 100 µl of trypsin to cover the cells and incubate at 37°C for 2 min. This will ensure efficient trypsinization.
7. Add 3 ml of pre-chilled culture medium (on ice) to neutralize the trypsin. Transfer half of the detached myotubes to a 15-ml tube and spin at 769 x g (2000 rpm, Cat 3-16K, Sigma) for 3 min, at 4°C (Save the half of cells for protein assay and westerrn blot).
8. Resuspend the cells gently in an eppendorf tube in 250 µl of ice-cold medium D (see below for recipe).
9. Mix 250 µl of cell suspension with 250 µl of ice-cold medium D with proper amount of digitonin (Sigma, Cat. D-141) (The titration of digitonin starts from 50ug/ml to 400ug/ml).
10. Shake the tube gently in ice-water bath for exactly 3 min and stop the reaction by adding 500 µl of ice-cold BSA (3 mg/ml in medium D, Calbiochem, Cat# 126575);
11. Pellet cells by centrifuge at 769 x g (2000 rpm) for 3 min at 4ºC.
12. Resuspend the cells in 290 µl medium D and place the tube on ice.
13. Transfer 30 µl of the cell suspension into an eppendorf tube, and pellet the cells at 14,000 rpm for 3 minutes (based on the protein assay, this speed seems ok for the cells). Resuspend the cells in 10 µl of 2X protein sample buffer and boil for 5 min at 100ºC for future protein determination and/or Western blot.

Mitochondrial respiration

14. Calibrate the Oxygen monitor before testing.
15. Add the 270 µl cell suspension into the respiration chamber with proper amount of substrates (5 mM pyruvate and 2 mM malate, or 5 mM succinate) (Prepare the stock concentration for succinate at 500 mM, pyruvate and malate at 500 mM and 200 mM, respectively. Store in -20^0C), and let it stabilize till the value becomes stable (It will take about 10 min).
16. Add 2.5 µl of ADP (10 mM, Biochemika, Cat# 01905) to a final concentration of 0.1 mM ADP and monitor oxygen consumption for 5 min. To pipette 2.5 µl without adding air bubbles, we need to set the pipette to 2.8 µl.
17. Add 2.5 µl of ADP (100 mM) to a final concentration of 1 mM ADP and monitor oxygen consumption for 5 min.

18. Add 2.5 µl of oligomycin (1 mg/ml, Biochemika, Cat# 75352) to a final concentration of 10 µg/ml and monitor oxygen consumption for 5 min.
19. Remove the suspended cells and wash the chamber completely with diluted bleach (1:10 dilution of bleach in ddH_20 followed by ddH_20 three times after the completion of the experiments.

Reagents

Medium D:

Stock	Volume	Final Conc.
2.5 M Sucrose (Sigma, S0389)	10 ml	250mM
100 mM $MgCl_2$ (Sigma, M2393)	10 ml	10 mM
125 mM KH_2PO_4 (Sigma, P5655)	10 ml	12.5mM
200 mM Hepes (Sigma, H4034)	10 ml	20 mM
H_2O	60 ml	
Adjusted to pH 7.1		

Differentiation Medium:

Stock	Volume	Final Conc.
DMEM (High Glucose)	460 ml	1X
100% horse serum	10 ml	2%
10X penicillin/streptomycin	5 ml	1X
1 M HEPES, pH 7.4	25 ml	50 mM
4 mg/ml transferrin	1.25 ml	10 µg/ml
4 mg/ml insulin	1.25 ml	10 µg/ml