**Protocol for measuring mitochondrial bioenergetics in fish liver using the Oroboros – OC 03/05/2023**

**Goal:** Measure mitochondrial respiration from liver homogenate in fish using a series of drugs to elicit different respiration states.

**General timeline:**

Day of assay:

1. Prepare media and reagents as needed (e.g., PBS, isolation media, respiration media, substrates, etc.).
2. Air calibration on Oroboros – this must be done every day prior to collecting data.
3. Tissue preparation – tissues must be processed and used immediately following collection. Centrifuge must be chilled prior to use (takes about 5 minutes).
4. Data collection with Oroboros.
5. Freeze aliquot of tissue sample used in Oroboros – this is used to determine the amount of protein in the homogenate. Respiration values are corrected by the amount of tissue. Data from the Oroboros cannot be used without knowing the amount of tissue.
6. Cleaning the Oroboros.

After the assay:

1. Bradford assay – to determine protein content.
2. Data extraction from Oroboros to calculate respiration values.

**Methods:**

1. **Prepare media and reagents.** 
   1. Phosphate buffered saline (PBS – want 1M)

*Used for rinsing tissue.*

1. For 500 mL of 10M PBS:

8.9 g of Na2HPO4\*2H20 (100mM) +

1.2 g of KH2PO4 (18mM) +

40g of NaCl (1.37M) +

1 g KCl (27mM).

1. For 1 L of 1M PBS:

100 ml of 10x PBS +

900ml of ddH2O

* 1. Mitochondria isolation media

*Isolation media is used for processing tissue homogenate. Should be made in sterile conditions to avoid contamination.*

* + 1. Sucrose (250 mM) – Sigma Aldrich 84097

Molecular weight = 342.30 g/mol

Concentration = 1.0 mol/L \* 342.3 g/mol

1M concentration = 342.3 g/L

1mM concentration = 0.3423 g/L

250mM concentration = 0.3423 g/L \* 250 = 85.575 g/L

* + 1. EGTA (1 mM) – Sigma Aldrich E4378

Molecular weight = 380.35 g/mol

Concentration = 1.0 mol/L \* 380.35 g/mol

1M concentration = 380.35 g/L

1mM concentration = 0. 38035 g/L

* + 1. HEPES (5 mM) – Sigma Aldrich H7523

Molecular weight = 238.30 g/mol

Concentration = 1.0 mol/L \* 238.30 g/mol

1M concentration = 238.30 g/L

1mM concentration = 0. 23830 g/L

5mM concentration = 5 \* 0.23830 = 1.1915 g/L

* + 1. Directions for 500 mL:
       1. Sucrose: 42.7875 g +
       2. EGTA: 0.1902 g +
       3. HEPES: 0.5958 g +
       4. Dissolve in 500 mL of ddH2O. Use stir bar and may need to heat to get sucrose to go into solution.
       5. pH to ~7.5 while ice cold using KOH.
  1. Respiration media – used for measuring respiration in the Oroboros.
     1. Use Mir05 kit or make from scratch – details in document *MiPNet22.10\_MiR05-Kit.*
  2. Pyruvate (Make 0.5M stock for final concentration: 5mM if 5ul used in 500ul chamber) – Sigma Aldrich P2256
     1. Make fresh day of assay.
     2. Molecular weight = 110.04 g/L

1M = 110.04 g/L = 110.04 mg/mL

0.5M = 55.02 mg/mL

* + 1. Add 55.02 mg pyruvate to 1 mL of respiration media for 0.5 M stock.
    2. Use correction to account for actual measurement:
  1. Malate (Make 0.2M stock for final concentration 2mM if 5ul used in 500 ul chamber) – made from L-(-)Malic acid (Sigma Aldrich M1000)
     1. Molecular weight: 134.09 g/L

1M = 134.09 g/L = 134.09 mg/mL

0.2M stock = 26.818 mg malic acid to 1 mL of ddH2O

* + 1. Add 35.61 mg malate to 1 mL of respiration media for 0.2 M stock.
    2. To make 5 mL total: add 178.05 mg of malic acid to 5 mL of ddH2O.

PH to ~7.1 using KOH at room temperature.

Aliquot 250 ul and store at -20°C.

* 1. Succinate (make 1.0M stock for final 10mM if 5ul used in 500 ul chamber) – Sigma Aldrich S2378
     1. Molecular weight: 270.14 g/L

1M = 270.14 g/L = 270.14 mg/mL

* + 1. Add 270.14 mg succinate to 1 mL of respiration media for 1.0 M stock.
    2. To make 5 mL total: add 1.351 g of succinate to 5 mL of respiration media. Aliquot 250 ul and store at -20°C.
  1. ADP (Make 0.2M stock for final concentration 2mM if 5ul used in 500 ul chamber) – Sigma Merck A5285 (Adenosine 5’-diphosphate monopotassium salt dihydrate)
     1. Molecular weight: 501.32 g/L; 1M = 501.32 g/L = 501.322 mg/mL
     2. Add 100.264 mg ADP to 1 mL of respiration media for 0.2M stock.
     3. To make 5 mL total: add 501.32 mg of ADP to 5 mL of ddH2O.

pH to ~6.3-6.9 at room temperature using KOH. (can we pH such a small volume?)

Aliquot 250 ul and store at -80°C (no freeze/thaw cycle; so one use per tube).

* 1. Oligomycin (make 125 uM stock for final concentration 2.5uM – 10 ul used in Oroboros)
     1. Molecular weight: 791.00 g/L; 1M = 791.062 g/L = 791.062 mg/mL

1mM = 0.791062 mg/mL

125mM = 0.791062 mg oligomycin to 1 mL of respiration media.

For 1.25mM: dilute 10ul of 125 mM stock into 990 ul of assay buffer.

For 125 uM: dilute 100 ul of 1.25 mM stock into 900 ul of assay buffer.

* + 1. Aliquot 100 ul and store at -20°C.
  1. FCCP (0.5 uM per step of 5ul so need 50uM stock solution for 500ul chamber) – Sigma Merck C2920
     1. Molecular weight: 254.17 g/L

1M = 254.17g/L = 254.17mg/mL

1mM = 0.25417 mg/mL

50mM = 12.7085 mg/mL

Dilution 1: 10 ul of 50 mM stock solution into 990 ul of ddH20 (0.5mM)

Dilution 2: 10 ul of Dilution 1 (0.5mM) into 990 ul of ddH20 (5uM)

Dilution 3: 100 ul of Dilution 2 into 900ul of ddH20 (0.5uM)

* + 1. To make 5 mL: 500 ul of Dilution 2 into 4.5 mL ddH2O
    2. Aliquot 250 ul and store at -20. Only use aliquots once. Is it light sensitive?
  1. Antimycin A (for final concentration 2.5uM) - Sigma A8674
     1. Molecular weight: 532 g/mol

Note: This product is a mixture of antimycins, each with a unique molecular weight. The percentage of each component are lot specific and determined by HPLC – information found on certificate of analysis. Average molecular weight for this lot is 532 g/mol.

* + 1. df
  1. H2O2 – injections used if oxygen level drops in chambers before the end of sampling.
     1. Use 25 ul of 50% H2O2 to increase oxygen in the chamber by ~25uM
     2. H2O2 is light sensitive and should be kept in the dark.

1. Air calibration on Oroboros
   1. Air calibrations take about 60 minutes and should be performed every day prior to data collection.
   2. After initial calibration, calibration settings are automatically applied.
   3. See *MiPNet06.03\_POS-Calibration-SOP* document for full details.
   4. Methods are straightforward but need to be summarized here.
2. **Tissue preparation.**
   1. Start timer as soon as enclosure is disturbed. Record the amount of time between disturbance and liver dissection.
   2. Euthanize fish.
   3. Remove whole liver.
   4. Rinse in 1 mL of ice-cold phosphate-buffered saline (1X) in Eppendorf tube by putting liver in tube and taking it out again with forceps. Repeat.
   5. Add liver to test tube with 1 mL of ice-cold isolation media.
   6. Homogenize in test tube on ice using Potter Elvehjem homogenizer. Use three gentle hand passes to homogenize.
   7. Transfer homogenate to clear Eppendorf tube on ice.
   8. Centrifuge tubes at 750 g, 4°C for 10 minutes. Mitochondria are in the supernatant. Remove supernatant and save in clean Eppendorf tube.
   9. Repeat step H (Centrifuge tubes 750 g, 4°C for 10 minutes. Mitochondria are in the supernatant. Remove supernatant and save in clean Eppendorf tube).
   10. Centrifuge at 10,000 g, 4°C for 10 minutes. Mitochondria are in the pellet. Take off and dispose of supernatant.
   11. Gently resuspend pellet in 540 ul of respiration media equilibrated to ~30°C (or temperature of assay).
3. **Data collection with Oroboros.** 
   1. Baseline oxygen consumption (not used in analyses).
      1. Add: NA
      2. Allow temperature and oxygen consumption to stabilize ~5 minutes.
   2. Non-phosphorylating state (state 2)
      1. Add: 5 ul of 500mM pyruvate, 5 ul of 200 mM malate, and 5 ul of 1.0M succinate
      2. Allow reading to stabilize ~5 minutes.
   3. OXPHOS (state 3)
      1. Add: 5 ul of 0.2 M stock ADP
      2. Allow reading to stabilize.
   4. Non-phosphorylating state, inhibit ATP synthase (state 4)
      1. Add: 10 ul of 125 uM Oligomycin stock.
      2. Allow reading to stabilize.
   5. Maximal uncoupling
      1. Add: 5 ul of FCCP in serial titration until maximal respiration is reached (no further response to FCCP)
      2. Allow final reading to stabilize.
   6. Non-mitochondrial respiration
      1. Add: x ul of antimycin A
      2. Allow reading to stabilize.
4. **Freeze aliquot of tissue sample used in Oroboros.**
   1. Remove 250 ul of sample from Oroboros chamber after measurements are collected.
   2. Store in Eppendorf tube at -20C.
5. **Cleaning the Oroboros.**

*Cleaning the chambers in between samples (if drugs other than substrates were used) or following a zero calibration is incredibly important to avoid contamination. For example, the last drug used to measure respiration is often Antimycin A which destroys mitochondrial membranes. Trace amounts of this can kill the next sample. If contamination like this is suspected, add a second 100% EtOH wash (step bv below).*

* 1. Prior to use:
     1. Remove 70% EtOH from chambers.
     2. Rinse three times with ddH2O.
     3. Rinse stoppers with ddH2O including capillaries.
  2. In between samples (these cleaning steps must be carefully followed):
     1. Remove sample from chamber.
     2. Rinse three times with ddH2O.
     3. First 70% EtOH wash: fill chambers with 70% EtOH, let stopper slide into chamber (do not push in) while siphoning off excess EtOH. Stir for five minutes.
     4. Second and third EtOH washes: fill chambers with 70% EtOH (no stopper). Stir for five minutes.
     5. 100% EtOH wash: fill with 100% EtOH, slide stopper in, siphon off excess. Stir for 15 minutes.
  3. After last sample:
     1. Follow cleaning protocol as for 6b.
     2. After 100% EtOH wash, fill with 70% EtOH for storage.
     3. Slide stoppers in and cover with cover slips.

1. **Bradford assay.**
   1. See *Pierce Bradford Assay Kit* document.
   2. Methods are straightforward but need to be summarized here.
2. **Data extraction from Oroboros.**
   1. See *MiPNet24.06\_Oxygen\_flux\_analysis\_DatLab\_7.4* document.
   2. Methods need to be summarized here.