Measurement of oxidative phosphorylation

A. **Isolated mitochondria** (page 1-3)
B. **Intact cells** (page 4-6)
C. **Permeabilized cells** (page 7-9)

A. Measurement of oxidative phosphorylation in isolated mitochondria

To analyze oxidative phosphorylation (OxPhos) of isolated mitochondria the high-resolution respirometer OROBOROS Oxygraph-2K is used supplied with OROBOROS DatLab software for the data acquisition and analysis (Innsbruck, Austria) [1, 2]. The mitochondria are used in the amount of 0.4-0.8 mg/ml.

Procedure:

1. Fill the Oxygraph chambers with 2.2 ml of MiR05 respiration buffer. Under constant stirring the buffer is warmed to 37^{o}C and gets saturated with oxygen. Both oxygen concentration (blue) and rate of oxygen consumption (red) traces has to reach the steady state condition (horizontally aligned).
2. Add the mitochondria suspension in a volume of 10-50 µl. Immediately close the stoppers. Ensure no bubbles are formed in the buffer.
3. The mitochondria isolated from cultured cells have limited endogenous substrates and therefore will not respire until the substrates are added. However, mitochondria isolated from most of the tissues will immediately start consuming oxygen reaching state 1 in which the rate limiting factor is ADP [3].
4. To assess the rates of different respirometric complexes sequential stimulation of mitochondria with low and high amounts of ADP is applied in the presence of different metabolic substrates. For complex I add 10 mM glutamate, 10 mM pyruvate, or 10 mM L-lactate, all with 2 mM malate. For complex II add 10 mM succinate, along with the inhibitors of NADH dehydrogenase (1 µg/ml rotenone), adenine nucleotide transporter (5 µM carboxyatractilozide).

There are certain approaches to evaluate OxPhos in cell and tissue samples [4-7]. The one presented in **Figure 1** is based on the activation of the complex I first by adding the

corresponding substrates. Then the low amount of ADP is added to transiently activate OxPhos.

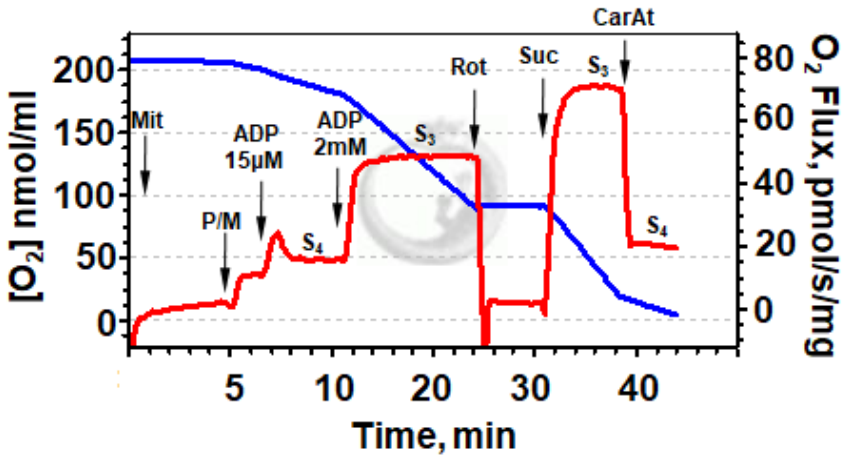

Figure 1 The respirogram demonstrates the protocol used to sequentially evaluate the OxPhos in complexes I and II. The 0.4 mg/ml mitochondria protein (Mit) was used per 2 ml oxygraphic chamber. The order of additions enables the examining of state 3 (S_3, active respiration) and state 4 (S_4, resting respiration) for complex I first. After inhibition of complex I with rotenone, the state 3 of complex II is obtained. To evaluate the state 4 of complex II, a high amount of ADP needs to be eliminated. This is achieved by inhibiting the adenine nucleotide transporter. Therefore, the order of state 3 and 4 for complexes I and II is opposite. *Abbreviations*: P/M, 10 mM pyruvate/2 mM malate; Rot,1μg/ml rotenone, an inhibitor of complex I; Suc, 10 mM succinate; CarAt, 5 μM carboxyatractilozide, an inhibitor of adenine nucleotide transporter.

The non-saturating low amount of ADP could vary in the range of 0.2-200 μM [6, 8, 9] to enable rapid phosphorylation of low amounts of ADP to ATP and maintain the resting respiration (state 4) by Chance [10]. For the mitochondria isolated from cultured cells that were found to have a limited reserve of endogenous substrates the 15-20 μM ADP was optimal to assess the state 4 respiration after a quick phosphorylation. The following addition of a high amount of ADP (2 mM) leads to a high rate of sustained respiration (state 3).

The complex II respiration is addressed similarly by the addition of succinate in a separate run.

However, complex II respiration can be measured in the same experiment along with evaluation of complex I. For that, as demonstrated in **Figure 1**, after obtaining the states 4 and 3 of the complex I, the complex is inhibited by the addition of its specific inhibitor

rotenone. Next, succinate is added to activate complex II respiration. Because the excess amount of ADP is present in the chamber, the achieved rate of respiration of the complex II corresponds to its state 3 (which is opposite to the order of states when only one complex is evaluated). To measure the state 4 of complex II, the high amount of ADP needs to be eliminated. For that, carboxyatractilozide is added to inhibit ADP entry through the adenine nucleotide transporter.

References

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B. **Measurement of oxidative phosphorylation in intact cells**

The experiments with intact cells can be performed using the cell growth medium. We use our custom-made buffer to have a better controlled environment. Cells are harvested by trypsinization and rinsed with and resuspended in a modified Krebs buffer:

Extracellular Buffer

Chemical	**Mw, g**	**[C], mM**	**Amount, g/500ml**	**Amount g/300ml**	**Sigma Catalog #**
NaCl	58.44	137	4	2.4	60129
KCl	74.55	2	0.075	0.045	S7653
$MgCl_2x6H_2O$	203.30	2	0.203	0.122	M9272
KH_2PO_4	136.09	1	0.068	0.04	60216
MOPS	209.26	20	0.29	0.174	M1254
$CaCl_2$	110.98	2	0.111	0.067	429759

Buffer Osmolarity: ~311 mOsm Adjust **pH 7.4** at 37°C with NaOH

The recommended amount of cells per chamber is in the range of 1-5 mln cells/ml. The number can vary depending on cell type. Thus, cancer cells are highly oxidative and 1 mln/ml cells give a good respiratory signal. However, injured cells or none-cancerous cells, that have lower mass of mitochondria per cell and lower rates of respiration, have to be taken in amount of 2-3 time higher to obtain sufficient respiratory signal. Muscle cells are highly oxidative and during the experiment opening the chamber for a short time to additionally infuse the buffer/medium with oxygen is required [1].

Due to a limited permeability of the plasma membrane to the mitochondria effectors, the basic protocol used to evaluate respiration of intact cells differs from that of isolated mitochondria. **It is based on sequential challenging of cells with inhibitor of ATPase oligomycin and following stepwise titration with low doses of protonophore FCCP** (Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone) or **CCCP** (Carbonyl cyanide m-chlorophenyl hydrazone). This protocol is presented on a respirogram in **Figure 2**.

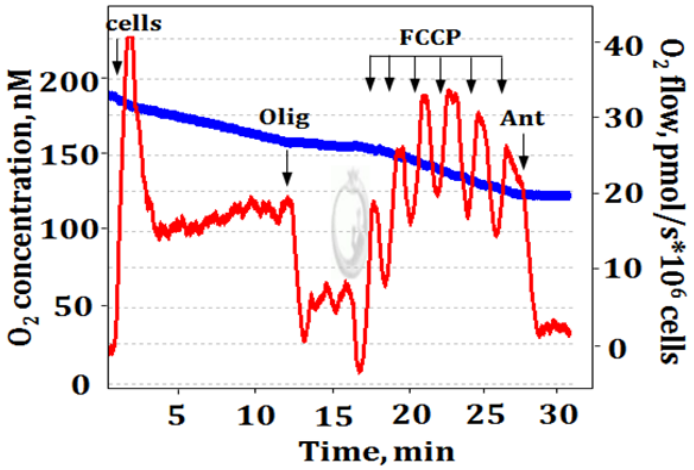

Figure 2 The respirogram for evaluation of OxPhos in intact cells. *Abbreviations*: *Basal* unperturbed respiration; *Leak*, oligomycin inhibited residual respiration; *Uncoupled*, the protonophore stimulated respiration, reaching the maximal respiratory capacity of mitochondria, obtained by stimulation with 20 nM steps of FCCP until the highest rate of respiration is achieved.

Procedure:

1. Fill the Oxygraph chambers with 2.2 ml of modified Krebs buffer. Under constant stirring the buffer is warmed to 37^{o}C and gets saturated with oxygen. Both oxygen concentration (blue) and rate of oxygen consumption (red) traces have to reach the steady state condition (horizontally aligned).
2. Add the desired amount of cells (counted and resuspended in no more than 50 µl of buffer/medium right before measurements). Once cells are added to the chambers, the stoppers need to be closed as soon as possible, as cells start consuming the oxygen immediately.
3. Wait until rate of oxygen consumption gets stabilized (red traces become horizontal). This is a *Basal* rate of respiration.
4. Add 1 µg/ml oligomycin to inhibit ATP synthase. This enables evaluation of the proton *Leak* across the mitochondria inner membrane [2]. Wait until the red traces become horizontal (get stabilized).
5. Add protonophore FCCP (or CCCP) by 20 nM steps until the activated respiration starts declining.

Protonophore “substitutes” the inhibited ATPase and allows protons to be delivered back to the matrix stimulating respiration to its maximal rate. This is an uncoupled respiration, meaning that the oxygen consumption is not associated with the synthesis of ATP. After reaching the maximum possible respiration rate, the oxygen consumption declines as the higher doses of protonophore deplete the inter-membrane proton reservoir. The maximal respiratory capacity can vary in different cell types based on their metabolic activity and pathologic changes. Therefore, it is important to do low dose protonophore titration rather than challenging cells with one high dose of FCCP (or CCCP).

This protocol enables the evaluation of the changes in the activity of ATPase and the electron transport enzymes along with the alterations in the membrane lipid bilayer.

References

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C. Measurement of oxidative phosphorylation in permeabilized cells

The intact cells do not allow for the studying of the full range of mitochondria functions due to impermeability of the plasma membrane to most of the mitochondria modulators. The use of permeabilized cells is a solution. This method helps to preserve intracellular mitochondria heterogeneity and mitochondria network integrity along with the mitochondria physical and functional connection with other intracellular components, including ER and cytoskeleton. Yet, it is suitable for studies of very small biological samples, which do not allow the isolation of mitochondria. The limitation is that only the active state 3 (but not state 4) respiration of mitochondria enzymes can be addressed. **Figure 3** demonstrates the permeabilized cells protocol [1].

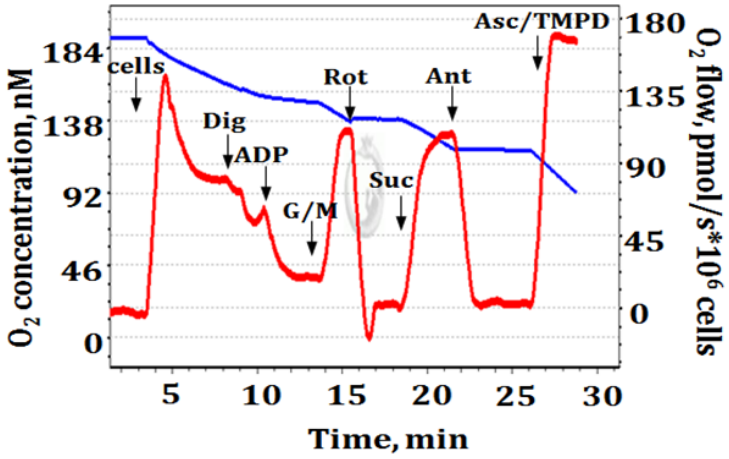

Figure 3 The respirogram demonstrates sequential evaluation of mitochondria complexes I, II, and IV in digitonin permeabilized cells. *Abbreviations*: Dig, digitonin; G/M, glutamate/malate; Rot, rotenone; I, Suc, succinate; Ant, antimycin; ASC/TMPD, ascorbate/ N,N,N′,N′-Tetramethyl-p-phenylenediamine.

Due to a compromised, by detergent (digitonin or saponin), plasma membrane, the growth medium cannot be used in this study as its ionic composition differs significantly from the intracellular environment; especially harmful for mitochondria is the 2 mM concentration of calcium that will cause immediate irreversible dissipation of the membrane potential. The buffer used in permeabilized cell experiments mimics the mitochondria ionic habitat.

The level of calcium ions must be strictly controlled! The intracellular buffer composition is as follows:

Intracellular Buffer

Chemical	Mw, g	[C], mM	Amount, g/500ml	Amount g/300ml	Sigma Catalog #
KCl	74.55	120	4.47	2.68	60129
NaCl	58.44	10	0.29	0.174	S7653
$MgCl_2x6H_2O$	203.30	2	0.203	0.122	M9272
KH_2PO_4	136.09	1	0.136	0.082	60216
MOPS	209.26	20	2.092	1.255	M1254
EGTA	380.35	1	0.190	0.114	03777
$CaCl_2$	110.98	0.7	0.039	0.023	429759

Buffer Osmolarity: ~290.1 mOsm Adjust **pH 7.2** at 37°C with KOH

Store the buffer at 4 or -20°C preferably in glass bottles

To control calcium concentration or to vary it with accordance to experimental tasks, the calcium is added in the presence of its chelator EGTA. The MaxChelator program is available to calculate $CaCl_2$ and the corresponding EGTA concentrations:
https://web.stanford.edu/~cpatton/downloads.htm

Procedure:

1. Fill the Oxygraph chambers with 2.2 ml of intracellular buffer. Under constant stirring the buffer is warmed to 37°C and gets saturated with oxygen. Both oxygen concentration (blue) and rate of oxygen consumption (red) traces have to reach the steady state condition (horizontally aligned).
2. Add the desired amount of cells (counted and resuspended in no more than 50 µl of buffer/medium right before measurements). Once cells are added to the chambers, the stoppers need to be closed as soon as possible, as cells start consuming the oxygen immediately.
3. Wait until the rate of oxygen consumption gets stabilized (red traces become horizontal). This is a *Basal* rate of respiration.

4. Add 10 μM digitonin. The optimal concentration of digitonin has to be obtained experimentally for each type of cell to ensure that only plasma membrane and not the mitochondria membrane is compromised. The respiration rate will drop due to the disturbance caused by permeabilization and dilution of the intracellular pool of adenine nucleotides. Wait until the signal is stabilized (red traces become horizontal).
5. Add a non-limiting amount of ADP (1-2 mM) and then respiratory substrates to the complex I, 10 mM glutamate, 10 mM pyruvate, or 10 mM L-lactate, all with 2 mM malate. The complex I respiration will achieve its maximal rate. Wait for the red trace to become horizontal.
6. Inhibit complex I with 1 μg/ml rotenone. Wait for the signal to get stabilized and add succinate to activate the complex II. Wait for the maximal complex II respiration.
7. Add 2.5 μM antimycin to inhibit the complex II. When the signal is stable, add the complex IV substrates 1mM Ascorbate and the artificial electron donor TMPD (N,N,N′,N′-Tetramethyl-p-phenylenediamine) that transfers electron to cytochrome *c*.

This protocol can be used without the addition of inhibitors to obtain combinatorial activities of the complexes. In addition, the combination of substrates can be used depending on the experimental tasks.

References

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