

Functional assays

The following pages describe a very simple protocol to obtain classical data about the mitochondrial function using a clark electrode.

1. Before the addition of mitochondria

Calibrate the response of your clark electrode using a reducing agent such as dithionite hydrosulfite.

2. Preparation of the oxygraphic vessel

Add 3 ml of the buffer designed to study oxidative phosphorylation presented in the second part of the

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«buffers composition» section. If you want to study the oxidative phosphorylation supported by complexe I, add Glutamate (5mM) + Malate (1mM) or Pyruvate (5mM) + Malate (1mM). If you want to study oxidative phosphorylation supported by complexe II, add succinate (5mM) + Rotenone ($2\mu\text{g.ml}^{-1}$, an inhibitor of complexe I used to avoid reverse electron transfer from complexe II to I). You can also study the functioning of the respiratory chain supported by both complexe I and II by adding Pyruvate or Glutamate (5mM) + Succinate (5mM) + Malate (1mM). Add P1,P5-Di (adenosine-5)pentaphosphate (AP5A, $20\ \mu\text{M}$) to inhibit the activity of the

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adenylate kinase. This enzyme binds to external mitochondrial membrane and can use 2 ADP to make 1 ATP and 1 AMP, and reciprocally. Consequently this enzyme needs to be inhibited to correctly study the oxidative phosphorylation. Add 60 μM of ADP to prepare the warm-up of the oxidative phosphorylation.

Remarque: If possible, avoid to use the same vessel for the study of complexe I and complexe II supported oxidation rates because rotenone is highly difficult to remove.

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3. Injection of mitochondria

When your vessel is ready, you can add your mitochondria to obtain a final concentration of mitochondrial protein in the vessel closed to 0.3 mg/ml. As ADP is already present in the vessel, the first oxidation rate you will obtain is quite fast. A short moment after, you will observe a decrease of the oxidation rate meaning that all the added ADP is now phosphorylated. Your mitochondria reached the state IV oxidation rate (i.e. oxidation rate when mitochondria are at rest, and more precisely when mitochondria are not phosphorylating).

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4. Determine the maximal ADP-induced oxidation rate

To determine the maximal ADP-induced oxidation rate, add $500\mu\text{M}$ of ADP when you are using complex I substrates or $250\mu\text{M}$ of ADP when you are using succinate. You will observe a fast increase of the oxidation rate: your mitochondria reached the state III oxidation rate (i.e. the oxidation rate when mitochondria phosphorylate at its maximal activity). After several minutes (depending on the amount of mitochondria added at the onset of the experiment), you will observe a decrease of the oxidation rate: your mitochondria reached again the state

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IV oxidation rate. If you want, you can perform an other addition of ADP.

5. Determine the maximal activity of the respiratory chain

Even though during the state III oxidation rate the membrane potential decreases, it always exerts an inhibitory effect on the respiratory chain activity. So, to investigate the maximal oxidation rate of your mitochondria, you need to release this inhibition by collapsing the membrane potential using an uncoupler. You therefore have to add carbonyl cyanide m-chlorophenylhydrazone (CCCP, $1\mu\text{M}$) for example to abolish the membrane

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potential. The resulting oxidation rate corresponds to the maximal activity of the respiratory chain.

6. Extract useful information from this simple experiment

By following the described protocol you will obtain the state III and state IV oxidation rates. I added the figure 1 for clarity. State III oxidation rate is used to approach the maximal capacity of the oxidative phosphorylation. State IV oxidation rate gives you an idea of the properties of the inner mitochondrial membrane, since the respiratory chain activity during state IV is only dedicated to compensate the

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proton leaks accross the inner mitochondrial membrane.

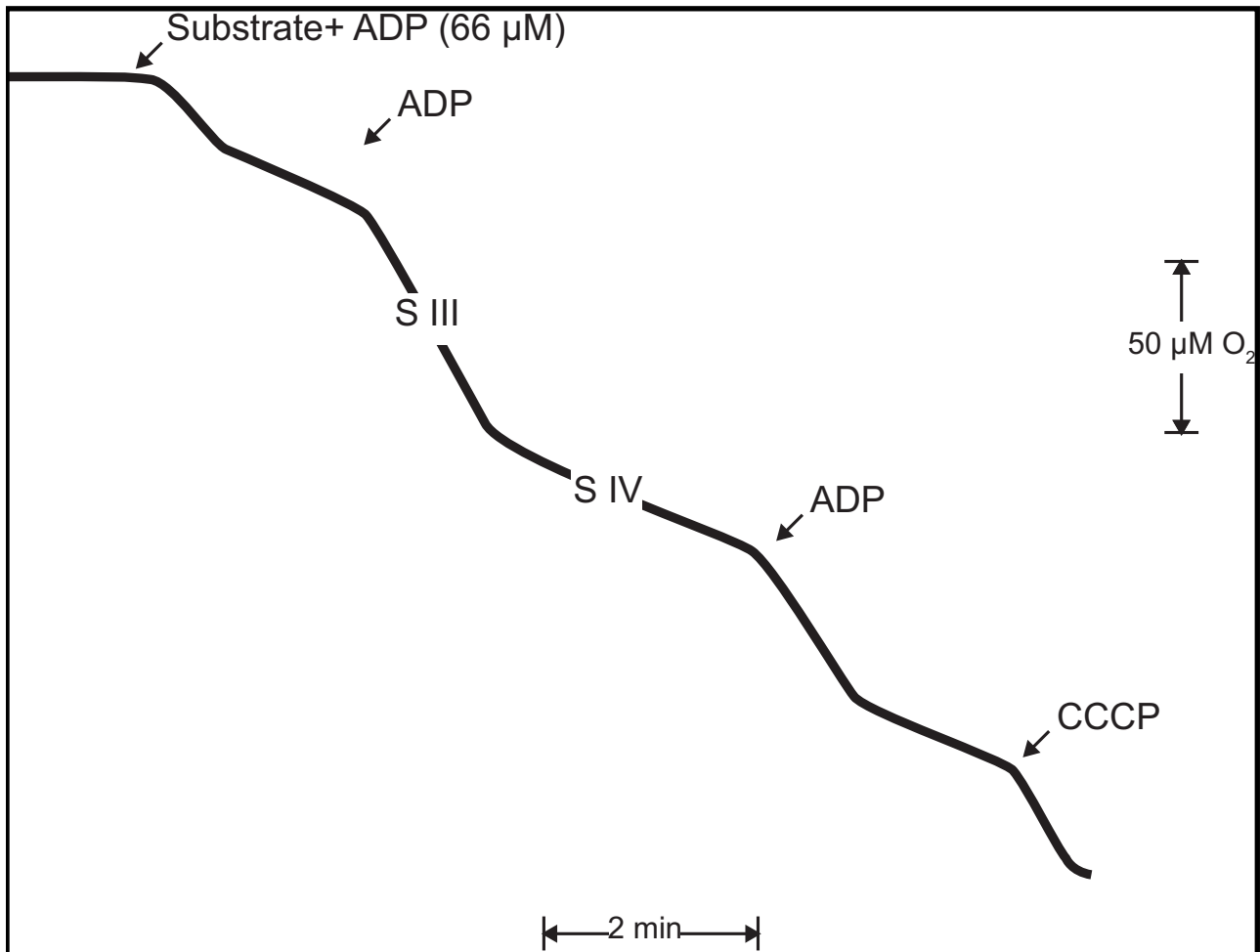


Figure 1 : Example of classical oxygraphic recording of respiratory chain activity.

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By dividing the state III oxidation rate by the state IV one, you can determine the respiratory control ratio (RCR):

$$\text{RCR} = \text{State III} / \text{State IV}$$

This ratio will give you an idea of the integrity of your mitochondria. For example, if you are studying mitochondria isolated from muscle of adult rats when succinate is used as substrate, your RCR should be approximately equal to 5 to ensure the integrity of your preparation.

The oxidation rate obtained when an uncoupler is added gives you the

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maximal capacity of the respiratory chain.

Finally, as you know the amount of ADP you added to obtain the state III oxidation rate, and as you can easily determine the amount of oxygen used by your mitochondria to phosphorylate this amount of ADP, you can calculate the ADP to O ratio by the following equation:

$$\text{ADP/O} = \text{Q.ADP}/(\text{Q.O}_2 * 2)$$

where Q.ADP is the amount of added ADP and Q.O₂ is the amount of O₂ used by your mitochondria during state III.

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This ratio will allow you to determine how many moles of oxygen are required for the phosphorylation of one mole of ADP when mitochondria are under state III conditions. In other words, you will obtain the maximal coupling efficiency of your mitochondria.

By following this simple protocol, you have now determined important parameters of the oxidative phosphorylation functioning.

The next point will give you a list of inhibitors frequently used in the oxygraphic field to obtain useful data about the mitochondrial function.

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7. List of inhibitors and their respective targets

Inhibitor	Target
Rotenone	Complexe I
Malonate	Complexe II
Antimycin A	Complexe III
myxothiazol	Complexe III
KCN	Complexe IV
Oligomycin	ATP synthase
Atractyloside	ANT

Hope you found/will find this protocol useful!