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O2k-Fluorometry: HRR and H_2O_2 production in mouse brain mitochondria.

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1. Introduction

High-resolution respirometry [1] is extended by O2k-MultiSensor modules allowing the simultaneous measurement of oxygen consumption and additional parameters (mt-membrane potential, pH, Ca^{2+}, NO [2-5]). Fluorometric methods are available for a wide range of analytical parameters of major interest in mitochondrial physiology: H_2O_2, mt-membrane potential, intracellular pH, Ca^{2+}, Mg^{2+} , and NADH levels.

Several highly important advantages are offered by the *simultaneous* measurement of multiple parameters, particularly in a single respirometric chamber, or in multiple O2k-Chambers operated in parallel:

- Quality control of mt-preparations by respirometric performance.
- Optimization of protocols, i.e. evaluation of inhibitory or uncoupling effects of TPP^+, safranin or other fluorophores; evaluation of effective concentrations of applied substrates, uncouplers and inhibitors.
- Temperature and oxygen levels are controlled. The O_2 dependence can be studied, particularly important for H_2O_2 production.
- Elimination of artifacts of normalization for a mitochondrial marker when combining two methods in a single chamber.
- Additional information is acquired for a limited amount of sample.
- Very economical: The O2k-Core with O2k-Fluorescence LED2-Module offers an inexpensive alternative to conventional fluorometers.

The O2k-Fluorescence LED2-Module is introduced here with an application for the simultaneous measurement of respiration and H_2O_2 production of mouse brain mitochondria.

2. The O2k-Fluorescence LED2-Module

The O2k-Fluorescence LED2-Module is a LED and filter based fluorometry add-on module of the O2k [3]. Optical sensors are inserted through the front window of the O2k chambers (Fig. 1), for measurement of hydrogen peroxide production (Amplex® UltraRed), ATP production (Magnesium green™), mt-membrane potential (Safranin), Ca^{2+} (Calcium green™), and numerous other applications open for O2k-user innovation [4].

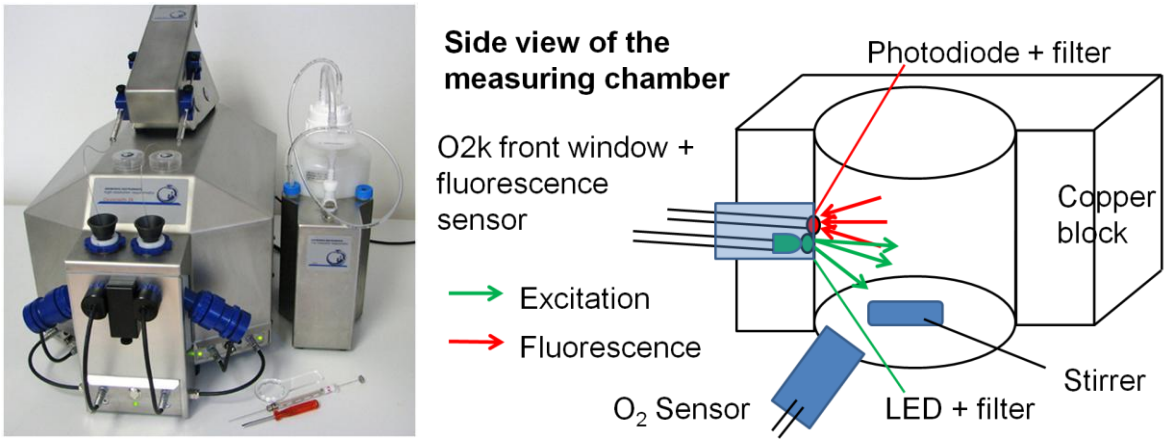

Figure 1. OROBOROS Oxygraph-2k with O2k-Fluorescence LED2-Module. Excitation: Green LED (520 nm) or blue LED (465 nm) with short pass filter. Detector: Photodiode and long pass filter. Recording: Amperometric channel of Oxygraph-2k and DatLab.

3. Filter Optimization

Excitation and emission filters were selected for applications with Amplex® UltraRed to ensure that only emission (linear relationship with analyte concentration, positive signal) but not absorption phenomena (logarithmic relationship with analyte concentration, negative signal) contribute to the signal change detected by the photodiode (Fig. 2).

The photodiode (Fig. 2A) was replaced by a 600 µm light guide, connected to a mini spectrometer. Fluorescence was induced by the addition of resorufin, which is the reaction product of H_2O_2 formed with Amplex® UlraRed in the presence of horse raddish peroxidase (Fig. 2B). Difference spectra for 0 and 3 µM resorufin in 100 mM potassium phosphate buffer (pH=7) were obtained for different filter configurations (Fig. 2B-D).

The same approach was applied to select suitable filter combinations for applications with safranin (mt-membrane potential [5]), Magnesium green™ (ATP production) and Calcium green™ (free Ca^{2+}).

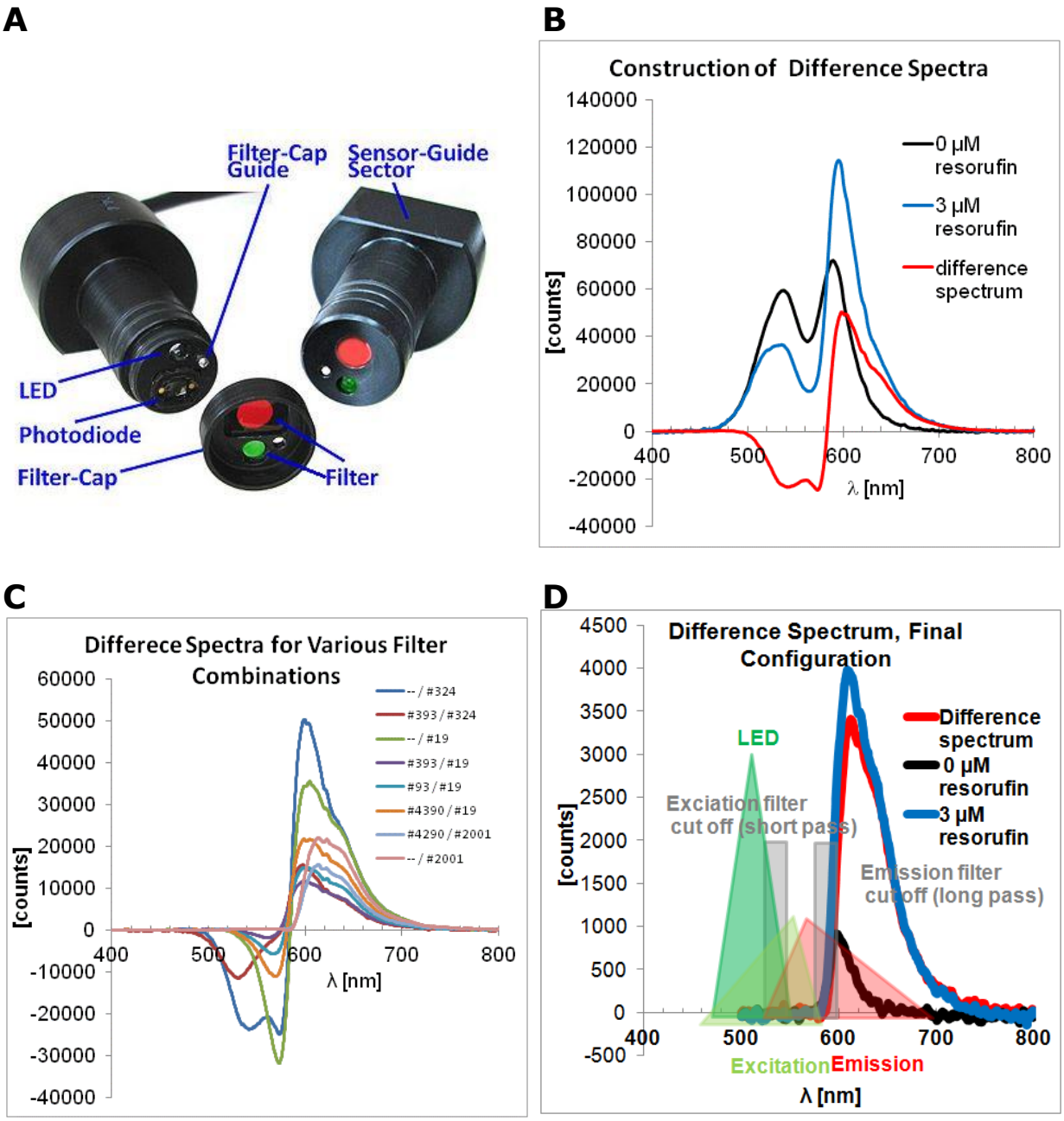

Figure 2. Optimization of Fluo-Sensor filters for dectection of H_2O_2.
A: Fluo-Sensor Green, showing the light source (LED), the detector (photodiode), the excitation filter (green, covering the LED), and the emission filter (red, covering the photodiode).
B: The difference spectrum obtained with an initial filter combination reveals a distinct absorption (negative) contribution.
C: Difference spectra of different filter combinations.
D: The finally optimized filter configuration: The difference spectrum (red) shows no negative contribution. The spectra are overlaid with schematic representations of the LED spectrum (dark green), the excitation (green) and emission (red) spectra of resorufin and the cut-off regions of the excitation and emission filters (grey), respectively.

4. Application: Respiration and H_2O_2 production

Fig. 3 shows an example of OXPHOS analysis in isolated mouse brain mitochondria with a coupling/substrate control SUIT protocol [1,6].

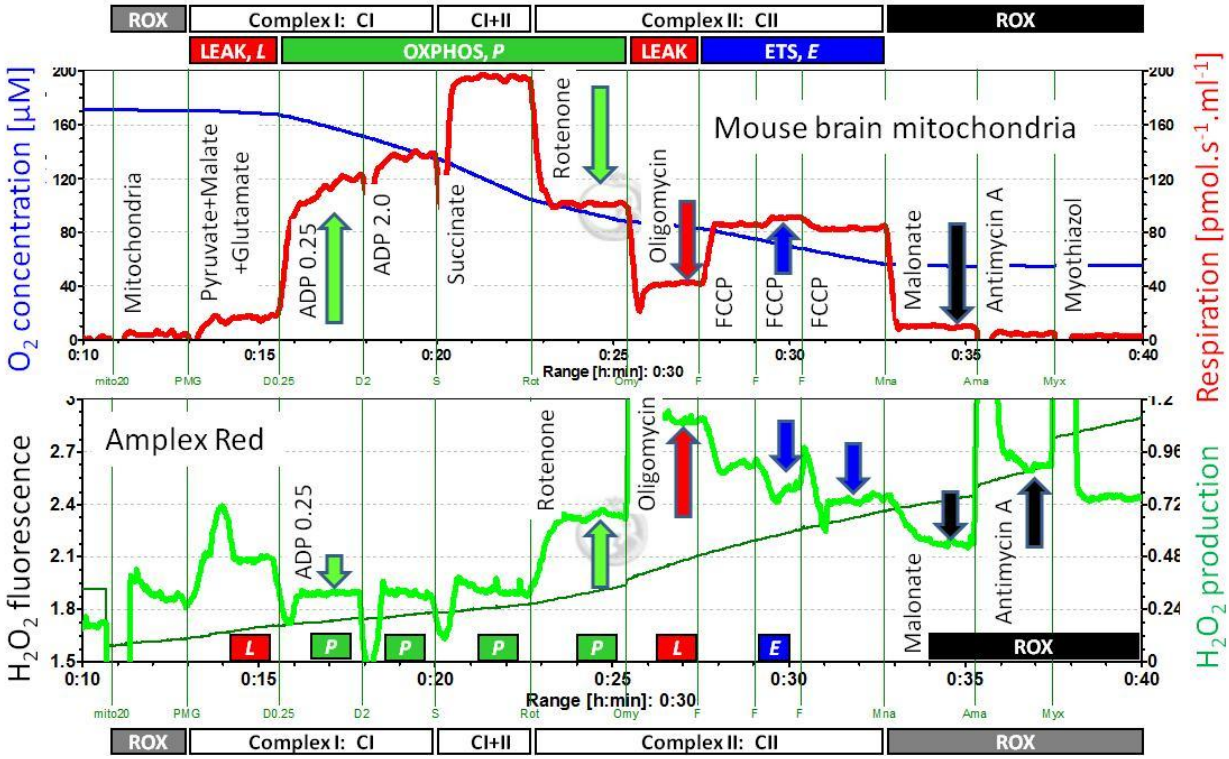

Figure 3: Simultaneous measurement of respiration (top) CI-linked in the LEAK state followed by ADP titration, and substrate control in the OXPHOS state from CI, CI+II- to CII-linked states, sequential oligomycin and FCCP titration and inhibition to the ROX state. H_2O_2 production (bottom) is independent of respiratory rate and is a function of metabolic state, decreasing with stimulation by ADP and uncoupling (FCCP), but increasing with inhibition by rotenone, oligomycin and Antimycin A.

5. References

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6. Acknowledgements

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