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O2k-Fluorometry – a *MitoCom* Project

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high-resolution respirometry
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1. Introduction

High-resolution respirometry [1] is extended by MultiSensor techniques allowing the simultaneous measurement of oxygen consumption and one additional parameter (mitochondrial membrane potential, pH, Ca^{2+}, or NO concentration [2, 3]). Fluorometric methods are available for a wide range of analytical parameters of major interest in mitochochondrial physiology: H_2O_2 production, mitochondrial membrane potential, intracellular pH, Ca^{2+}, Mg^{2+} , and NADH levels.

Advantages of the simultaneous measurement of additional parameters in a single respirometric chamber:

- Quality control of preparations by respirometric performance.
- Objective exclusion criteria for protocol related problems, e.g. overtitration of uncouplers, side effects of TPP^+ or fluorophores.
- 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.
- Oxygen dependence of parameters can be studied.

Here we introduce the O2k-Fluorescence LED2-Module, describe one selected stage in its development, and show its 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 to be used together with the O2k [5]. Optical sensors are inserted through the front window of the O2k chambers, 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.

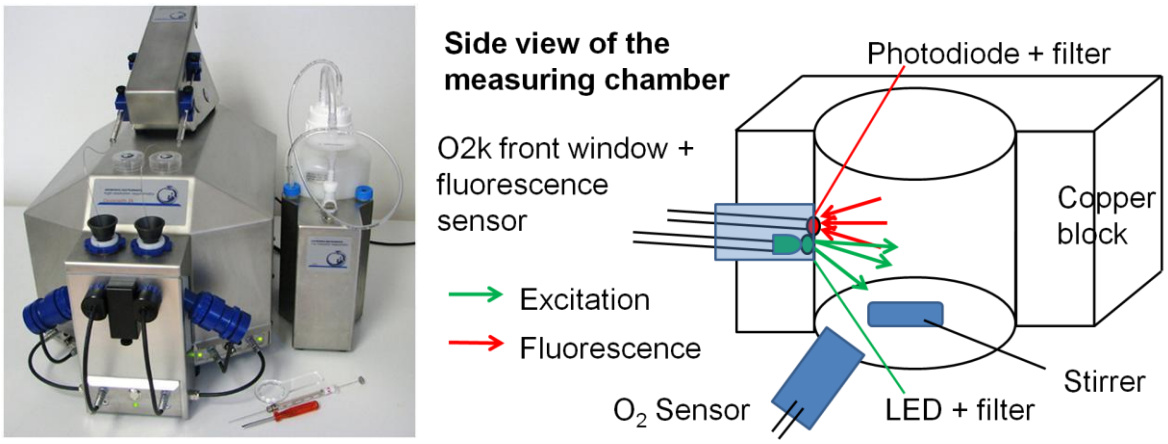


Fig. 1. OROBOROS Oxygraph-2k with O2k-Fluorescence LED2-Module, Excitation: green (520 nm) or blue (465 nm) LED + short pass filter; detector: photodiode + long pass filter, recording: amperometric channel of Oxygraph-2k.

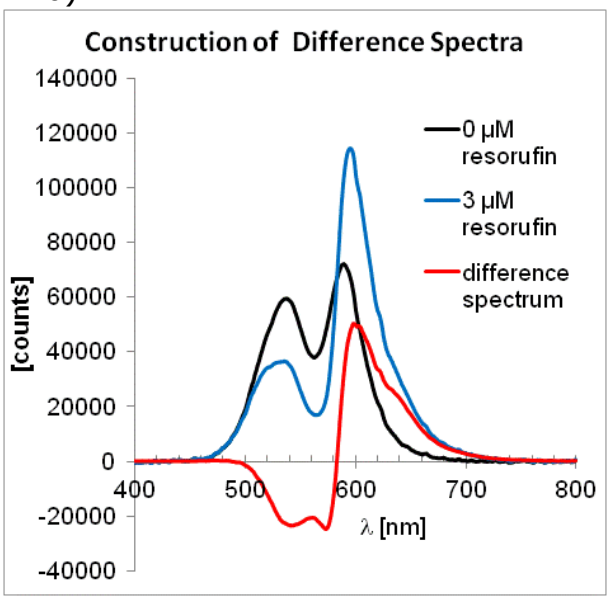
3. Filter Optimization

The challenge was to select excitation and emission filters for the application "Amplex® UltraRed" that 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.

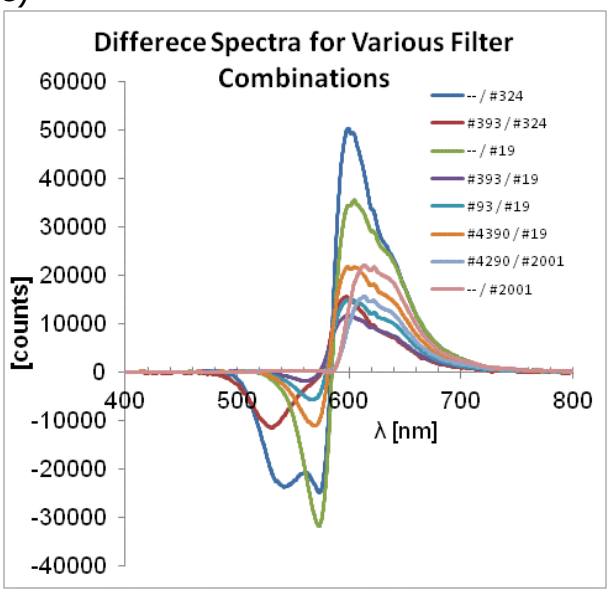
Method: The photodiode in the set up shown in Figure 1 was replaced by a 600 µm light guide, connected to a mini spectrometer. Fluorescence was induced by the addition of resorufin, the reaction product of Amplex® UlraRed and H_2O_2. Difference spectra for c (resorufin) = 0 and 3 µM in 100 mM potassium phosphate buffer (pH=7) were obtained for different filter configurations, see Figure2.
The same approach was followed to find suitable filter combinations for the other featured applications.

a)

b)



c)



d)

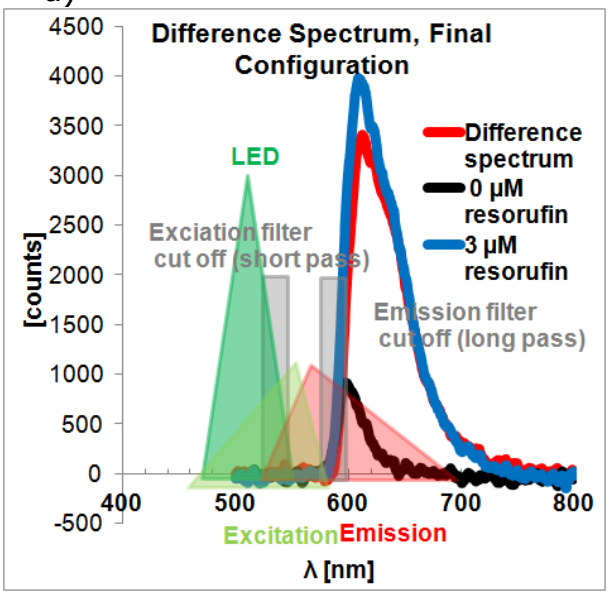


Figure 2.
a) Fluorescence Sensor Green, showing the light source (LED), the detector (photodiode), the excitation filter (green, covering the LED), and the emission filter (red, covering the photodiode). **b)** Construction of difference spectra: The difference spectrum from an initial filter combination reveals a distinct absorption (negative) contribution. **c)** Difference spectra of different filter combinations. **d)** Finally selected 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. For detailed explanations of the substrate-uncoupler-inhibitor-titration (SUIT) protocol, see ref. [1,6].

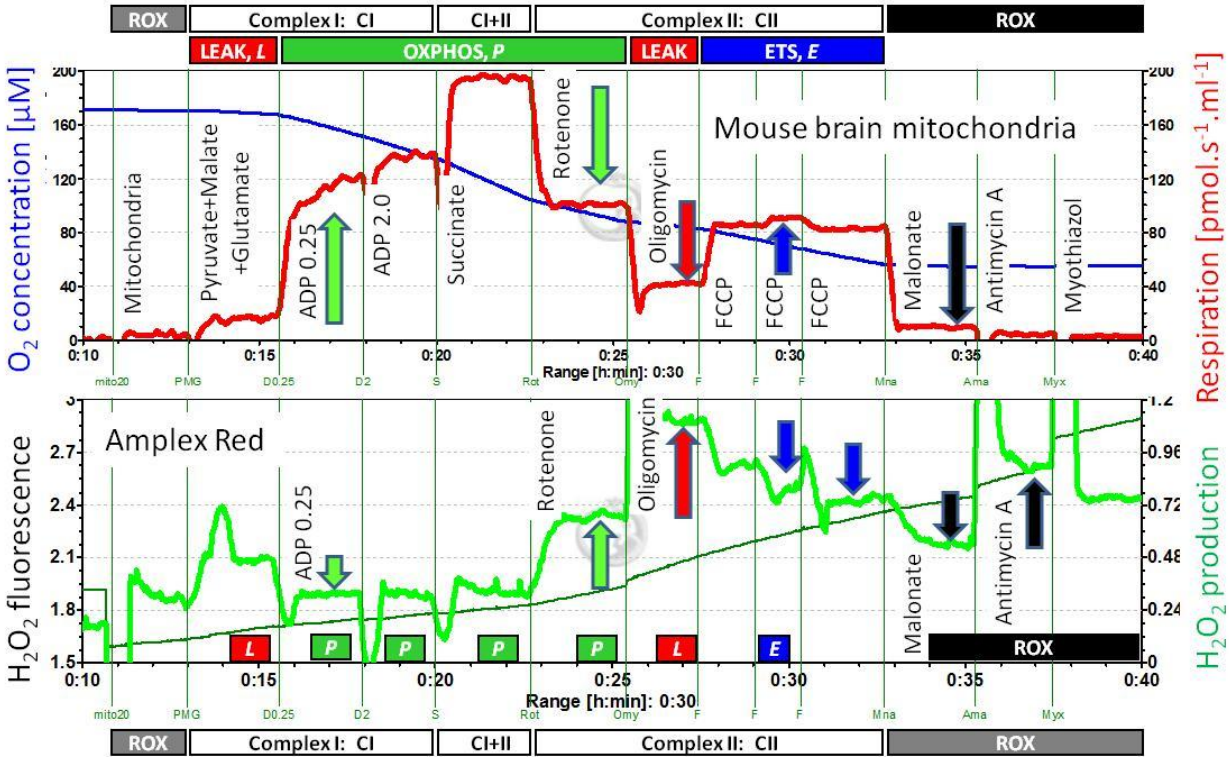


Fig. 3: Simultaneous measurement of respiration (top) CI-linked in the LEAK state followed by ADP titration, and substrate control in the OXPHOS state from CI, CI+II- to CII-linked states, sequential oligomycin and FCCP titration and inhibition to the ROX state. H_2O_2 production is independent of respiratory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

5. References

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6. Acknowledgements

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