



O2k-Fluorometry

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O2k-Fluorometry: HRR and simultaneous determination of mt membrane potential with safranin.

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1. Introduction

The general methodological outline, potential caveats to be observed, and an example of a hypothetical application of combined high-resolution respirometry (HRR) and fluorescence-based determination of mitochondrial transmembrane potential ($\Delta\Psi_{mt}$ or mtMP) are described in the publication by Krumschnabel et al. [1]. Here, a step-by-step description of data acquisition and analysis is described based on the example given in Fig. 9.6 of the publication.

2. The O2k Demo experiment

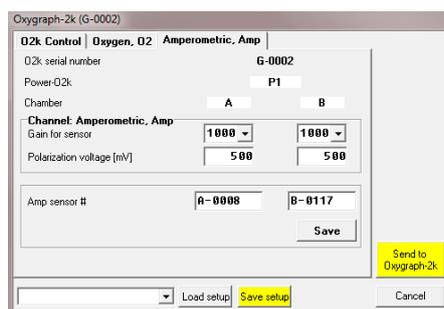
A substrate-uncoupler-inhibitor titration (SUIT) protocol is applied that examines Complex II-linked respiration of mouse brain first spin homogenate at different coupling states and the concurrent changes of mtMP as assessed by the use of safranin (Saf). Sample preparation as well as the characteristics of the different coupling states elicited by additions in the SUIT protocol are described in detail in [1], [2] and [3].

3. Setting the instrument for data acquisition

Instrumental setup and preparation with regard to measuring respiration are outlined in [4]. In addition, the O2k-Fluorescence LED2-Module

consisting of the Fluorescence-Control Unit and, in the case of using Saf, two Fluorescence Sensors Blue, are connected to the O2k (for details see: [5]) and switched on by pressing the power switch on the front panel of the Fluo Control Unit. Next, DatLab is started and the O2k Control window is opened by pressing F7.

Control of LED-intensity and Amplification: In the Control window the Amperometric tab window is opened and the sensor numbers are defined for documentation. Then, Polarization voltage and Gain for sensor are selected, which for the experiment to be described were 500 mV and 1000, respectively. In general, the ideal settings for polarization voltage



need to be determined in preliminary experiments. Settings are optimal when a signal is obtained which is both large enough to avoid limitation of resolution by digital noise but also well below the range maximum of 10 V, keeping in mind that the maximum signal obtained in the course of an experiment may be larger than the initial one but must also not exceed this value.

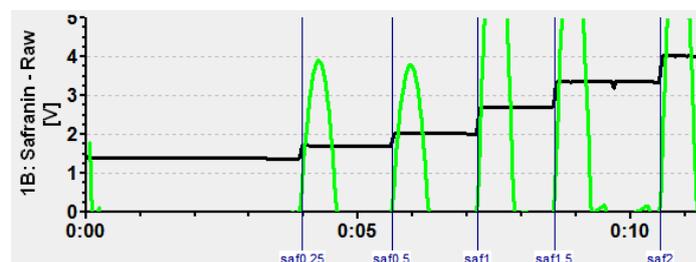
Selecting the appropriate Layout: During data acquisition the experimenter should be able to assess whether the signal obtained is within the desired range and if it stable or has attended a new steady state following any experimental manipulation. Thus, in the case of using Saf it is appropriate to initially select the layout 'Safranin raw signal' which displays data related to respirometry according to the standard layout '05 Flux per Volume corrected' and the Saf signal as plots of the raw signal 'Amp Raw' [V] just below each related plot of the oxygen signal.



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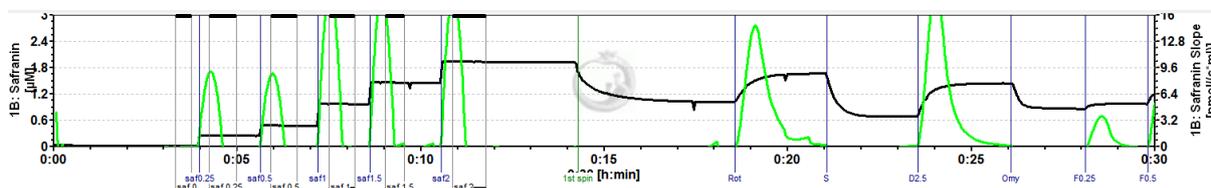
4. Data acquisition

Following these initial adjustments the chambers containing respiration medium MiR06Cr were closed and the chamber lights were switched off.



After recording a baseline, Saf was added from a 200 μM stock in 5 consecutive steps to obtain final Saf concentrations of 0.25, 0.5, 1, 1.5, and 2 μM . Given the excellent linearity of

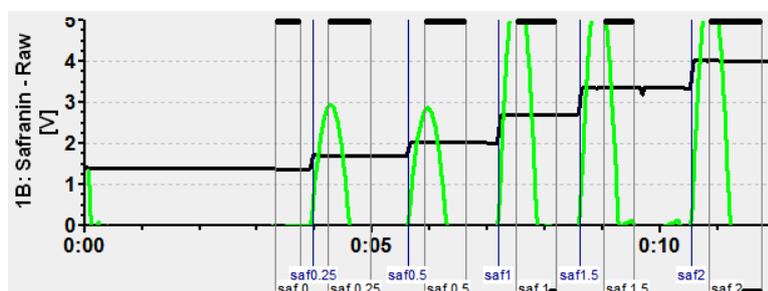
fluorescence signal over this concentration range (see Fig. 9.1 of [1]), a single addition of 2 μM Saf may be regarded sufficient if a simple conversion of the raw signal to Saf concentration is desired.



Subsequently, mouse brain first spin homogenate (Thom) was injected, resulting in an uptake of the dye and a corresponding decline of Saf fluorescence and this was followed by additions of SUIT chemicals causing changes in respiratory rate and of mtMP as described in [1]. If the experimenter wants to observe Saf fluorescence converted to Saf concentration in real-time, calibration can be done while the experiment is running. Alternatively, calibration can be done during data analysis, both following the same procedure as outlined next.

5. Data analysis

For calibration of the Saf signal the appropriate plot of the raw signal of chambers A or B are



selected and marks are set to data regions before and after stepwise addition of Saf. Clicking into the black bar on the top or bottom of the marks, each mark should be named according to

the final concentration of Saf obtained and the concentration entered in 'Value'. Next, the Calibration window is opened by selecting 'Calibration' and 'Amperometric, Amp' and the marks created are displayed in the center of the window. The marks desired to be included in the calibration

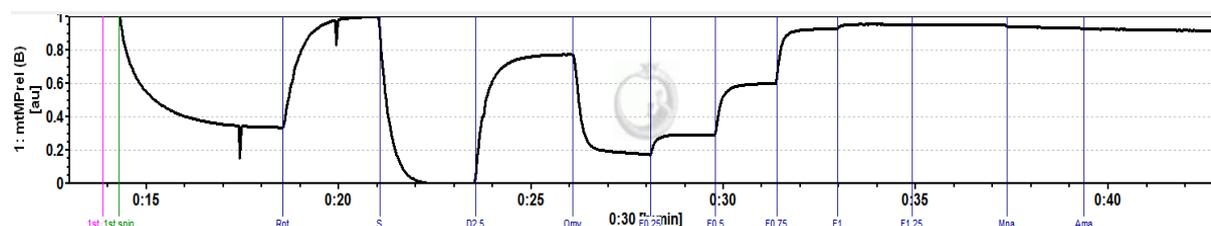
Select marks	Name	Time	Signal [V]	Slope	Conc.
<input type="checkbox"/>	saf 0				
<input checked="" type="checkbox"/>	saf 0.25	00:04:36	1.70603	-0.16779	0.25000
<input checked="" type="checkbox"/>	saf 0.5	00:06:16	2.02022	-0.12087	0.50000
<input checked="" type="checkbox"/>	saf 1	00:07:51	2.69305	-0.17955	1.00000
<input checked="" type="checkbox"/>	saf 1.5	00:09:17	3.36142	-0.25867	1.50000
<input checked="" type="checkbox"/>	saf 2	00:11:19	4.01059	-0.26207	2.00000

Gain [V/μA] 1000 Polarization voltage [mV] 500

Sensitivity [V/μM] 1.3659
Intercept [V] 1.3628 R²=0.99998

are selected by ticking the field next to each mark name and names and Saf concentrations will appear on the right side of the window. DatLab then calculates the parameters of a linear regression analysis through these data and the values for Sensitivity [V/μM] and

the Intercept as well as the coefficient of determination, R^2 , are displayed in the lower part of the window. For a visual inspection of the calibration the data can be viewed by pressing 'Show graph'. Individual calibration marks may now be selected or deselected as required and the final selection is then used for calibration by pressing 'Calibrate'. All data of the selected plot are now converted to Saf concentration and are being displayed as 'Safranin [μM]'. Conversion of changes in fluorescence or Saf concentration into mV changes of mtMP may be achieved using the K^+ selective ionophore valinomycin, adding known amounts of KCl solution and applying the Nernst equation, see [6].



Alternatively, the signal may be normalized to detect changes within minimum and maximum fluorescence observed during the experiment, which in the present experiments corresponds to the Saf signal elicited in the CII-linked LEAK state, $S(\text{Rot})_L$, and CII-supported ETS capacity, respectively. Regarding the latter, it should be kept in mind that complete collapse of mtMP may only be observed at an uncoupler concentration surpassing that required for stimulation of maximum uncoupled respiration which may actually already become inhibitory again. Of note, we found that fluorescence may show an apparent drift in the uncoupled state such that the observed maximum signal is not seen in the fully uncoupled state but during Rot-inhibited respiration of tissue homogenate, i.e. in the initial ROX state.

The step-by-step procedure to obtain this normalized plot is as follows:

- * Select plots to display the fluorescence signals as either raw signal or calibrated signal
- * Set marks on the traces corresponding to the minimum and maximum signal observed during the experimental part of the data, NOT the calibration part.
- * Display the marks and keep a note of the minimum and maximum value (or copy and paste marks to Excel)
- * Start the scripting function by pressing [ctrl+Alt+D]
- * Press 'Generate plot', then 'Load macro'; alternatively, if the macro is not available in your program, enter it manually as displayed below; in addition, on the right hand side of the macro window right mouse click on the channel '1B: Safranin - Raw' and select 'A' to name the variable for the macro function. Next, press 'Save macro' if you want to re-use it for subsequent experiment analysis.
- * Select 'mtMPrel(A).DLC' and open it

Edit macro

Name Unit

Editor | Debugger | Help

```

1 var min,max: Float;
2 min:= 2.3048;
3 max:=3.6393;
4
5
6 FuncResult:= (A-min) / (max-min);

```

Save macro | Load macro

- 1A: O2 Raw Signal
- 1A: O2 Concentration
- 1A: O2 Pressure kPa
- 1A: O2 Pressure mmHg
- 1A: O2 Slope uncorr.
- 1A: O2 Flux per V
- 1A: O2 Flux per unit
- 1B: O2 Raw Signal
- 1B: O2 Concentration
- 1B: O2 Pressure kPa
- 1B: O2 Pressure mmHg
- 1B: O2 Slope uncorr.
- 1B: O2 Flux per V
- 1B: O2 Flux per unit
- 1: Block Temp.
- 1: Barom. Pressure
- 1: Peltier Power
- 1A: pX - Raw
- 1A: pX
- 1A: pX - slope
- 1B: pX - Raw
- 1B: pX
- 1B: pX - slope
- 1A: Safranin - Raw
- 1A: Safranin
- 1A: Safranin - slope
- A: 1B: Safranin - Raw
- 1B: Safranin
- 1B: Safranin Slope

- * Enter noted values of min and max for Chamber A into the corresponding lines and click 'Update plot'
- * Press 'Generate plot', then 'Load macro'
- * Select 'mtMPrel(B).DLC' and open it
- * Enter noted values of min and max for Chamber B into the corresponding lines and click 'Update plot'
- * Finally, in the 'Select plot' menu tick 'Calc' '1: mtMPrel(A)' and '1: mtMPrel(B)' for displaying the normalized plots of chambers A and B, respectively.

6. References

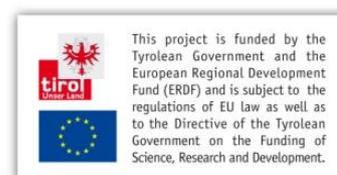
1. [Krumshnabel G, Eigentler A, Fasching M, Gnaiger E \(2013\) Combined high-resolution respirometry and fluorometry. Validation of safranin for determination of mitochondrial membrane potential. Methods Enzymol 542: 163-81.](#)
2. [Pesta D, Gnaiger E \(2012\) High-resolution respirometry. OXPHOS protocols for human cells and permeabilized fibres from small biopsies of human muscle. Methods Mol Biol 810: 25-58.](#)
3. [Gnaiger E \(2014\) Mitochondrial pathways and respiratory control. An introduction to OXPHOS analysis. 4th ed. Mitochondr Physiol Network 19.12. OROBOROS MiPNet Publications, Innsbruck: 80 pp.](#)
4. [Gnaiger E \(2014\) An experiment with high-resolution respirometry: Phosphorylation control in cell respiration. Mitochondr Physiol Network 10.04\(07\): 1-12.](#)
5. [MiPNet17.05 O2k-Fluorescence LED2-Module](#)
6. [Vercesi AE, Rodrigues CO, Uyemura SA, Zhong L, Moreno SN \(1998\) Respiration and oxidative phosphorylation in the apicomplexan parasite Toxoplasma gondii. J Biol Chem 273: 31040-7.](#)

7. Acknowledgements



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