O2k-FluoRespirometry: HRR and simultaneous determination of mt-membrane potential with safranin or TMRM

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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 Succinate-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]. All steps are equally valid when using TMRM, an alternative fluorophore suitable for detecting mtMP, an exception being the choice of different FluoSensors (see below).

## 3. Setting the instrument for data acquisition

Instrumental setup and preparation with regard to measuring respiration are outlined in [4]. Start DatLab and connect the instrument with the computer. In addition, the integrated Fluorescence-Control Unit must be activated by ticking 'Amperometric, Amp' in the O2k configuration window popping up upon starting DatLab and, in the case of using Saf, two Smart Fluo-Sensors Blue are connected to the O2k. When TMRM is used instead of Safranin, two Smart Fluo-Sensors Green are connected to the O2k. In either case, the Smart Fluo-Sensors will be recognized by the O2k and their series number is automatically displayed and stored in the O2k configuration window.

Control of LED-intensity and Amplification: In the O2k Control window the Amperometric tab window is opened and Polarization voltage and Gain for the sensors are selected, which for the experiment to be described were 230 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 not exceed this value.
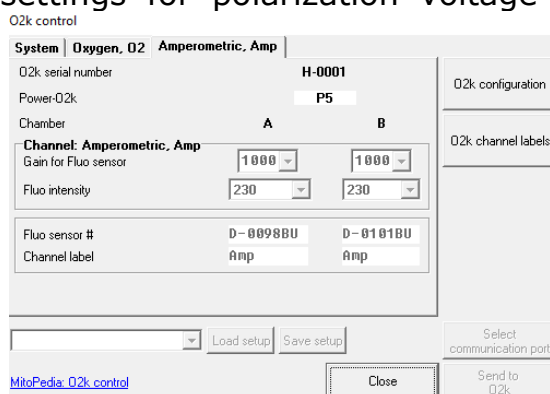
Selecting the appropriate Layout: During data acquisition the experimenter should be able to assess whether the signal obtained is within the desired range and if it is stable or has attended a new steady state following any experimental manipulation. Thus, in the case of using Saf it is appropriate to initially select the layout '01 Amp Amperometric_Raw signal' which displays data related to respirometry according to the standard layout '04a Flux per Volume' and the Saf signal as plots of the raw signal 'Amp raw' [V] just below each related plot of the oxygen signal. The Y-axis scaling may need adjustment, depending on the gain and polarization voltage chosen.

4. Data acquisition
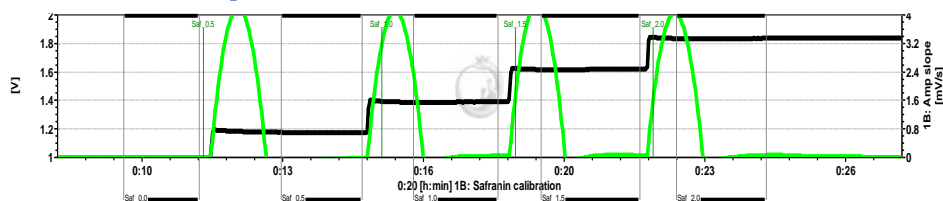
Following these initial adjustments the chambers containing respiration medium MiR06Cr were closed and the chamber lights ('Illumination in chamber' in the O2k Control window) were switched off. After recording a baseline, Saf was added from a 500 µM stock solution in

4 consecutive steps to obtain final Saf concentrations of 0.5, 1, 1.5, and 2 µM in the 2-mL O2k-Chamber. 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 suffcient if a simple conversion of the raw signal to Saf concentration is desired.
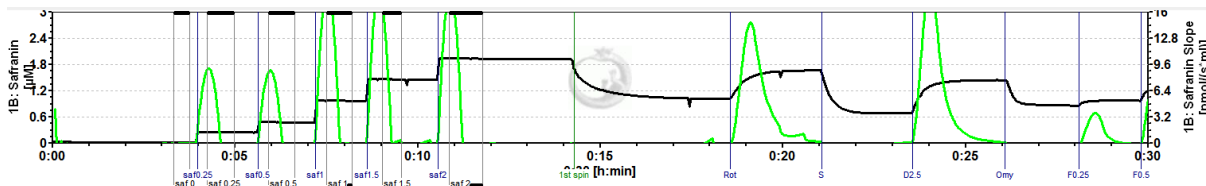
Subsequently, mouse brain first spin homogenate (Thom) was injected, resulting in an uptake of the dye and a corresponding decline of Saf fluorescence and this was followed by additions of SUIT chemicals causing changes in respiratory rate and of mtMP as described in [1]. If the experimenter wants to 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 chamber A or B are selected and marks are set to data regions before and
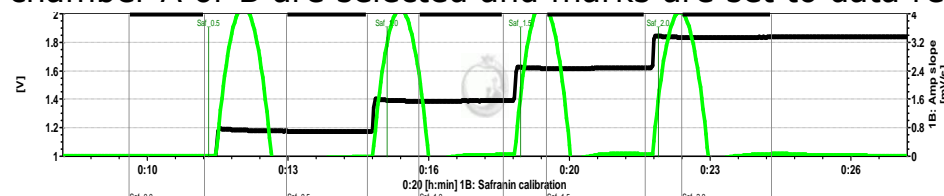
after stepwise addition of Saf. Clicking into the black bar on the top or bottom of the marks, each mark should be named according to the final concentration of Saf obtained and the concentration entered in 'Final conc.'. Next, the Calibration window is opened by selecting 'Calibration' and 'Amperometric, Amp' and the marks created are displayed in the center of the window.

The marks desired to be included in the calibration are selected by ticking the field next to each mark name. The mark 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], the Intercept and 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 'Amp raw [µM]'. To change the Y-axis label, select menu 'Oroboros O2k' and then 'O2k channel label'. 'Amperometric, Amp Channel label' can now be changed to e.g. 'Safranin' and data will be 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].
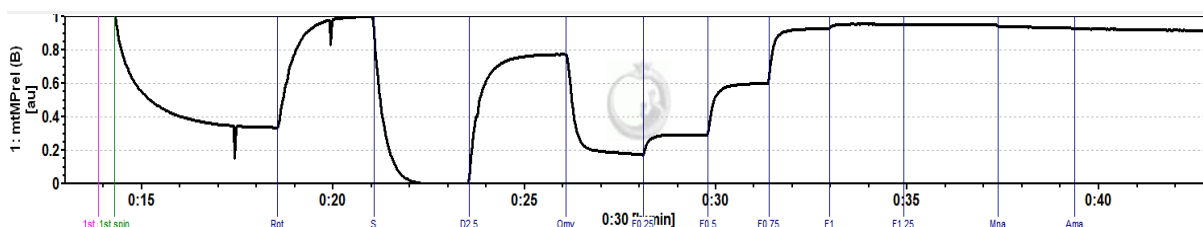
Here we can only provide instructions on how the signal can be normalized to detect changes within minimum and maximum fluorescence observed during the experiment, which in the present experiments corresponds to the Saf signal elicited in the Succinate-linked LEAK state, $S(Rot)_L$, and Succinate-supported electron transfer (ET) capacity, respectively. Regarding the latter, it should be kept in mind that complete collapse of mtMP may often only be observed at an uncoupler concentration surpassing that required for stimulation of maximum uncoupled respiration which may 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'; if the macro is not yet available in your program, enter it manually as displayed below; enter values of min and max as determined for Chamber A into the corresponding lines; in addition, on the right hand side of the macro window right mouse click on the channel '1A: Safranin - Raw' and select 'A' to name the variable for the macro function. Next, name the macro, e.g.'mtMPrel(A)', name Units, e.g. 'au' for arbitrary units, and press 'Save macro' if you want to re-use it for subsequent experiment analysis.
* Click 'Run' and then 'Generate plot' and 'Close'.
* Repeat the procedure for the other channel and name it 'mtMPrel(B) ' for saving.
* Finally, in the 'Select plot' menu (either press Ctrl+F6 or go to Menu 'Graph' and then 'Select plot') tick the tab 'Calculated', activate the Y1-axis by ticking Y1 below the '1 Calc' header and click Ok.

6. References

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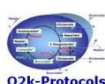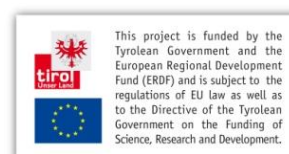
http://wiki.oroboros.at/index.php/O2k-Demo_experiments#O2k-Demo_O2k-MultiSensor

Acknowledgements