Oroboros **O2k-Protocols**
mt-Preparations

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O2k-Fluorometry: HRFR and simultaneous determination of H_2O_2 production with Amplex Red

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1. Introduction

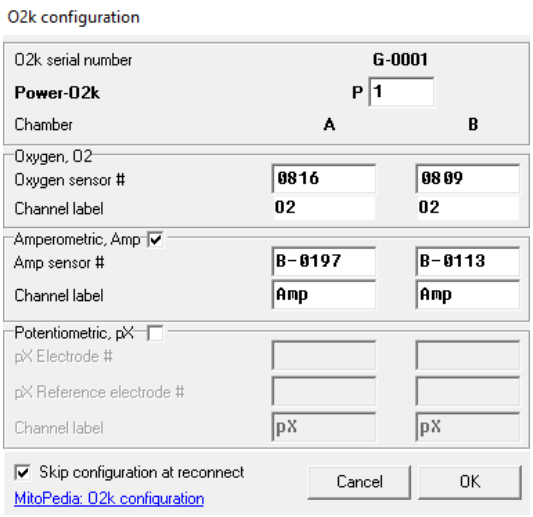
Basic methodological topics are presented for using Amplex Red (AmR) for fluorometric detection of H_2O_2 production in substrate-uncoupler-inhibitor titration (SUIT) protocols. Changes have to be addressed of chemical fluorescence background corrections and fluorescence sensitivity within an experiment [1,2]. These considerations are illustrated in a demo experiment on High-Resolution FluoRespirometry (HRFR) with mitochondria isolated from mouse heart [2] using DatLab 7.

2. The O2k-Demo experiment

Mitochondria were isolated following a standard protocol, using a glass/Teflon potter for tissue homogenization and subsequent differential centrifugation. A SUIT protocol was used in the succinate-pathway control state [2-4]. H_2O_2 titrations were performed repeatedly at various sections of the

experiment to analyze changes of fluorescence sensitivity over the course on the experiment.

3. Instrumental setup



This is an application of the O2k-FluoRespirometer. If the O2k-Fluo LED2-Module is connected to the O2k-FluoRespirometer[6], the Fluo Control Unit needs to be switched on at its front panel. For the use of Amplex Red (AmR), the Fluorescence Sensors Green are inserted through the windows of the O2k [1]. Click on **O2k configuration** in the Oroboros O2k menu and tick the **Amperometric, Amp** channel. Define the Amp sensor numbers for documentation. Save the settings by clicking **OK**.

Amplification and LED-intensity: Adjust the settings of the signal amplification (Gain: 1000) and light intensity of the LED (polarization voltage: 100 to 500 mV) in O2k control \ Tab: Amperometric, Amp [F7]. The light intensity may be optimized in test experiments to obtain signals which are large enough to minimize noise. The maximum raw signal of 10 V must not be exceeded during the experiment. Activate the settings by clicking L **Send to O2k**.

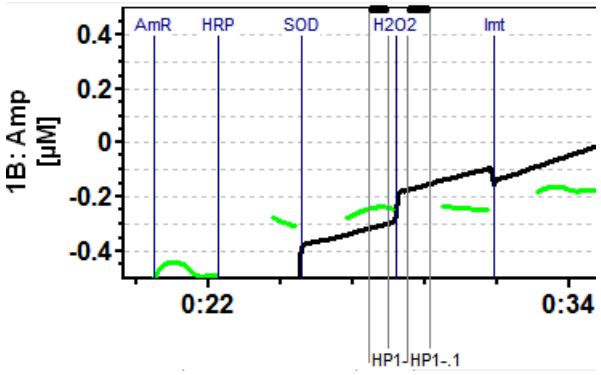
Graph layout Select the pull-down menu Layout \ O2&Amp, Standard layouts and ▶ **01 Amp Amperometric_Raw signal.** This displays respirometric data (see O2 standard layout '04a Flux per volume') with a graph below showing the raw signal 'Amp-Raw' [V] and its time-derivative 'Amp slope' [mV/s] without calibration.

4. Experimental procedure

The chambers containing respiration medium MiR05Cr were closed and the chamber illumination were switched off. Then the constituents of the AmR detection system for H_2O_2 production were added, i.e. AmR (final concentration, f.c., 10 µM), HRP (f.c. 1 U/ml), and SOD (f.c. 5 U/ml), and a baseline was recorded. Next, 0.1 µM H_2O_2 was injected from a concentrated stock solution, allowing for an initial calibration of the fluorescence signal. In order to

observe experimental data converted to concentration of the fluorescent AmR assay product (here referred to as AmR for simplicity, but actually being Resorufin or a derivative originating from the reaction of Amplex Ultra Red with H_2O_2) in real-time the following procedure was conducted:

- Select the plot for 'Amp raw' and mark a brief section immediately before and after addition of the calibration standard. Click into the top bar of the mark to open the window Mark information' and enter a name and concentration for each mark, which in the example would be 'HP1-.0' and '0.000', and 'HP1-.1' and '0.100' to indicate that AmR concentration was 0 and 0.1 µM at the first and second mark, respectively.
- Select 'Calibration' / 'Amperometric, Amp' and the marks created are displayed in the center of the window. To use the marks for calibration select them by ticking the box next to each mark name and names and values entered above will appear on the right side of the window. Now 'Slope' can be ticked next to each 'Conc.', which will make sure that the fluorescence change (the increase) of the signal within each marked section is taken into account for the calculation of the Sensitivity value ([V/µM]).
- Pressing **Calibrate** will convert the raw data of fluorescence to AmR concentration which is now displayed in the corresponding plot window as 'Amp [µM]'.
- Repeat the procedure for the other chamber.

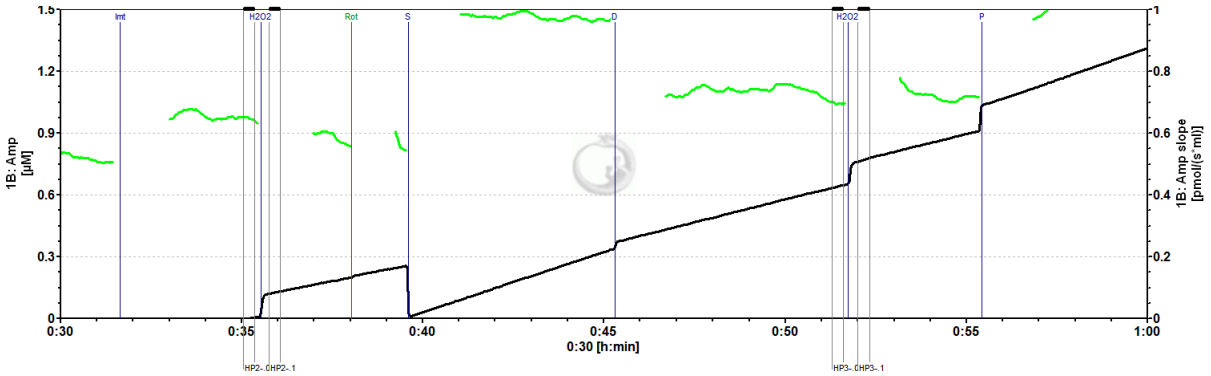


After this calibration step, mitochondria were injected (Imt), followed by another titration of 0.1 µM H_2O_2. This allows assessment of the optical effect of the sample on fluorescence sensitivity. The fluorescence changes that are subsequently recorded correspond to the apparent H_2O_2 production by the mitochondria in the absence of external substrates. In the above and subsequent images artefacts caused by injections of substrates and inhibitors have been deleted, leading to breaks in the slope plots.

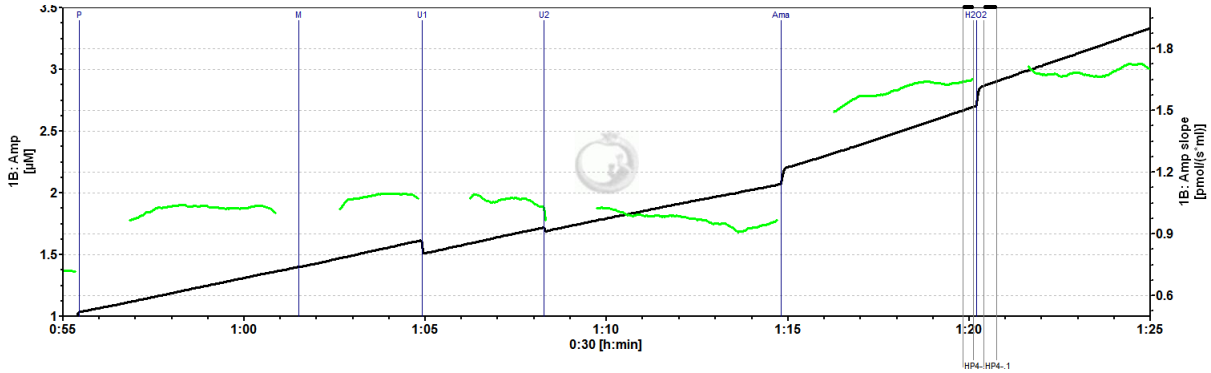
Repeated adjustment may be required of the scaling of the Y-axis ranges for the calibrated signal and the slope such that it is possible to clearly assess if the signal has reached stability or not before further injections are made.

In the next step 1 µM rotenone (Rot) was added to inhibit Complex I (CI), followed by addition of 10 mM succinate, which supports CII-linked

respiration. This caused an immediate increase of H_2O_2 production typical for the LEAK state, whereas the subsequent addition of ADP, inducing CII-linked OXPHOS, reduced H_2O_2 production. Another calibration with H_2O_2 standard was conducted. This was followed by addition of pyruvate (P) and malate (M). P caused another elevation of H_2O_2 production whereas M had no further effect.

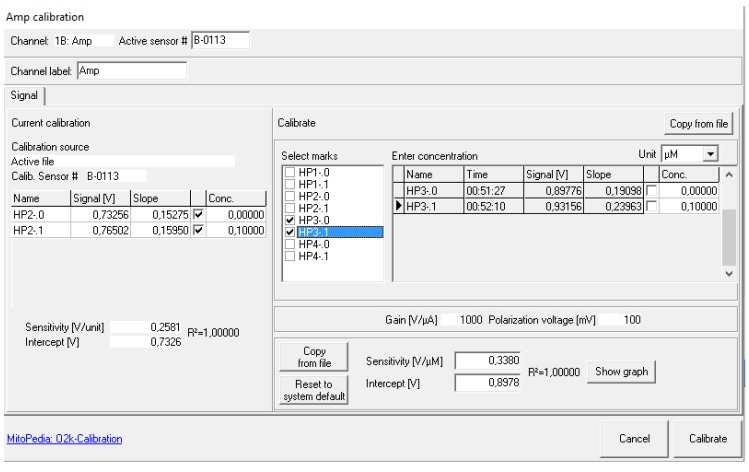


Similarly, adding the uncoupler CCCP left H_2O_2 production unaltered, while inhibition of CIII with antimycin A (Ama) increased it again. The experiment was ended with another final addition of H_2O_2 standard.



5. DatLab analysis

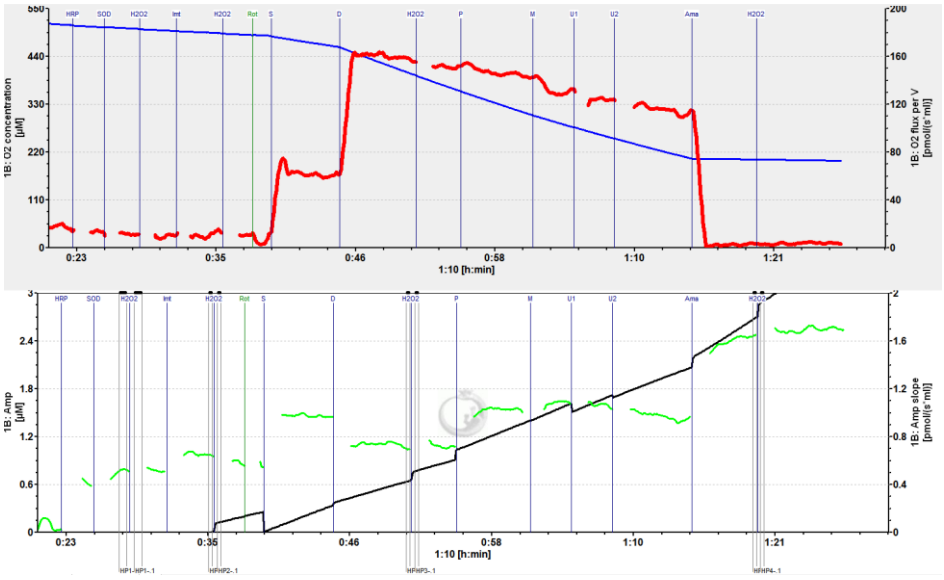
The experimental data shown above are displayed as fluorescence converted to [µM] concentration of the reaction product and as fluxes [pmol/s*ml], based on the calibration conducted before addition of the mitochondria and SUIT chemicals. In the paper by Krumschnabel et al. [1] it was shown that in the absence of mitochondria the sensitivity of the AmR assay over time is fairly constant in MiR05Cr (see Figure 4). In the present experiment repeated additions of a calibration stock of H_2O_2 were made



and thus again assay sensitivity over time could be evaluated. For this purpose, the step-by-step procedure described above was conducted to mark and name sections before and after addition of H_2O_2 for all additions, in each case assigning the marks set before and after addition the values '0.000' and '0.100', respectively, taking into account that the immediate conversion of added H_2O_2 by the AmR/HRP assay system will invariantly restore a concentration of H_2O_2 of zero. When all additions were marked in this way the calibration window 'Calibration' / 'Amperometric, Amp' was opened and the paired marks for each calibration were sequentially selected (including the correction of the slope in each case) and the resulting values for sensitivity [V/µM] and intercept were noted. A comparison of these calibrations indicated that the presence of mitochondria affected sensitivity by approximately 8% and 12% in the absence of external substrates and in the OXPHOS state, respectively, while the inhibition of CIII with Ama caused an increase of apparent sensitivity by about 30%. Thus, if H_2O_2 production rates at different Substrate control states or Coupling control states are of interest to the experimenter, it appears advisable to select the H_2O_2 addition most closely related in time and condition for calibration in each case. Importantly, changes in apparent assay sensitivity may depend on the medium used and it may be advisable to check if corresponding corrections are required in preliminary runs [7].

calibration	Sensitivity [V/µM]
before Imt	0.2723
with Imt (ROX1)	0.2615
S(Rot)_P (OXPHOS)	0.2408
Ama (ROX2)	0.3589

Suggestions for alternative approaches for analysis and calibration of AmR experiments by users are encouraged and may either be directly posted on our discussion page of the Amplex Red entry (www.bioblast.at/index.php/Talk:Amplex_red) or sent to the Oroboros team.



The full experiment showing oxygen-related data on the upper panel and AmR data on the lower one, allowing to correlate respirometric data and H_2O_2 production. The AmR signal was calibrated using the addition of H_2O_2

in the presence of mitochondria but in the absence of external substrate (marks 'HP2.-0' and 'HP2.-1').

6. References

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Acknowledgements

MitoFit mitochondria fit&well
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