O2k-Fluorometry: HRR and simultaneous determination of H₂O₂ production with Amplex Red.

Gerhard Krumschnabel[1], Marina Makrecka-Kuka[2], Sarawut Kumphune[3], Mona Fontana-Ayoub[1], Mario Fasching[1], Erich Gnaiger[1,4]

[1]OROBOROS INSTRUMENTS Corp
high-resolution respirometry
Schöpfstr 18, A-6020 Innsbruck, Austria
Email: erich.gnaiger@oroboros.at
www.oroboros.at
[2]Latvian Institute of Organic Synthesis Laboratory of Pharmaceutical Pharmacology
[3]Biomedical Res Unit in Cardiovascular Sciences, Dep Medical Technology
Faculty of Allied Health Sciences, Naresuan University
[4]D. Swarovski Research Laboratory, Dept. Visceral, Transplant and Thoracic Surgery, Medical University of Innsbruck, Austria
http://wiki.oroboros.at/index.php/K-Regio_MitoCom_Tyrol

1. Introduction

Various experimental and methodological issues need be considered when using Amplex Red (AmR) for combined high-resolution respirometry (HRR) and fluorescence detection of H_2O_2 production in experiments applying extended substrate-uncoupler-inhibitor titration (SUIT) protocols, including the drift of background fluorescence, the potential change in assay sensitivity over time, and the impact of medium composition. A principle assessment of these issues is given in Krumschnabel et al. [1]. Here, an example will be shown how an actual experiment can be conducted and analyzed taking these issues into consideration.

2. The O2k Demo experiment

The O2k demo experiment examines respiratory rates and parallel changes of H_2O_2 production in isolated cardiac mitochondria from mouse with a SUIT protocol on Complex II-linked respiration [2, 3]. Besides the addition of SUIT chemicals repeated calibrations of H_2O_2 production were performed to allow for the detection of changes in assay sensitivity. Sample preparation was done following a standard protocol using a

glass/Teflon potter for mouse cardiac tissue homogenization and subsequent isolation of mitochondria by differential centrifugation.

3. Setting the instrument for data acquisition

Instrumental setup and preparation for respiratory measurements are described in [4]. Further, the O2k-Fluorescence LED2-Module consisting of the Fluorescence-Control Unit and, for the use of AmR, two Fluorescence Sensors Green, 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 100 mV and 1000, respectively. The ideal settings for polarization voltage need to be determined in preliminary experiments and can be considered 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, a value that must never be exceeded in the course of an experiment.
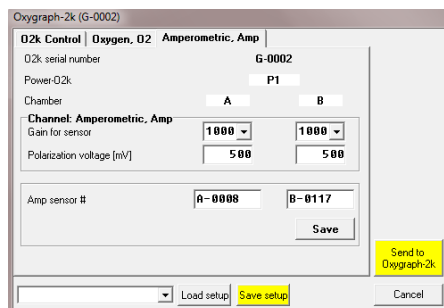
Selecting the appropriate Layout: The initial layout selected to observe the data during acquisition was 'B1 AmR1 – Raw signal' which displays respirometric data according to the standard layout '05 Flux per Volume corrected' and the AmR signal as plots of the raw signal 'Amp-Raw' [V] and its time-derivative, 'Amp slope' [pmol/(s*ml)] just below each related plot of the oxygen signal. Please note that the absolute value of the slope is meaningless at this point as it is calculated either on the basis of system default values for Sensitivity and Intercept or uses values from a previously stored experiment. However, the slope is still important to assess relative changes and signal stability.

4. Data acquisition

After adjusting the instrument settings, the chambers containing respiration medium MiR05Cr were closed and the chamber lights 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 AmR concentration 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 'Edit mark information' and enter a name and value for each mark, which in the example would be '01' and '0.000', and '02' and '0.100' to indicate that AmR concentration was 0 and 0.1 µM at the first and second mark, respectively.
* Select 'Calibration' and '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 field next to each mark name and names and value 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.
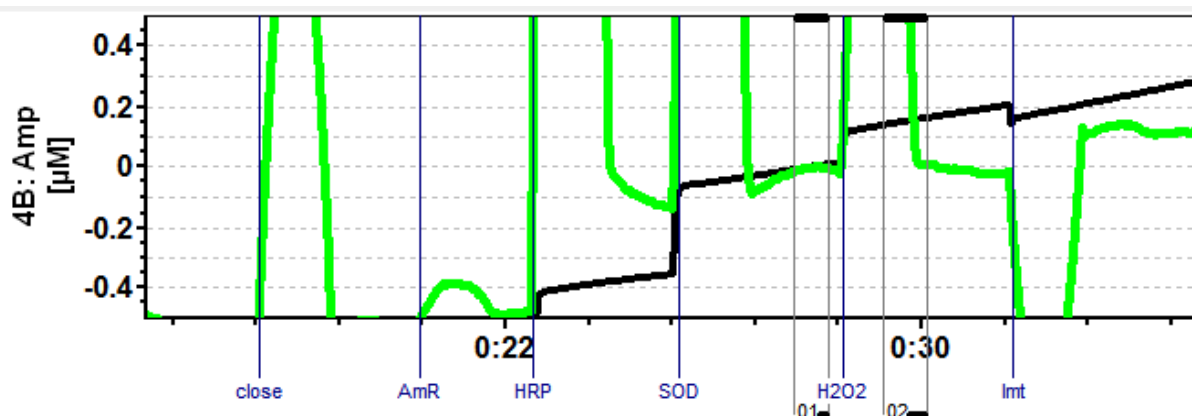
After this calibration step, mitochondria were injected (Imt), followed by another titration of 0.1 µM H_2O_2. This enables the experimenter to assess the effect of the presence of sample on assay sensitivity such as the impact of scavenging by mitochondria and/or impurities injected with the mitochondria. The fluorescence changes that are subsequently recorded correspond to the apparent H_2O_2 production by the mitochondria in the absence of any external substrates.

It is important to note here that repeated adjustment may be required for the scaling of the y-axis ranges both 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 again. Next, another calibration with H_2O_2 standard was conducted followed by addition of pyruvate (P) and malate (M). P caused another elevation of H_2O_2 production whereas M had no further effect.
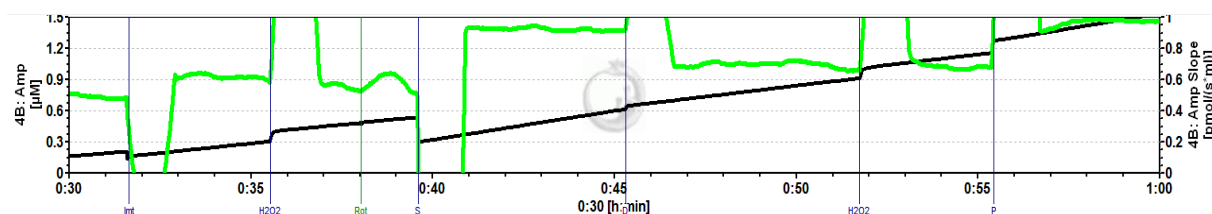

Similarly, adding the uncoupler CCCP left H_2O_2 production unaltered, while inhibition of CIII with antimycin A (Ama) increased it again. The experiment was ended with another final addition of H_2O_2 standard.
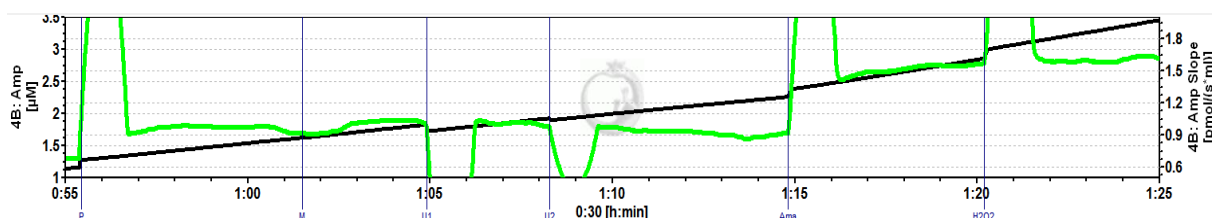
5. Data analysis

The experimental data shown above are displayed as fluorescence converted to [µM] concentration of the reaction product and as rates expressed in [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
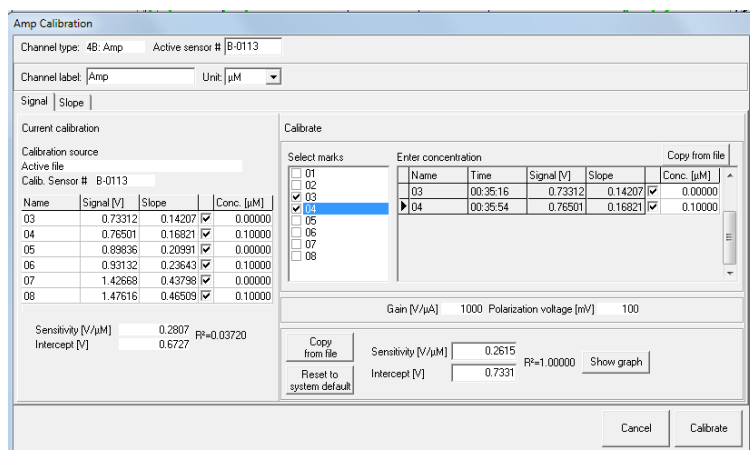

after addition of H_2O_2 for all additions, in each case assigning the marks set before and after addition the values '0.000' and '0.100', respectively, taking into account that the immediate conversion of added H_2O_2 by the AmR/HRP assay system will invariantly restore a concentration of H_2O_2 of zero. When all additions

were marked in this way the calibration window 'Calibration' and '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.

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 (http://www.bioblast.at/index.php/Talk:Amplex_red) or sent to the Oroboros team.
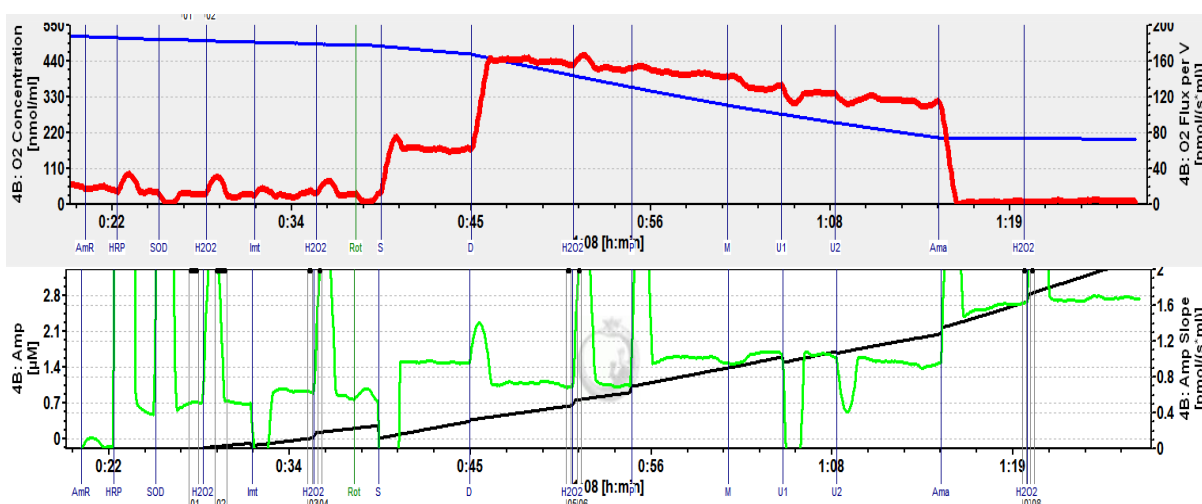
The full experiment showing oxygen-related data on the upper panel and AmR data on the lower one, allowing to correlate respirometric data and H_2O_2 production. The AmR signal was calibrated using the addition of H_2O_2 in the presence of mitochondria but in the absence of external substrate (marks '03' and '04').

6. References

1. Krumschnabel 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 biopisies of human muscle. Methods Mol Biol 810: 25-58.

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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

7. Acknowledgements