

O2k-FluoRespirometry: HRR and simultaneous determination of H₂O₂ production with Amplex UltraRed

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1. Introduction

Basic methodological topics are presented for using Amplex UltraRed (AmR) for fluorometric detection of H₂O₂ 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 respirometry (HRR) 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]. H₂O₂ 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

O2k configuration

O2k serial number	G-0001	
Power-O2k	P 1	
Chamber	A	B
Oxygen, O2		
Oxygen sensor #	0816	0809
Channel label	O2	O2
Amperometric, Amp <input checked="" type="checkbox"/>		
Amp sensor #	B-0197	B-0113
Channel label	Amp	Amp
Potentiometric, pX <input type="checkbox"/>		
pX Electrode #		
pX Reference electrode #		
Channel label	pX	pX
<input checked="" type="checkbox"/> Skip configuration at reconnect		
MitoPedia: O2k configuration		
		Cancel OK

This is an application of the O2k-Fluorometer. If the O2k-Fluo LED2-Module is connected to the O2k-Core (up to O2k Series G [6]), the [Fluo Control Unit](#) needs to be switched on at its front panel. For the use of Amplex UltraRed (AmR), the [Fluorescence Sensors Green](#) are inserted through the windows of the O2k [1]. Click on **O2k configuration** in the Oxygraph-2k menu and tick the **Amperometric, Amp** channel. Define the Amp sensor numbers for documentation. Save the settings by clicking **OK**.

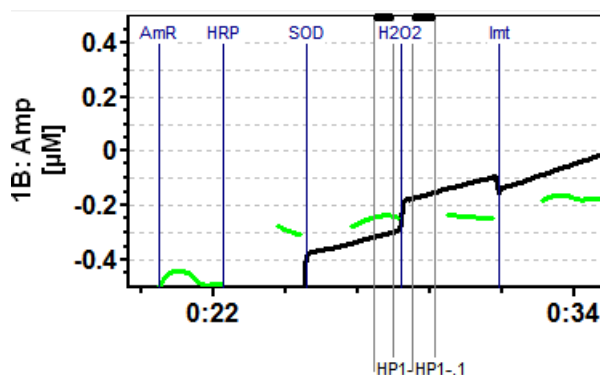
Amplification and LED-intensity: Adjust the settings of the signal amplification (Gain: 1000) and light intensity of the LED (polarization voltage: 100 to 500 mV) in **O2k control \ Tab: Amperometric, Amp [F7]**. The light intensity may be optimized in test experiments to obtain signals which are large enough to minimize noise. The maximum raw signal of 10 V must not be exceeded during the experiment. Activate the settings by clicking **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 was switched off. Then the constituents of the AmR detection system for H₂O₂ 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 H₂O₂ was injected from a concentrated stock solution, allowing for an initial calibration of the fluorescence signal. Experimental data are expressed as H₂O₂ concentration converted to the fluorescent AmR assay product (a derivative originating from the reaction of Amplex UltraRed with H₂O₂, similar to resorufin):

- Select the plot for 'H₂O₂ 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 H₂O₂ concentration was 0 and 0.1 μM at the first and second mark, respectively.
- Select 'Calibration' / 'Amperometric, Amp'. The marks 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. Then names and values entered above appear on the right side of the window. Now 'Slope' can be ticked next to each 'Conc.', to 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 ([V/μM]).
- Pressing **Calibrate** converts the raw data of fluorescence to AmR concentration which is now displayed in the corresponding plot window as 'H₂O₂ [μM]'.
- Repeat the procedure for the other chamber.

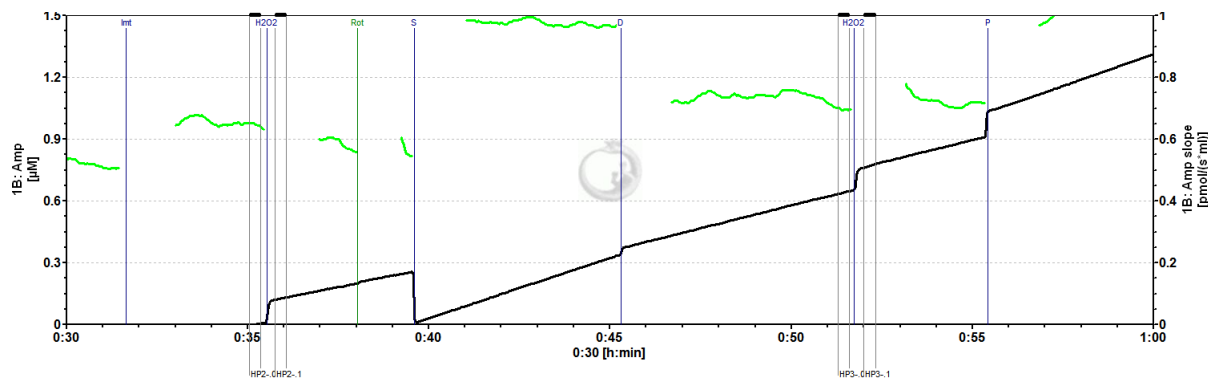


After this calibration step, mitochondria were injected (imt), followed by another titration of 0.1 μM H₂O₂. This allows assessment of the optical effect of the sample on fluorescence sensitivity. The fluorescence changes that are subsequently recorded correspond to the apparent H₂O₂ production by the mitochondria in the absence of

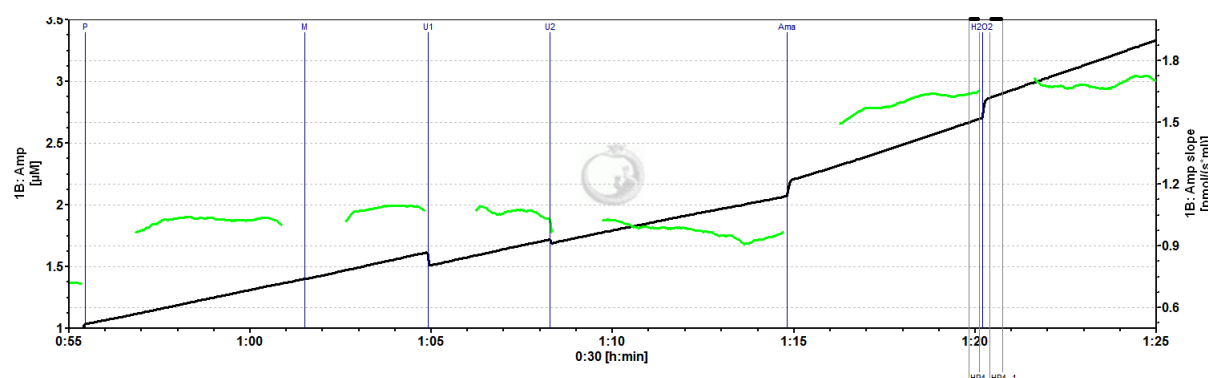
external substrates. In the above and subsequent images artefacts caused by injections of substrates and inhibitors have been deleted, leading to discontinuities in the slope plots.

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 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 (S), which supports S-linked respiration. This caused an immediate increase of H₂O₂ production typical for the LEAK state, whereas the subsequent addition of ADP, inducing S-OXPHOS, reduced H₂O₂ production. Another calibration with H₂O₂ standard was conducted. This was followed by addition of pyruvate (P) and malate (M) as NADH-linked substrates, inducing NS-linked respiration. P caused another elevation of H₂O₂ production whereas M had no further effect.



Similarly, adding the uncoupler CCCP left H₂O₂ production unaltered, while inhibition of CIII with antimycin A (Ama) increased it again. The experiment was ended with a final titration of H₂O₂ 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.

Amp calibration

Channel: 1B: Amp Active sensor #: B-0113

Channel label: Amp

Signal: |

Current calibration

Calibration source

Active file

Calib. Sensor #: B-0113

Name	Signal [V]	Slope	Conc.
HP2-0	0.73256	0.15275	0.00000
HP2-1	0.76502	0.15950	0.10000

Sensitivity [V/unit]: 0.2581 R²=1.00000

Intercept [V]: 0.7326

Calibrate

Select marks

Name	Time	Signal [V]	Slope	Unit	Conc.
HP1-0				µM	
HP1-1				µM	
HP2-0				µM	
HP2-1				µM	
HP3-0				µM	
HP3-1	00:51:27	0.89776	0.19098	µM	0.00000
HP3-2	00:52:10	0.93156	0.23963	µM	0.10000
HP4-0				µM	
HP4-1				µM	

Gain [V/µA]: 1000 Polarization voltage [mV]: 100

Copy from file

Sensitivity [V/µM]: 0.3380

Intercept [V]: 0.8978 R²=1.00000 Show graph

Reset to system default

MitoPedia_Q2k-Calibration

Cancel Calibrate

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 H₂O₂ were made and thus again assay

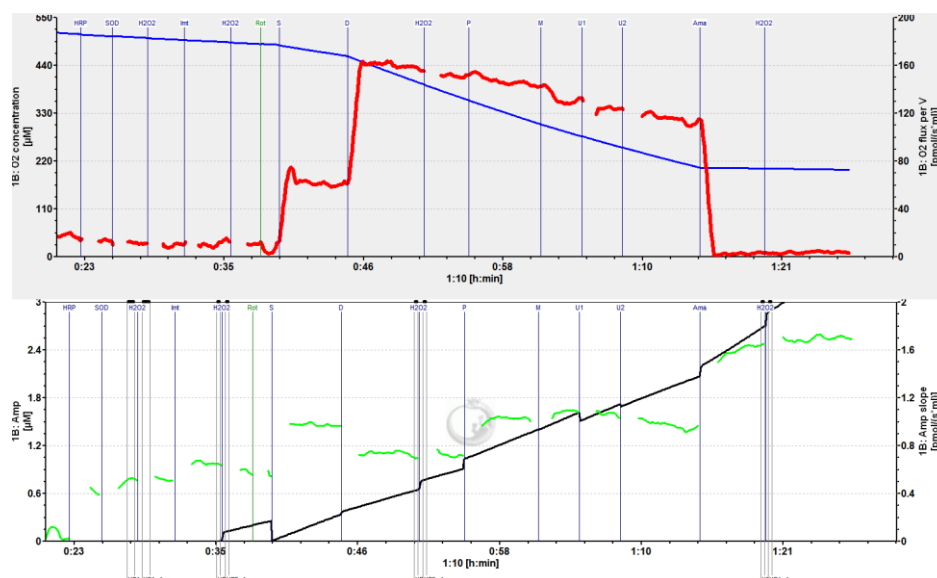
sensitivity over time could be evaluated. For this purpose, the step-by-step procedure described above was conducted to mark and name sections before and after addition of H₂O₂ for all additions, in each case assigning the marks set before and after addition the values '0.000' and '0.100', respectively, taking into account that the immediate conversion of

added H₂O₂ by the AmR/HRP assay system will invariably restore a concentration of H₂O₂ 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/ μ M] 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 H₂O₂ production rates at different pathway control states or coupling control states are evaluated, the H₂O₂ titration most closely related in time and condition for calibration should be used. 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/ μ M]
before lmt	0.2723
with lmt (ROX1)	0.2615
S(Rot)_P (OXPHOS)	0.2408
Ama (ROX2)	0.3589

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 H₂O₂ production rates at different pathway control states or coupling control states are evaluated, the H₂O₂ titration most closely related in time and

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 traces in the upper panel and AmR traces in the lower panel, allowing to correlate respiration, oxygen concentration, and H₂O₂ production. The AmR signal was calibrated using the addition of H₂O₂ in the presence of mitochondria but in the absence of external substrate (marks 'HP2.0' and 'HP2.1').

6. References

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O2k-Protocols

http://wiki.orooboros.at/index.php/O2k-Mitochondrial_preparations

Acknowledgements



MitoFit
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Standortagentur