O2k-Procedures: mtMP analysis

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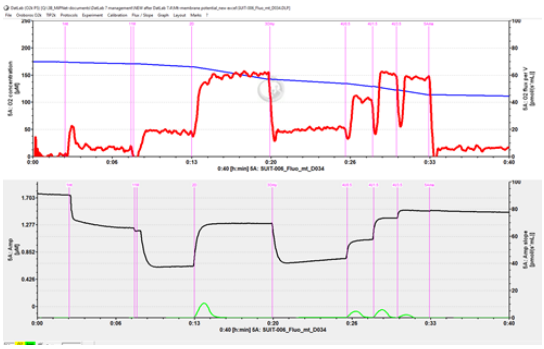
Updates: https://wiki.oroboros.at/index.php/MiPNet24.09_Data_analysis_of_mt-membrane_potential

Data analysis of mitochondrial membrane potential estimation using various fluorescence dyes



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1. General Information

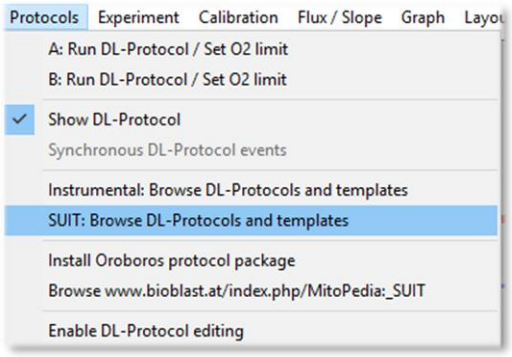
Substrate-uncoupler-inhibitor-titration (SUIT) protocols are designed to study respiratory control in a sequence of coupling and pathway control states induced by multiple titrations within a single experimental assay. DatLab 7.4 has been specifically designed to guide the user through SUIT protocols (DL-Protocols in DatLab). Excel templates (SUIT-###_Fluo_mt_D###_general.xlxs) are provided for data analysis of O_2 flux and relative mitochondrial membrane potential (mtMP) using different fluorescence dyes (*e.g.*, safranin, TMRM, Rhodamine123) for isolated mitochondria, tissue homogenate and permeabilized cells.

Use the SUITbrowser to find the best SUIT protocol for your research questions: »https://suitbrowser.oroboros.at/

This MiPNet provides instructions on how to use two different formats of templates to analyze data from O_2 flux and mitochondrial membrane potential measurements. The first are the templates available after installation of DatLab 7.4 (section 2), and the second are an improved version of these templates which integrate a correction for chemical background (section 3).

2. Data analysis with templates available with DatLab 7.4

In DatLab 7.4, select the menu [Protocols] and click on [SUIT:Browse DL-Protocols and templates].



Select your SUIT protocol and open the SUIT-###_Fluo folder. Inside this folder, you will find another folder for the specific DL-Protocol (named SUIT-###_Fluo_mt _D###). In each folder, four Excel files can be found:

- A blank template (named SUIT-###_Fluo_mt_D###_general.xlxs) to calculate the relative mtMP values.
- A demo version of the general template (named SUIT-###_Fluo_mt_D###_general_demo.xlsx), which provides an example of the file already with data showing the relative mtMP values.
- A blank template (named SUIT-###_Fluo_mt_D###_safranin.xlxs) to convert the fluorescence values measured by safranin into absolute mtMP values expressed as mV.
- A demo version of the specific safranin template (named SUIT-###_Fluo_mt_D###_general_demo.xlxs) which provides an example of the file already with data showing the mtMP values expressed in mV.

Create a copy of SUIT-###_Fluo_mt_D###_general.xlxs analysis template for your data analysis and rename it. You can rename the template by opening it and choosing the option ‘Save as´ in the archive top menu.

2.1. Calibration

1. Open the DatLab file containing the data from the calibration.
2. Open the menu [Calibration] and select 'Amperometric, Amp' to calibrate and convert the amperometric signal into the concentration of the fluorescence dye.
3. Select the first four marks (e.g., Saf0, Saf0.5, Saf1, Saf1.5, Saf2) for calibration. Check the r2 of the linear regression and press the 'Show graph' button to check the linearity of regression. The sensitivity [V/µM] can be also found in the same window.
4. Press 'Calibrate' and the [Y1: Amp raw] will turn into calibrated [Y1: Amp]. Adjust the scaling [F6] of [Y1: Amp].

2.2. Biological Experiment

In the Excel template you can select the setting by ticking the boxes `Titration volume correction´ and `Known sample concentration´. More information can be found here: »MiPNet24.06 Oxygen flux analysis with DatLab7.4

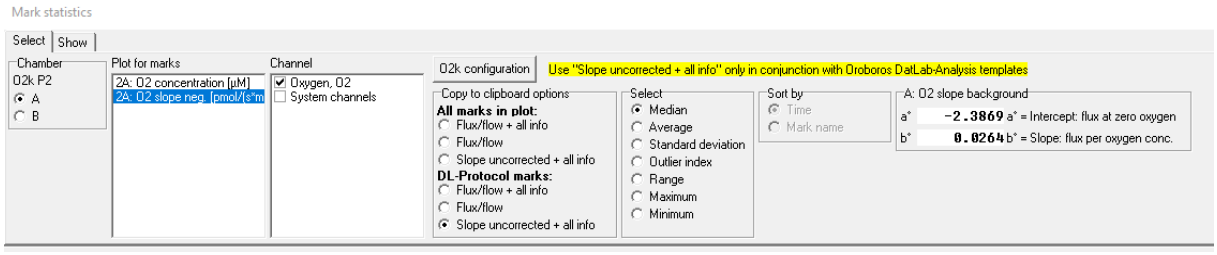
Oxygen flux analysis:

The calculations of the O_2 fluxes are provided under the following link complying with Oroboros transparency policy:

»https://wiki.oroboros.at/index.php/Flux_/_Slope#O2

In DatLab 7.4, after setting the marks separately for the O2 flux, go to Marks, and select Slope uncorrected + all info in DL-Protocol marks. In the new window, select:

1. Your chamber of interest.
2. Plot for Marks: O2 slope neg. [pmol/s*mL].
3. Channel: Oxygen, O2. Leave only this channel selected.
4. Select: Median.
5. Sort by: Time (default).
6. Then, click on Copy to clipboard to copy the selected values.



In the Excel template (SUIT-###_Fluo_mt_D###_general.xlxs): Click on the yellow cell A7 in the "Data" sheet (in the excel templates available online, select sheet "2-data") and paste these selected values (only O_2) from DatLab Ctrl+V.

The calculated values for the specific O_2 flux, specific O_2 flux (bc), *FCR* and *FCR* (bc), on each step of the protocol can be found from column K in the rows 24, 25, 27 and 28, respectively.

Paste the DatLab graphs showing the traces for the chamber:

1. In DatLab: Select the graph (left mouse click into the respirometry graph of interest) → select Graph\Copy to Clipboard\WMF.
2. In the Excel template: Click on the yellow cell A27: `Paste DatLab graph here', → press Ctrl+V to paste.
3. Select the graph (right click on the graph) → select Size and properties and set the width of the graph to 22 cm (8 inches).

Membrane potential analysis

In DatLab 7.4, copy the calibration values of the fluorescence signal from the previous calibration file: Open the menu [Calibration] and select 'Amperometric, Amp', press 'Copy from file' to open the calibration file and copy the sensitivity value. Then press the 'Calibrate' button in the 'Amp calibration' window.

1. Select Y1: Amp as the active plot for setting marks and place marks according to your protocol as done for the O_2 flux.
2. Use the macro in DatLab 7.4 as explained in MiPNet20.13 to obtain normalized (calculated) fluorescence plots.
3. Copy marks from the original amperometric trace to the calculated one: select the calculated trace and open the menu Marks and select Copy marks from\5A: Amp [µM] or 5A: Amp raw.
4. Open Marks window and select Slope uncorrected + all info; in the new window select channel Calculated and use DL Protocol marks: Slope uncorrected + all info to display data for the marked regions.

5. Export data with Copy to Clipboard.
6. Paste the calculated fluorescence values in the Excel template into the yellow cell A42.
7. The calculated mtMP values can be found in row 50 starting with column J.
8. Copy the Amp graph as it is explained in the previous section for the O_2 trace and paste on the yellow cell A63.

3. Data analysis with templates available online at Bioblast

The templates are available for download here:
» https://wiki.oroboros.at/index.php/Mitochondrial_membrane_potential#Calculations

3.1. Calibration

1. In DatLab 7.4. open the calibration file and apply the calibration as described in section 2.1.
2. Open Marks window, select DL-Protocol marks and Slope uncorrected + all info; in the new window select channel Amperometric,Amp and use DL-Protocol marks: Slope uncorrected + all info to display data for the marked regions.
3. Export data with Copy to Clipboard.
4. Paste the calibration values in the Excel template into the yellow cell B5 in the sheet "1-calibration and chemical BG".

3.2. Chemical background

Compounds capable of absorbing a portion of either the excitation or emission energy will affect the fluorescence intensity. Therefore, high-resolution measurement of fluorescence signal depends on chemical background correction. The DLPs that will guide you through the chemical background measurement can be found in the specific SUIT-###_Fluo_mt_D### folder with the name SUIT-###_Fluo_mt_D###_chemical background.DLP.

1. Open the DatLab file containing the data from the chemical background.
2. Copy the calibration values of the fluorescence signal from the previous calibration file: Open the menu Calibration and select Amperometric, Amp for your chamber of interest. Press Copy from file to open the calibration file and copy the sensitivity value. Then press the Calibrate button in the Amp calibration window.
3. Select Y1: Amp as the active plot for setting marks and place marks according to your protocol.
4. Open the menu Marks and select Slope uncorrected + all info to display the data for the marked regions. Select channel Amperometric, Amp for data output and Amp [µM] as a Plot for marks.
5. Export the data with Copy to Clipboard.
6. Open the Excel template (SUIT-###_Fluo_mt_D###_general.xlxs) and select the excel tab '1-calibration and chemical BG'. Place the cursor on cell B18, marked in yellow and insert the copied chemical background data (Ctrl+V).
7. Still using '1-calibration and chemical BG', check the volumes for all injections (row 34), starting with columns K (after the calibration). This step is necessary to determine the chemical background. The volumes for the pre-injection marks should be zero.

8. The values for background correction can be found in row 40, starting with column K and in consecutive columns. Each value in row 40 denotes a correction value calculated from its own column and the column to the left. If you have a column 'pre-D' to the left and to the right a column 'D' the value in row 40 of the column 'D' will be the correction voltage for ADP injection. Numbers in the 'pre-D' and similar columns have no experimental meaning. The correction in step change of injections is marked in blue.
9. The values for chemical background correction in row 40 (post-injections, marked in blue) are automatically used in the '2-data' row 54.
10. Over time the chemical fluorescence background will show which injections do need a correction. If the necessary corrections are reproducible for identical experimental conditions, it may no longer be necessary to run a separate chemical fluorescence background experiment for each biological experiment. If the effect is obviously caused only by the carrier and not the substance itself, correction values for the carrier can be measured and used for all titrations of such substances dissolved in this carrier.

Results: The results from the calibration phase are only used internally for the calculation of substance specific correction values.

3.3. Biological Experiment

Oxygen flux analysis:

Follow the same instructions as under section 2 ('Data analysis with templates available with DatLab 7.4')

Membrane potential analysis:

1. In DatLab 7.4, copy the calibration values of the fluorescence signal from the previous calibration file: Open the menu Calibration and select Amperometric, Amp, press Copy from file to open the calibration file and copy the sensitivity value. Then press the Calibrate button in the 'Amp calibration' window.
2. Select Y1: Amp as the active plot for setting marks and place marks according to your protocol as done for the O_2 flux.
3. Open Marks window, select DL-Protocol marks and Slope uncorrected + all info; in the new window select channel Amperometric,Amp and use DL-Protocol marks: Slope uncorrected + all info to display data for the marked regions.
4. Export data with Copy to Clipboard.
5. Paste the calculated fluorescence values in the Excel template into the yellow cell A42 in the sheet "2-data".
6. The calculated relative mtMP values can be found in row 62 starting with column L.
7. Copy the Amp graph as it is explained previously and paste in the yellow cell A63.

4. Further Information

» MitoPedia: Mitochondrial membrane potential
» Flux / Slope
» Safranin
» TMRM
» Rhodamine123

5. References

Cardoso LHD, Komlodi T, Doerrier C, Garcia-Souza LF, Gnaiger E, Sobotka O (2020) Oxygen flux analysis with DatLab 7.4. Mitochondr Physiol Network 24.06(02):1-11. – »Bioblast link

Krumschnabel G, Fasching M, Gnaiger E (2019) O2k-FluoRespirometry: HRR and simultaneous determination of mt-membrane potential with safranin or TMRM. Mitochondr Physiol Network 20.13(03):1-5. - »Bioblast link

6. Acknowledgements

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