

Data analysis of mitochondrial membrane potential estimation using safranin

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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-Protocol in DatLab](#)). Excel templates (SUIT-###_Fluo_mt_D###_safranin.xlsx) are provided for data analysis of O₂ flux and mitochondrial membrane potential (mtMP) using safranin for isolated mitochondria, tissue homogenate and permeabilized cells. Additionally, assessment of mtMP can be adapted for permeabilized fibers upon the implementation of some modifications. It is important to highlight

that the partition coefficients would be different for other fluorescence dyes, therefore, the values obtained will differ.

Each DL-Protocol is defined with a unique D- number (D###), for a detailed list see:

- https://www.bioblast.at/index.php/SUIT_protocol_library#List_of_SUIT_protocols_with_D-numbers

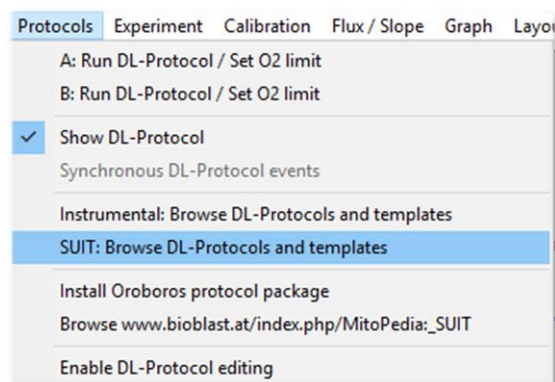
Use the SUITbrowser to find the best SUIT protocol for your research questions:

- <https://suitbrowser.oroboros.at/>

2. Starting data analysis

After finishing the real time respirometry measurement in DatLab 7.4 or anytime while doing the DatLab analysis, open our Excel template to analyze your data in a time efficient way.

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



2. Select your SUIT protocol, open 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. A blank template (named SUIT-###_Fluo_mt_D###_general.xlsx) to calculate the relative mtMP values.
 - b. 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.
 - c. A blank template (named SUIT-###_Fluo_mt_D###_safranin.xlsx) to convert the

fluorescence values measured by safranin into absolute mtMP values expressed in mV.

- d. A demo version of the specific template (named SUI-###_Fluo_mt_D###_general_demo.xlsx) which provides an example of the file already with data showing the mtMP values expressed in mV.
3. Create a copy of SUI-###_Fluo_mt_D###_safranin.xlsx 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.

3. Calibration

3.1. Fluorescence signal calibration

- 3.1.1. Open the DatLab file containing the calibration data.
- 3.1.2. Open the menu [Calibration] and select 'Amperometric, Amp' to open the Amp calibration window (be sure that you select the one for your trace of interest, *i.e.*, A or B).
- 3.1.3. Select the first four marks (Saf0, Saf0.5, Saf1, Saf1.5, Saf2) for calibration. Check the r^2 and press the 'Show graph' button to check the linearity of regression. The sensitivity [V/ μ M] is also shown in the same window.
- 3.1.4. Press 'Calibrate' and the [Y1: Amp raw] will turn into calibrated [Y1: Amp]. Adjust the scaling [F6] of [Y1: Amp].
- 3.1.5. Open the menu [Marks] and select 'Slope uncorrected + all info' to display the data for the marked regions. In the new window select only channel 'Amperometric, Amp' for data output and Amp [μ M] as a 'Plot for marks'.
- 3.1.7. Export the data with 'Copy to Clipboard'.
- 3.1.8. Open the Excel template (SUI-###_Fluo_mt_D###_safranin.xlsx) and select the sheet '1-calibration and chemical BG'. Paste the calibration data (Ctrl+V) in the yellow cell B5.
- 3.1.9. Fill in/check the fields marked yellow in row 1 and 2 describing your system.

4. 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.

- 4.1.1. Open the DatLab file containing the data from the chemical background.
- 4.1.2. 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.
- 4.1.3. Select [Y1: Amp] as the active plot for setting marks and place marks according to your protocol.
- 4.1.4. 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'.
- 4.1.5. Export the data with 'Copy to Clipboard'.
- 4.1.6. Open the Excel template (SUIT-###_Fluo_mt_D###_safranin.xlsx) 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).
- 4.1.7. 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.
- 4.1.8. 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.

- 4.1.9. The values for chemical background correction in row 40 (post-injections, marked in blue) are automatically copied and pasted in the '3-biol exp-calculation sheet' row 18 (marked in yellow).
- 4.1.10. *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.

5. Biological Experiment

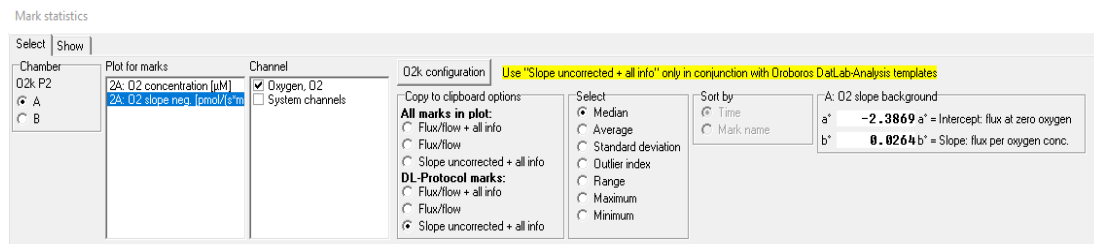
In the excel template you can select the setting by ticking the boxes 'Titration volume correction' and 'Known sample concentration'. More information can be found here: [MiPNet24.06 Oxygen flux analysis with DatLab7.4](#)

5.1. Oxygen flux analysis

The calculations of the O₂ fluxes are provided under the following link complying with Oroboros transparency policy:

<https://wiki.orooboros.at/index.php/Flux / Slope#O2>

- 5.1.1. In DatLab 7.4, after setting the marks separately to the O₂ flux, go to [Marks], and select 'Slope uncorrected+all info' in 'DL-Protocol marks'. In the new window select:
 - a. Your chamber of interest.
 - b. Plot for Marks: 'O2 slope neg. [pmol/s*mL]'.
 - c. Channel: 'Oxygen, O2'. Leave only this channel selected.
 - d. Select: 'Median'.
 - e. Sort by: 'Time' (default).
 - f. Then, click on [Copy to clipboard] to copy the selected values.



5.1.2. In the Excel template select '2-biol exp DATA': Click on the yellow cell A7 and paste these selected values (only O₂) from DatLab [Ctrl+V].

5.1.3. The calculated values for the specific O₂ flux, specific O₂ 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.

5.1.4. Paste the DatLab graphs showing the traces for the chamber:

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

5.2. Membrane potential analysis

5.2.1. 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.

5.2.2. Select [Y1: Amp] as the active plot for setting marks and place marks according to your protocol as done for the O₂ flux.

5.2.3. Go to 'Marks' and select 'Slope uncorrected + all info'. In the new window select: 'Amp [µM]' in 'Plot for Marks'.

5.2.4. Channel: 'Amperometric, Amp'. Leave only this channel selected.

5.2.5. Select: 'Median'.

5.2.6. Sort by: 'Time' (default).

5.2.7. Then, click on [Copy to clipboard] to copy the selected values.

5.2.8. In the Excel template select '2-biol exp DATA' (SUIT-###_Fluo_mt_D###_safranin.xlsx) and insert the copied data: Click on the yellow cell A42 and paste [Ctrl+V] Amp slope from the DatLab.

- 5.2.9. Copy the Amp graph as explained in the 5.1.4 and paste on the yellow cell A63.

6. Calculation of membrane potential values

The calculations of the mtMP values are provided under the following link complying with Oroboros transparency policy:

<https://bioblast.at/index.php/Safranin>

- 6.1 In the excel template select the tab '3-biol exp-calculation'.
- 6.2 Fill in/check cells C4 to C11 with the specific system parameters (described in orange). Some values, such as binding parameters and mitochondrial volume, may have to be estimated from literature data.
- 6.3 For isolated mitochondria set P_c (total cellular protein content) = P_{mt} (total mitochondrial protein content).
- 6.4 If available, correction voltages for the chemical background will be obtained using '1- calibration and chemical BG'. These values are incorporated on row 18 (starting from column P).
- 6.5 The titrated volumes will be connected to the '2-biol exp DATA' tab and filled in row 22. These data are used for the dilution correction.
- 6.6 An accurate setting of marks exactly as previously described is crucial for this process. The dilution correction for safranin is applied from the first titration (usually the sample), *i.e.* in column O. However, the dilution correction for the sample only applies for the first titration AFTER the sample has been included, *i.e.* in column P. If an experimental sequence does not fit to this Excel template (*e.g.*, an additional titration before the introduction of the sample is required) the template will have to be adapted accordingly.
- 6.7 If a homogeneous sample is used and not diluted with further titrations (*e.g.*, permeabilized fibers) the values for P_{mt} , P_c , and V_{mt} (abs) (total mitochondrial volume in the system) in rows 23, 24 and 25 will be the values set in cells in C6, C7, and J8, respectively.

Results: Delta Psi values are given in row 33, differences (delta delta Psi) to a reference region defined in K38 are given in row 36.

7. References

1. Cardoso LHD, Antunes D, Iglesias-Gonzalez J, Komlodi T, Doerrier C, Garcia-Souza LF, Gnaiger E, Sobotka O (2019) Oxygen flux analysis with DatLab 7.4. Mitochondr Physiol Network 24.06(01):1-5. – »[Bioblast link](#)«

2. Fasching M, Gnaiger E (2019) mtMP calculation. Mitochondr Physiol Network 24.11(01):1-10. - [»Bioblast link«](#)
3. 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«](#)



- » [MitoPedia: _Mitochondrial_membrane_potential](#)
- » [Flux / Slope_O2](#)
- » [Calculations of mtMP](#)

Acknowledgements



The project NextGen-O2k has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 859770.

