Oxygen flux analysis with DatLab 7.4

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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). An Excel template is provided for data analysis (O2 analysis template DL7.4). Each DL-Protocol is defined with a unique D-number, 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/

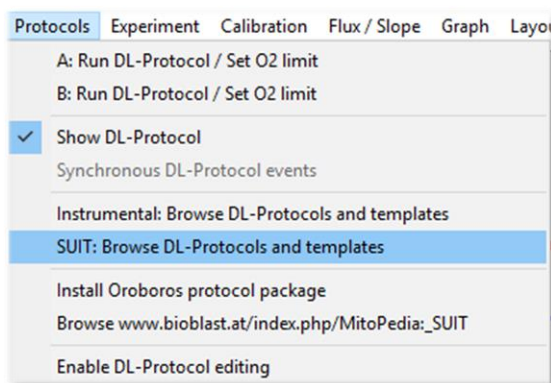An updated version of O2 analysis template DL7.4 can be downloaded from:

➢ https://wiki.oroboros.at/index.php/MiPNet24.06_Oxygen_flux_analysis_-_DatLab_7.4

2. Instructions

2.1. Starting a new data analysis

After finishing the real time respirometry measurement in DatLab 7.4 software or anytime while doing the DatLab analysis, the O2 analysis template DL7.4 allows the user to analyze the 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. Create a copy of the O2 analysis template DL7.4 for the data analysis and rename it. You can rename the template by opening it and choosing the option 'Save as'. The latest up-to-date version of this excel template is always available on:
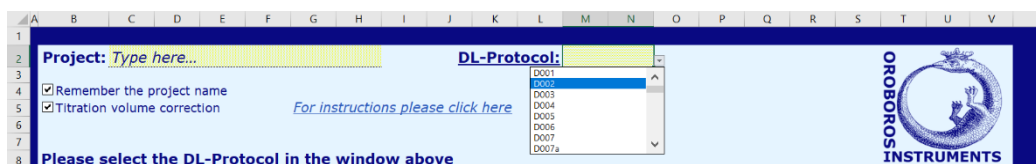
➢ https://wiki.oroboros.at/index.php/MiPNet24.06_Oxygen_flux_analysis_-_DatLab_7.4.

2.2.   Protocol setup

The template will open by default on the 'Protocol page' worksheet. In this worksheet, some settings for the data analysis can be selected, as described step-by-step below.

1. Type the name of your project in the upper left yellow window ('*Type here…*', cell C2).

2. Choose the specific DL-Protocol which was used for the respirometric measurements. To do so, write the D-number in the upper right yellow window (cell M2) or click inside this window to choose it from the dropdown list. The D-number is a unique code for every variation of SUIT protocols and you can find it at the end of the SUIT protocol name (D###). For example, SUIT-001 exists in four different variations: SUIT-001_O2_mt_D001, SUIT-001_O2_pfi_D002, SUIT-001_O2_ce-pce_D003, and SUIT-001_O2_PBMC-PLT_D004. Each one has an unique code and it is designed for the following samples: D001 for isolated mitochondria, tissue homogenate or cells permeabilized previously to addition to the chamber, D002 for permeabilized muscle fibres, D003 for cells that are permeabilized in the chamber and D004 for blood cells, permeabilized in the chamber. In each of these SUIT-001 protocols the sequence of steps is slightly different, therefore it is crucial to choose the correct D-number.

3. After choosing the DL-Protocol, The 'Preview' section will show the titration steps, mark names, states, and reference and baseline states from the chosen protocol.

a. The 'Reference state' and 'Baseline state' are the parameters used for baseline correction and *FCRs* (Flux Control Ratios) calculation in further steps. These values are specific and pre-set for each DLP, being defined as:
   i. Reference state is the respiratory state showing the highest O_2 flux.
   ii. Baseline state is the state showing the lowest O_2 flux.
b. The 'Description' section provides a brief summary of the selected protocol.

4. Select the settings by ticking the boxes:
   a. Remember the project name: your project name will be assigned to every set of data copied from the DLD file. This option is recommended for users who will analyze a dataset from one project in one excel file. By selecting this option, the template will automatically copy the project name from the 'Protocol page' worksheet and paste it to the 'Paste Data' worksheet for all measurements that will be pasted from DatLab. However, if you want to use this template for several projects together, leave this box unchecked and type the project name directly to the 'Paste Data' worksheet. If you wish to leave this option unchecked, it is not necessary to type anything in the 'Project' window at the top of the 'Protocol page' worksheet.
   b. Titration volume correction: Specific flux and *FCRs* will be corrected for the sample dilution effect generated during the stepwise titration. Titration volume correction is required only for samples in suspension (*e.g.,* isolated mitochondria, suspended cells, tissue homogenate). For samples that are not in suspension (*e.g.*, permeabilized mucle fibers), this option should be left unchecked. Remember: if there were changes in titration volumes, or in user-defined protocols, the titration volume must be saved in the .DLD file during or after the measurement.

☞ **Caution**: Remember that you have to select your protocol by its D-number.

☞ **Caution**: Only one protocol should be used in each Excel template copy.

2.3. Paste data

1. In DatLab 7.4, after setting the marks, go to 'Marks' and select 'Slope uncorrected+all info´ or press 'F2'. In the new pop-up 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. Copy to clipboard options: 'Slope uncorrected + all info'.
   e. Select: 'Median'.
   f. Sort by: 'Time' (by default).

   Then, click on [Copy to clipboard] to copy the selected values.
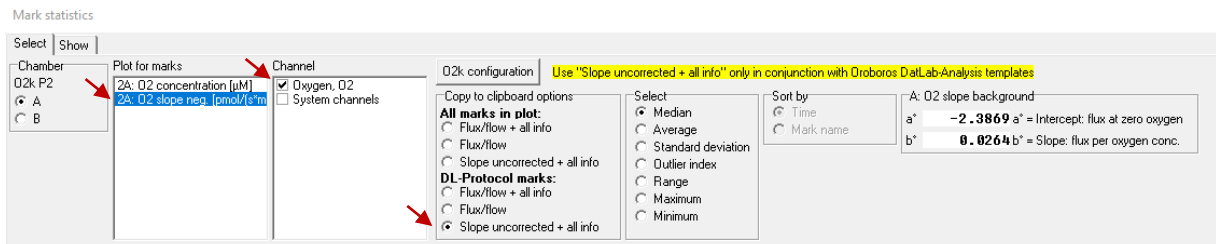
2. In the O2 analysis template DL7.4 select the worksheet 'Paste Data' and paste the copied values in the the cell F7. Data from extra experiments with the same DL-Protocol can be pasted in the consecutive yellow cells below (column F). Up to 250 independent measurements can be pasted in a O2 analysis template DL7.4. Check whether the sequence of steps in the protcol copied is the same as in the protocol chosen.
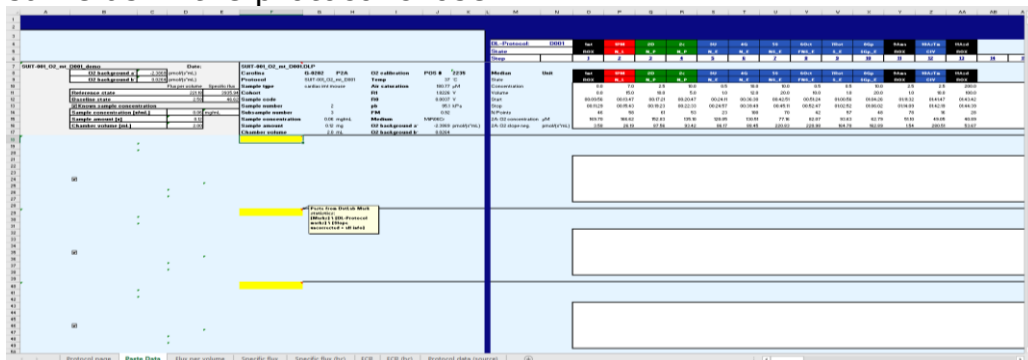
3. If the sample concentration is not yet known, the box 'Known sample concentration' can be unchecked, and the concentration will be considered by default as 1, with units $[x \cdot mL^{-1}]$. In this way, flux values can be normalized and *FCRs* can be obtained even if the sample concentration is unknown (for more information, see: https://www.bioblast.at/index.php/Flux_control_ratio).

4. The grey area (columns A to E) on the 'Paste Data' worksheet involves white editable cells (in columns C and D) for: O_2 background, sample concentration/amount and chamber volume. Once the data is copied from DatLab into the excel template, these white windows will be loaded with values from the pasted data. If you want to change these data later (*i.e.* sample protein amount was measured after the O2k traces were acquired) it is not necessary to edit the DLD file and copy/paste data again. The user can directly edit the values in these white cells of the O2 analysis template DL7.4.

5. 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

2.4. Visualization of the results

1. In the O2 analysis template DL7.4, a summary of your results can be visualized in the following worksheets: Flux per volume', 'Specific flux', 'Specific flux (bc)', '*FCR*' and '*FCR* (bc)' (where bc means baseline corrected).
   a. In this template, the specific flux is given in the following unit: $[pmol \cdot s^{-1} \cdot x^{-1}]$, taking in consideration that the sample concentration is $[x \cdot mL^{-1}]$. The unit x must be substituted by the unit used in your project, *e.g.,* mg of protein or a million

cells, resulting in the following units: [pmol·s^{-1}·mg^{-1}] or [pmol ·s^{-1}· Mill cell] which equals to [amol·s^{-1}·cell^{-1}].

2. On each of the tabs with the results, an incorporated filter function (row 3) allows you to visualize the results by different variables (*e.g.,* date, file name, user, sample type, cohort, sample code). Click on the arrow for the variable by which the results should be filtered and choose the data that should be kept or removed by ticking the boxes. From theses tables the data can be easily copied and pasted to any statistical program or be used to generate graphs.

2.5.   Data trust protection

O2 analysis template DL7.4 has several quality control steps implemented to obtain maximal protection in your data analysis in case of user mistake. To see the overview of all warnings and alarms implemented in this excel template please see the supplementary chapter below. The Excel template will detect any discrepancies in your data caused by for example using the wrong protocol, having a wrong titration steps, copying the wrong data from DatLab or having a non-compatible DatLab version.

2.6.   User defined protocols

The O2 analysis template DL7.4 is provided ready to be used for data analysis with all the SUIT protocols (for O$_2$) provided with the software. The information regarding these protocols is stored in the worksheet 'Protocol data (source)' tab. Each row of this worksheet contains data for one of the predefined SUIT protocols, and is locked for editions. However, the user can also create an *ad hoc* protocol to analyze with the O2 analysis template DL7.4 ('user defined protocol').

1. To create an user-defined protocol, add the information about this new protocol into the yellow row at the bottom of the worksheet

(row 56). The following information is necessary for correct data processing by the O2 analysis template DL7.4:

  a. The name of each step (columns C to V).

  b. Total number of steps ('# steps', column V).

  c. Baseline and reference state (name of the step and number). The name of the step that will be chosen as reference or baseline (columns AS and AU) must be typed identically to what was typed before (at the columns C to V). The number of these chosen steps must be typed at the columns AR and AT.

It is not essential, however it is possible to complete the user protocol with the following information:

  a. States related to each step of the protocol (columns X to AQ) [1]. For support, a link with the basic terminology used for states in DL-Protocols is provided below the table on the worksheet 'Protocol data (source)'.

  b. Protocol name (column A), DLP# (column B), short name and notes (columns AV and AW).

  c. Description of the protocol (column AX).

  d. Name and code of the protocol (columns A and B).

2. One user defined protocol can be created and saved in each Excel file. After being created, these protocols can be chosen in the 'Protocol page' under the name 'DLPu' or the new name edited by the user.

3. References

1. Gnaiger E, Aasander Frostner E, Abdul Karim N, Abdel-Rahman EA, Abumrad NA, Acuna-Castroviejo D, Adiele RC, *et al* (2019) Mitochondrial respiratory states and rates. MitoFit Preprint Arch doi:10.26124/mitofit:190001.v5. - »Bioblast link«

  » MitoPedia: Respirometry
  » Flux / Slope

Acknowledgements

Supplement: Further details

1.   Warning messages

DatLab 7.4-O2 analysis template has several warnings and alert messages implemented to control optimal setup for your data analysis:

- O2 analysis template DL7.4 is only compatible with DatLab 7 versions and older, which allow the option 'copy slope uncorrected+all info'. Therefore, copying different set of data from DatLab will cause the rows dedicated for oxygen concentration and slope uncorrected to turn red.

- *Alert: Incorrect DL-Protocol detected! Check the DLP measured and the DLP setup of this Excel template* - appearing on top in the 'Protocol page' worksheet and the text on the 'DL-Protocol' cell turning red with yellow background;
  Alert: *Multiple protocols detected! Check the DLP used and the protocol setup of this excel template* - appearing on the top of the 'Paste Data' worksheet, together with the name of the selected protocol turning red with yellow background (column G) and the reference state and baseline state cells (column B) appearing with red background:

  The alerts above appear if the DL-Protocol used in the DLD file differs from the one selected in the 'Protocol page' worksheet. Check the exact D-code number used in the measurements and select it in the 'Protocol page' worksheet.

- *Alert: Data detected in a 'Paste Data' worksheet. Please select the DL-Protocol in a yellow window bellow to continue* and *Please select the DL-Protocol in the window above* - appearing at the 'Protocol page' worksheet:

  These warning messages will be visible if data is copied into the 'Paste Data' worksheet, but the DL-Protocol in the 'Protocol page' worksheet is not selected. Select the correct DL-Protocol in the 'Protocol page' worksheet to proceed with data analysis.

- *Alert: 'Known sample concentration' is turned OFF!* - appearing on the 'Paste Data' worksheet:

  The alert appears if you uncheck the 'Known sample concentration' option for any set of copied experimental data in the 'Paste data' worksheet. With this selection, the sample concentration will be considered at 1 x/mL. Therefore the sample concentration/amount and the chamber volume will be no longer visible on the editable area.

- *Alert: The project name is missing! Check the 'Remember the project name' option or type it directly into the yellow cell in column A* – appearing in the 'Paste Data' worksheet:

  The alert will appear if you do not check 'Remember the project name' in the 'Protocol page' and do not write a name for each dataset copied.

- *Alert: Chamber Volume is missing, cannot calculate Specific Flux*! – appearing on 'Paste Data' worksheet;
  Alert: Sample amount is missing, cannot calculate Specific Flux! – appearing on 'Paste Data' worksheet;

  The alerts above will appear if values necessary to calculate specific flux are missing. Accordingly, if the chamber volume is missing, the first alert will appear and a red background will appear on the chamber volume cell (column D and/or G), please fill it with the calibrated chamber volume. Similarly, if the sample amount is missing, the second alert will pop-up and in this case, a red background will appear on the sample amount cell (column D and/or G). Consequently, the sample concentration cannot be calculated, and it will be considered equal to 1 with arbitrary units.

- *Alert: Incorrect DatLab sample concentration* – appearing on 'Paste Data' worksheet;

  This alert will appear if the sample concentration values on the editable area do not match with the ones from the DatLab copied data. Additionally, the text containing the sample concentration units turns red with yellow background (column H). The same colour alert will occur if the units for the sample concentration on the editable area do not match with the ones from the data copied from DatLab.

- *Alert: O2 slope neg. missing!* - appearing on the right side of the 'Paste Data' worksheet;

  This warning message appears if values different than 'O2 slope neg.' (*e.g.,* Flow per cells, Flux per volume) are copied from the DLD file. In this case, the Flux per volume, specific flux and *FCR* values will not be calculated neither included on the other worksheets of the O2 analysis template DL7.4.

- *Alert: Error in titrations!* - appearing on the right side of the 'Paste Data' worksheet;

This alert will appear if the titration sequence of the selected protocol differs from the one copied from the DLD file. The O2 analysis template DL7.4 specifically recognizes discrepancies in the mark names. In this case, the flux per volume, specific flux and *FCR* values will be neither calculated nor included on the other worksheets for the missing titration step/ mark.