NextGenO2k-Manual: NADH-Module

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NextGenO2k: NADH-Module manual

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NextGen-O2k

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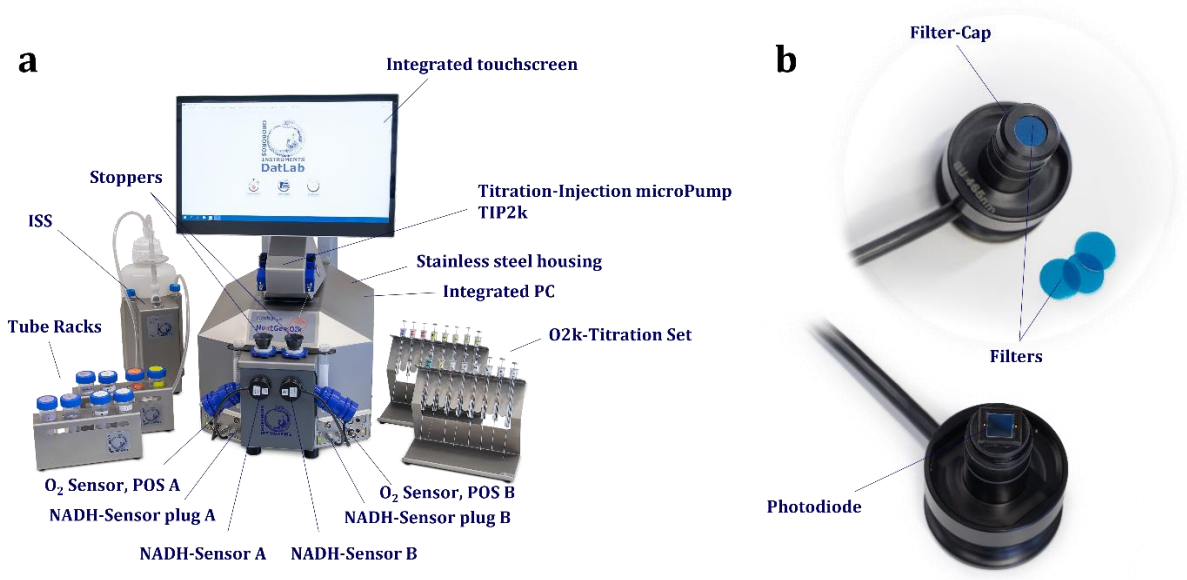
1. Introduction

The co-enzymes NAD+ and NADH are key regulators of cellular energy metabolism. In the cytosol and in the mitochondrial matrix, NAD^+ is reduced to NADH by the action of various dehydrogenases. The NADH generated in the cytosol is transported into the mitochondrial matrix through the malate-aspartate shuttle or glycerophosphate dehydrogenase Complex. Alternatively, lactate dehydrogenase catalyzes the conversion of pyruvate to lactate, oxidizing NADH to NAD^+. The mitochondrial NADH-pool is oxidized to

NAD^+ at the Complex I, feeding electrons into the N-junction. Alterations in the NAD^+/NADH redox states affect the cellular redox homeostasis resulting in various pathological conditions (e.g. aging, cancer, neurodegenerative diseases, diabetes). Therefore, monitoring the NADH-pool represents a crucial indicator of cellular metabolic state. The fact that NADH is intrinsically fluorescent and absorbs light at 340 ± 30 nm, while the oxidized form NAD^+ does not absorb light at this range, allows monitorization of the NADH autofluorescence. Experimentally, since light absorption and emission properties of NADH and NADPH are identical, it is not possible to distinguish the fluorescence spectra of both co-enzymes when measuring NADH autofluorescence. The NADH-Module allows simultaneous measurement of oxygen consumption and NAD(P)H autofluorescence, constituting an important tool for in-depth study of mitochondrial bioenergetics in health and disease.

2. NADH-Module setup

The NADH-Module is part of the NextGen-O2k which consists of an integrated ultraviolet (UV) LED light and NADH-Sensors. The UV light includes built-in lens (IN-C33ATNU2, Inolux, US) and short-pass glass filter (Asahi Spectra, Japan). The NADH-Sensors are composed of a photodiode and equipped with filters. The biological sample is illuminated with UV light (excitation 365 nm) and emits fluorescence at ~490 nm. Oxygen consumption and NAD(P)H fluorescence data are monitored real-time and acquired with the software DatLab 8 (Oroboros Instruments, Austria). The NextGen-O2k are UV light intensity-calibrated, and the UV light intensity can be set in DatLab from 0 mW to 30 mW. A pulsing feature is incorporated in the NADH-Module to minimize any potential negative effect of UV light on biological samples and medium.



The NextGen-O2k is a MultiSensor all-in-one instrument which includes a module to monitor simultaneously oxygen consumption and NAD(P)H autofluorescence: the **NADH-Module**. **(a)** The NADH-Module consists in NADH-Sensors and an integrated ultraviolet (UV) light. **(b)** NADH-Sensors are composed of a photodiode and filters.

NextGen-O2k Manual user information
» https://wiki.oroboros.at/index.php/MiPNet26.13_NextGen-O2k_manual

NADH-Service box contains:
3 Filter-set R370 for NADH (12/Pkg)
2 NADH-Sensors

2.1. Mounting a filter set of the NADH-Sensor

Each NADH-Sensor is delivered with a mounted filter set (three short pass supergel R370 Italian blue filters, Rosco, US). However, NADH-Sensors are equipped with removable Filter-Cap which allows to exchange the photodiode filters:

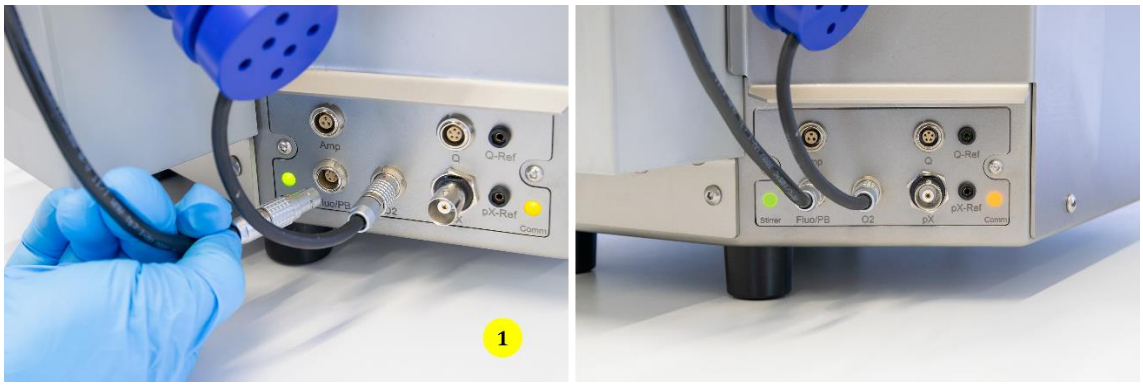
1. Pull the Filter-Cap straight from the sensor.
2. Remove all filters and store them in the Filter-set of the NADH-Service box.
3. Insert and fit the filters in the round window of the Filter-Cap.
4. Hold the sensor and the Filter-Cap in a vertical position. Align the Filter-Cap with the NADH-Sensor and press the Filter-Cap onto the sensor without rotational movements.

2.2. Assembly of the NADH-Sensor

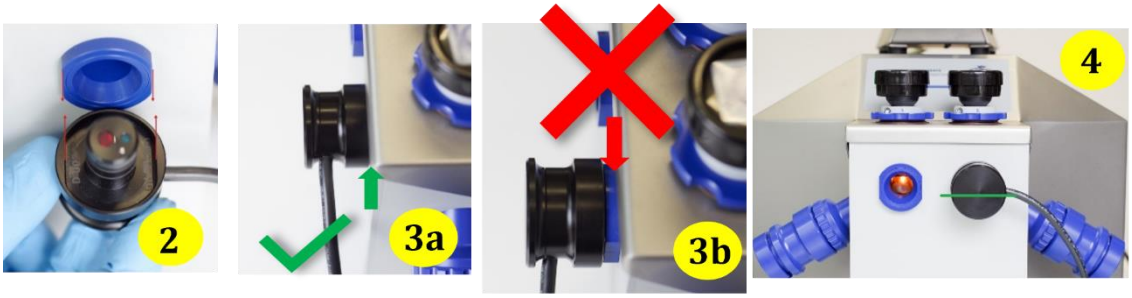
The NADH-Sensors are shipped in the NADH-Service box as an add-on module of the NextGen-O2k. NADH-Sensors are UV light intensity-calibrated with sensor-specific memory and direct input into DatLab 8.

The NADH-Sensors can be only used with the NextGen-O2k.

1. Connect the NADH-Sensor cable to the Fluo/PB plug located in the Main Unit of the NextGen-O2k by inserting the male plug of the cable into the female Fluo/PB plug (1). The red dot on the male plug faces straight upwards. Each NADH-Sensor can be used on either O2k-chamber A or B.



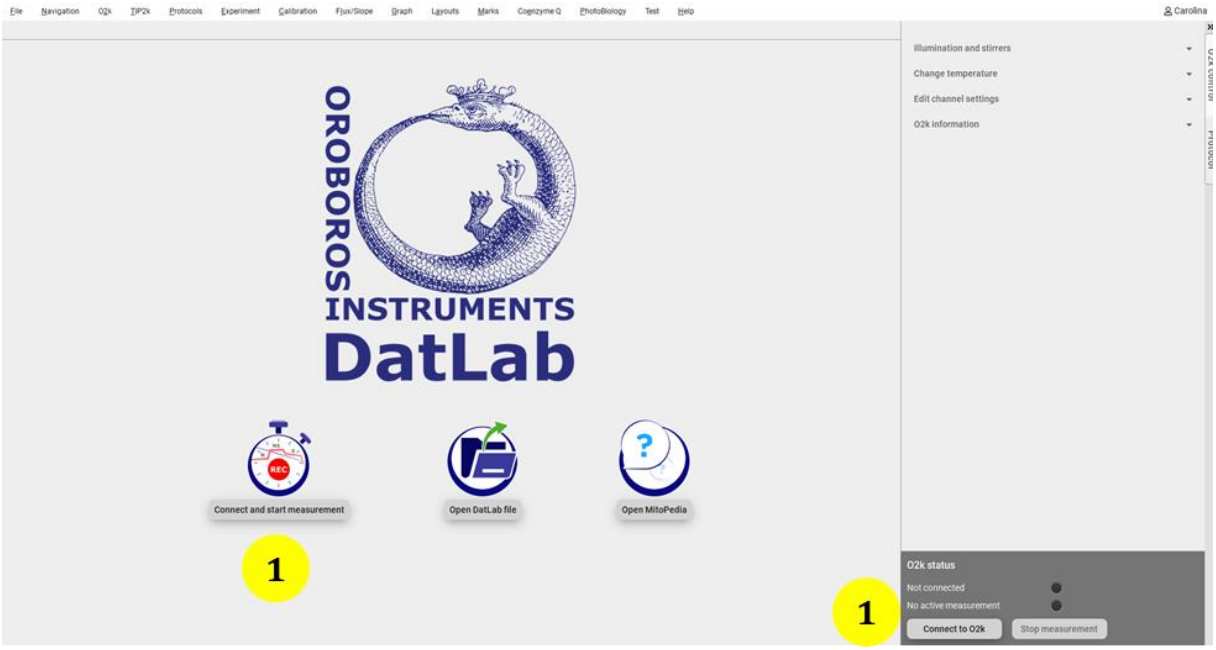
2. The blue frame of the chamber window and the NADH-Sensor are specially designed to only connect at a specific orientation, when the unbowed notches align (2). In this optimal position, the NADH-Sensor is carefully inserted in the window opening until the sensor covers the chamber window without gap (3a,b). In this position, the cable routing is horizontal (4).



3. Connect DatLab 8 and start the NADH measurements.

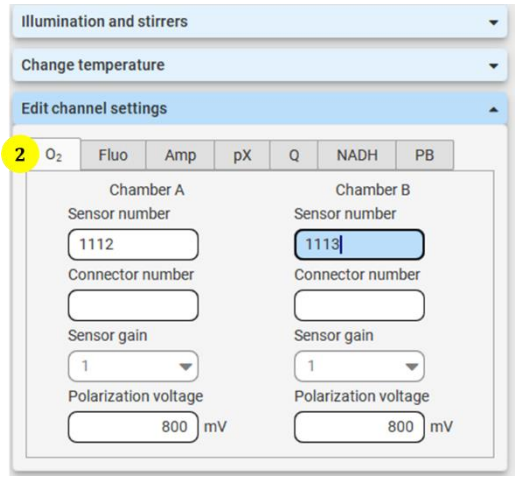
3. Operating instructions using DatLab 8

To start the measurement, click on "Connect and start measurement" or on "Connect to O2k" (1).



Click on ▼ in Edit channel settings (2) and fill the required information for the oxygen channel (O_2) and for the NADH channel (3).

Oxygen, O_2



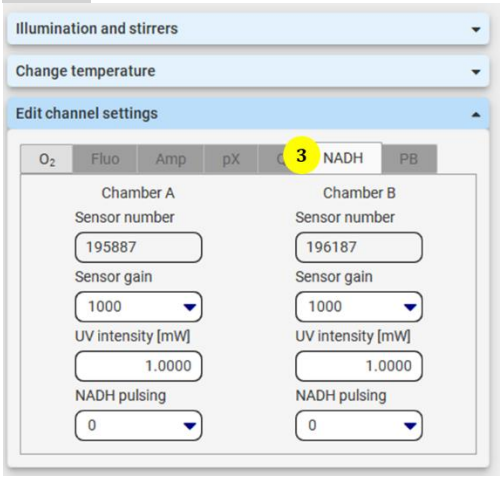
Sensor number: Enter the oxygen sensor number, which can be found on the side of the polarographic oxygen sensor (POS), for chambers A and B. The individual sensor number is included in the protocol for generating a database of calibration values (MiPNet06.03).

Connector number: Enter the connector number, which can be found on the side of the POS-Connector, for chambers A and B.

Sensor gain: The gain for sensor is set to 1 V/µA.

Polarization voltage [mV]: The default for the POS is 800 mV, which is maintained in all routine experiments. Any changes affect the calibration. The voltage can be modified in the range of – 2,000 to + 2,000 mV for scanning the plateau region of the POS, or for different types of sensors.

NADH



Sensor number: The NADH-Sensor number is automatically detected by DatLab 8 and set in the "Sensor number" field if the NADH-Sensor is connected to the O2k. The NADH-Sensor number can be found on the inside of the sensor.

Sensor gain: Select the amplification factor applied to increase the output NADH signal.

UV intensity [mW]: Set the light intensity of the UV LED (0-30 mW).

NADH pulsing: Select the pulsing mode (0-10) for alternating on-off switch of the UV light for a specific period of time during the measurement.

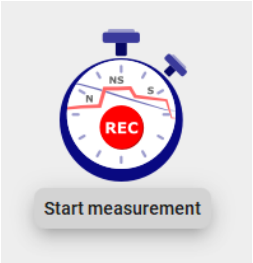
NADH pulsing modes (for data recording interval set to 2 s):

Mode 0: UV LED continuously on
Mode 1: UV LED off for 2 seconds
Mode 2: UV LED off for 4 seconds
Mode 3: UV LED off for 6 seconds
Mode 4: UV LED off for 8 seconds
Mode 5: UV LED off for 10 seconds
Mode 6: UV LED off for 12 seconds
Mode 7: UV LED off for 14 seconds
Mode 8: UV LED off for 16 seconds
Mode 9: UV LED off for 18 seconds
Mode 10: UV LED off for 20 seconds

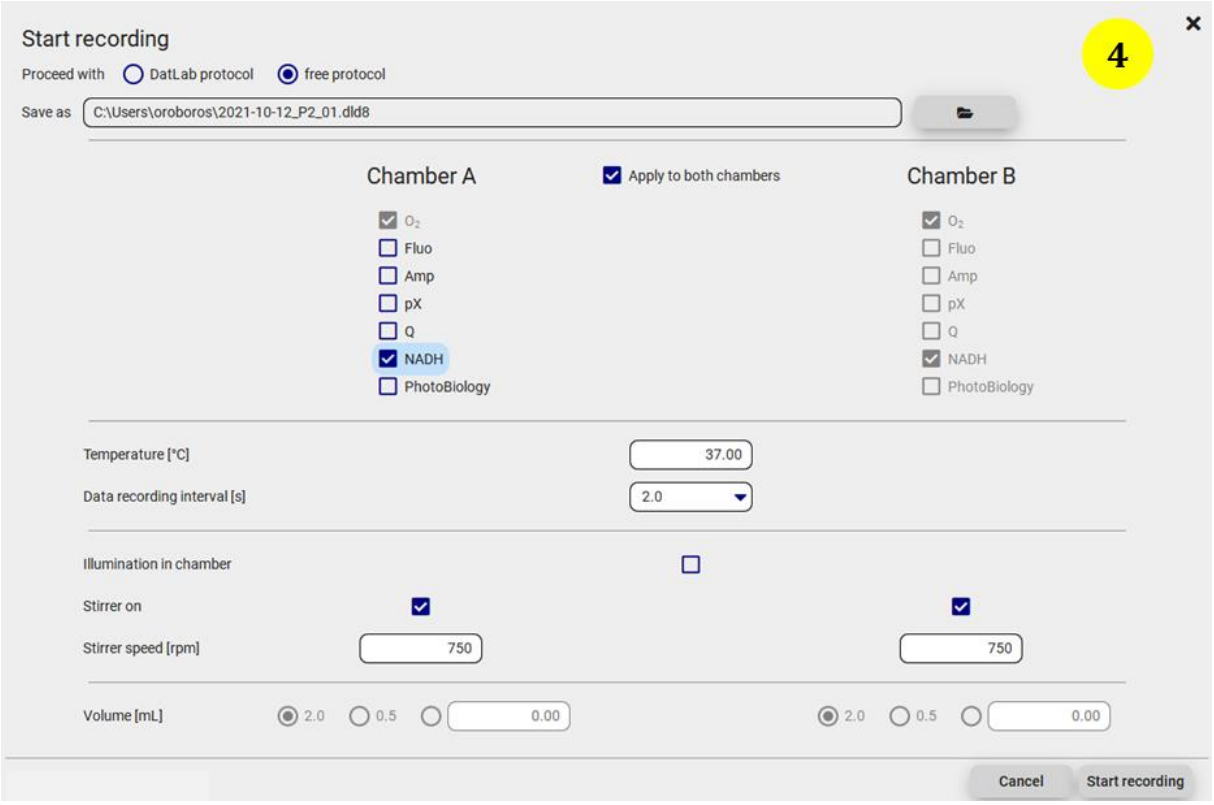
NADH pulsing settings for 2 s data recording interval.

Time [s]	2	4	6	8	10	12	14	16	18	20	22	24
Pulsing set to 0 UV LED (on/off)*	■	■	■	■	■	■	■	■	■	■	■	■
Pulsing set to 1 UV LED (on/off)*	■		■		■		■		■		■	
Pulsing set to 2 UV LED (on/off)*	■			■			■			■		
Pulsing set to 3 UV LED (on/off)*	■				■				■			
Pulsing set to 4 UV LED (on/off)*	■					■					■	
Pulsing set to 5 UV LED (on/off)*	■						■					
Pulsing set to 6 UV LED (on/off)*	■							■				
Pulsing set to 7 UV LED (on/off)*	■								■			
Pulsing set to 8 UV LED (on/off)*	■									■		
Pulsing set to 9 UV LED (on/off)*	■										■	
Pulsing set to 10 UV LED (on/off)*	■											■

*UV LED on.

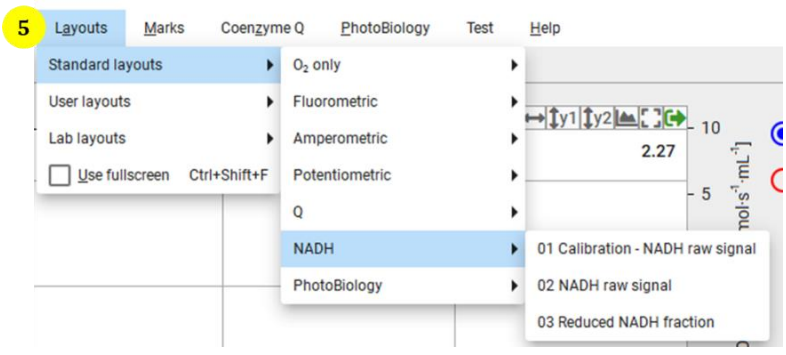


To open the "Start recording" window, click on **Start measurement**.



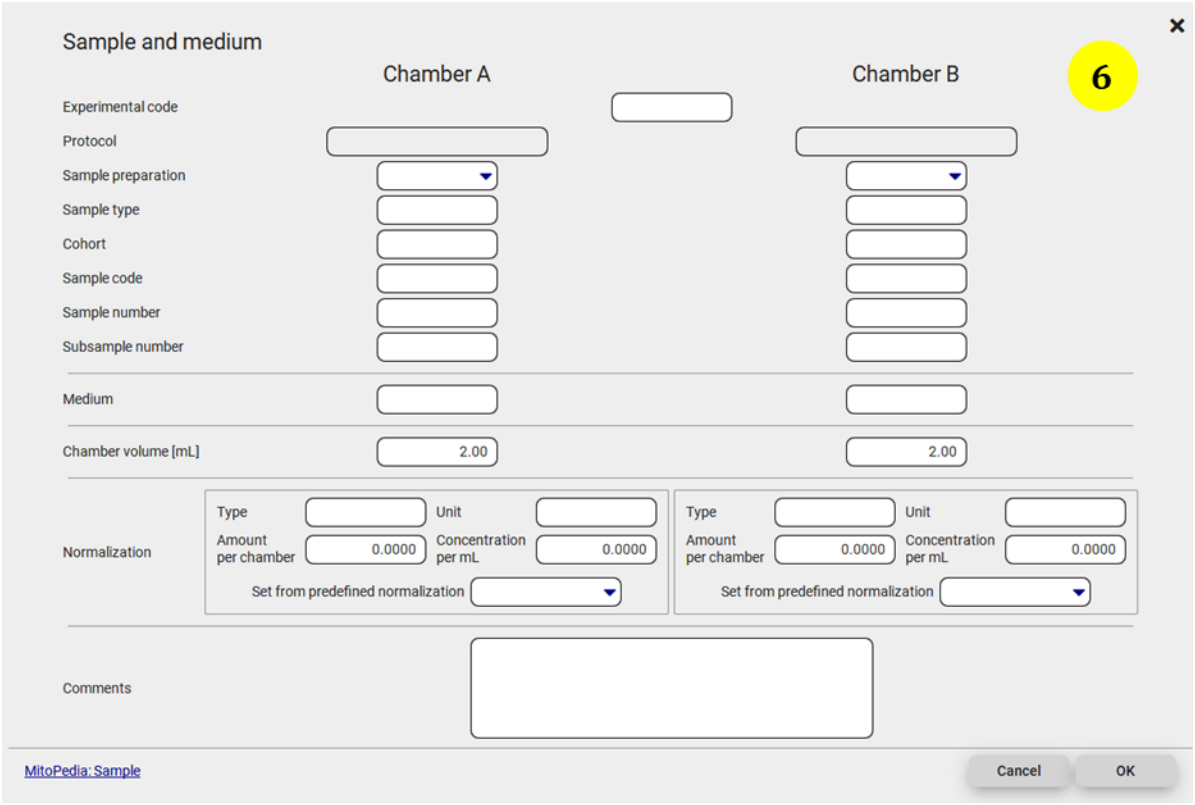
In the "Start recording" window **(4)** either a DatLab protocol (DLP) or a free protocol can be assigned to each chamber. Choose a directory to save the DatLab 8 file (.dld8). In a measurement using a free protocol, by clicking on the "NADH" channel, the illumination of both chambers will be switched-off automatically. Edit the optimal settings for the NADH measurement (i.e. experimental temperature, data recording interval, stirrer speed and chamber volume). In a measurement using a DLP, the pre-defined settings will be automatically loaded. Click on **Start recording**.

To display standardized graphs, plots and scaling of axis for NADH, in the menu **Layouts** **(5)**, select "Standard layouts/NADH/02 NADH raw signal". "02 NADH raw signal": Graphs 1 and 3 show the O2 concentration (blue line; left axis) and O2 flux per volume (red line; right axis) for



chamber A (left) and B (right), respectively. Graphs 2 and 4 show the fluorometric raw signal (black line; left axis) expressed as [nA].

Fill-in the window sample and medium (6). This information will remain for quality control purposes.



NADH settings can be modified by editing Edit channel settings (2) in the side menu.

Proceed with the desired experiment.

To stop the simultaneous measurement of oxygen consumption and NAD(P)H autofluorescence, click on Stop measurement. The dld8. file will be automatically saved. The UV light will switch-off.

4. Demo experiment

4.1. Materials

- Isolated mitochondria (mt): isolated mitochondria from mouse liver. Final concentration: 0.1007 mg/mL.
- Respiration medium: Mitochondrial respiration medium, MiRK03 without BSA (-BSA) (see reference: https://wiki.oroboros.at/index.php/MiRK03) was used.
- NADH: MW: 709.41 g·mol^{-1} (VWR/424237L). Stock solutions of 0.22 mM were freshly prepared every experimental day. 3.90 mg dissolved in 25 mL of 10 mM NaOH. For further details please see: https://wiki.oroboros.at/index.php/Nicotinamide_adenine_dinucleotide#Application_in_HRR.
- Hydrogen gas obtained from Oxia (oxygen regime controller from HyperOxia to HypOxia in the O2k).
- DatLab 8 software.

4.2. General settings

- Temperature [° C]: 37
- Data recording interval [s]: 2
- Illumination: off
- Stirrer: on
- Stirrer speed [rpm]: 750
- Chamber volume [mL]: 2

4.3 O_2 channel settings

- Sensor gain: 1
- Polarization voltage [mV]: 800
- Slope smoothing: 40

4.4. NADH channel settings

- Sensor gain: 1000
- UV intensity [mW]: 1
- NADH pulsing: 1
- Slope smoothing: 40

4.5. DL-Protocol

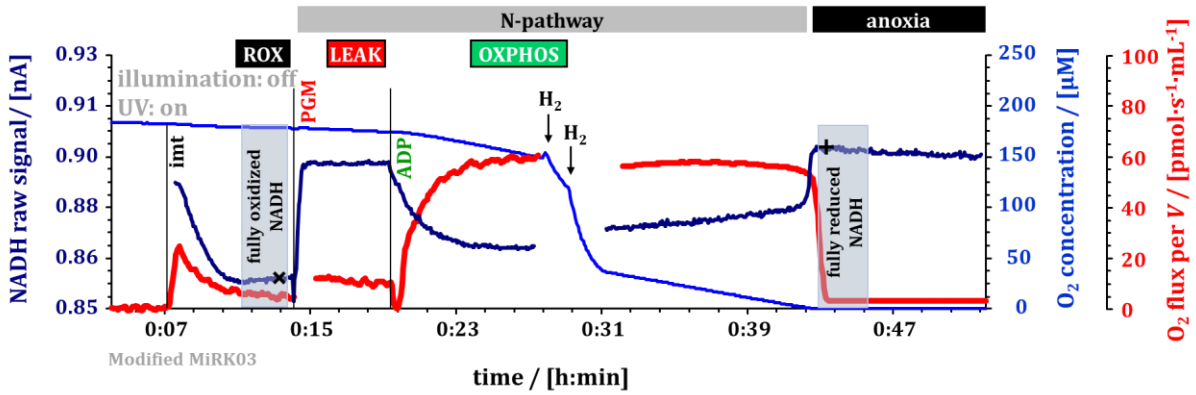
- DL-Protocol: SUIT-032 NADH mt D078

Abbreviation list and concentrations used:
imt: isolated mitochondria
P: pyruvate, 5 mM
G: glutamate; 10 mM
M: malate; 2 mM
D: ADP; 2.5 mM
H_2: Hydrogen gas

The measurements were performed in pre-calibrated 2 mL closed-chambers using modified respiration medium MiRK03(-BSA) under continuous stirring (750 rpm).

Simultaneous measurement of oxygen consumption and NADH autofluorescence starts after the addition of isolated mitochondria (imt). Once oxygen fluxes and NADH autofluorescence signal stabilized, NADH-linked substrates were added (pyruvate,

glutamate and malate, PGM) to support NADH-pathway in the LEAK state. In the LEAK state, we observed an increase in the NADH fluorescence which was attributed to a large reduction of mitochondrial NADH. The addition of kinetically saturating ADP concentration triggered a decrease in the NADH raw fluorescence signal which was attributed to the oxidation of NADH at Complex I. For calibration of the fully reduced NADH, NADH fluorescence was measured under anoxia. To decrease the oxygen levels, hydrogen gas (obtained from Oxia) was injected into the open O2k-chambers. When an oxygen concentration around 30 µM was reached, both chambers were closed. An increase in NADH fluorescence under anoxia showed the maximal reduction of the NADH-pool.

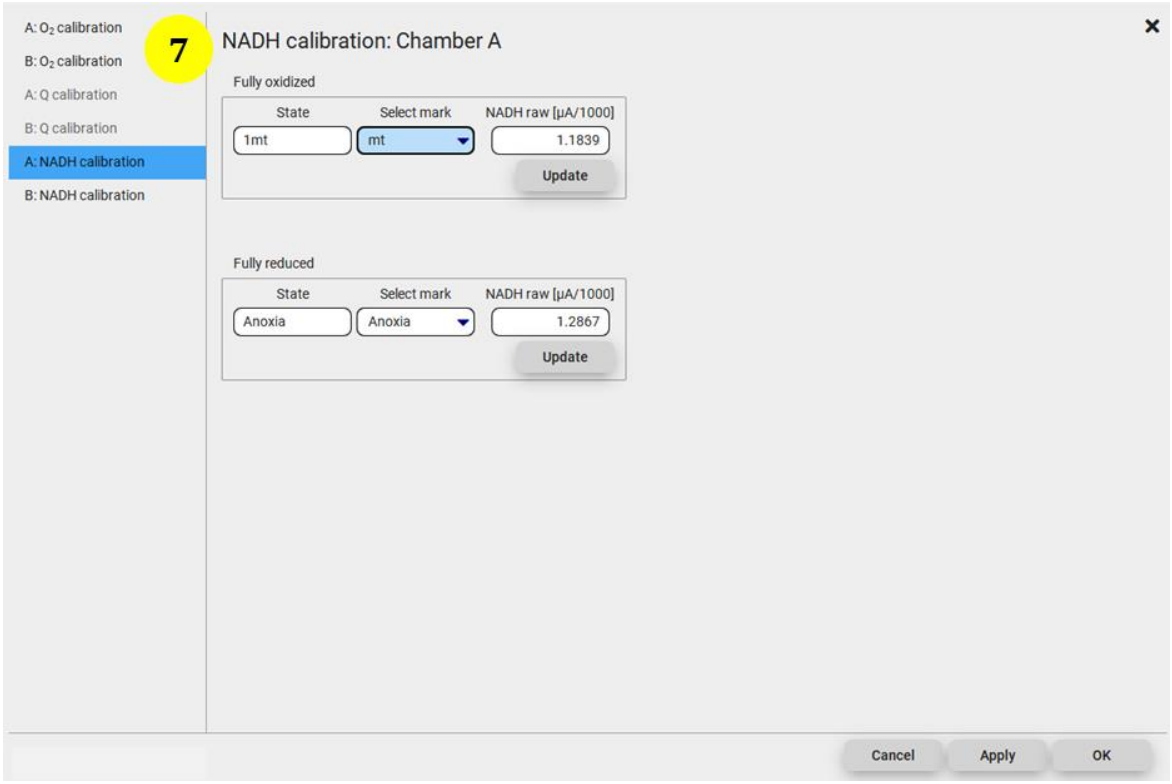


Simultaneous measurement of oxygen flux and NADH autofluorescence using mitochondria isolated from mouse liver. The experiments were carried out in MiRK03(-BSA), at 37 °C. Blue line: O_2 concentration [µM]; red line: O_2 flux per volume [pmol·s^{-1}·mL^{-1}]; dark blue line: NADH raw autofluorescence signal [nA]. DLD file: 2021-06-01 PN6-04.

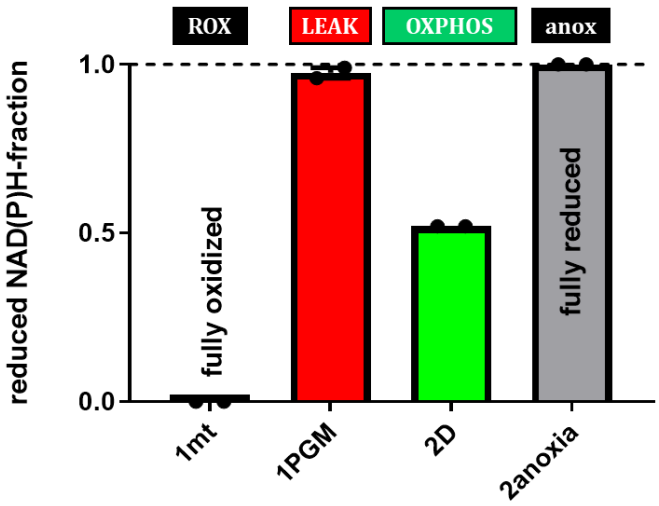
5. Analysis: reduced NADH-fraction

The NADH-Module allows the determination of NADH/NAD+ ratios by calibrating for minimum and maximum NADH fluorescence values: the fully oxidized and fully reduced NADH. Fully oxidized NAD$^+$ (taken as 0) was obtained in the presence of isolated mitochondria (in the absence of exogenous substrates), and fully reduced NADH under anoxia (taken as 1).

DatLab 8 allows the calibration of fully oxidized and fully reduced NADH. Click on the menu **Calibration** and select the tab "**A: NADH calibration**" (7). Select the mark corresponding to the fully oxidized and fully reduced NADH and press **Update**. Select **Apply** and repeat for chamber B.



To plot the reduced NADH fraction, in the upper menu **Layouts**, select"Standard layouts/NADH/**03 Reduced NADH fraction**".



Reduced NADH fraction using mitochondria isolated from mouse liver. For calibration fully oxidized NAD^+ was obtained in the presence of isolated mitochondria (1mt) and fully reduced NADH under anoxia (2anoxia).

The results of the reduced NADH fraction indicates that in the LEAK state (NADH-pathway using pyruvate, glutamate and malate as fuel substrates) 97 % of the NADH-pool was reduced, while in OXPHOS state the reduced NADH was 52 %.

7. References

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8. Author contributions

Doerrier C and Gnaiger E are responsible for the project and instrumental development. Doerrier C prepared the MiPNet. Komlódi T contributed to the preparation of the MiPNet. Haider M and Niedenzu W developed the DatLab 8 software. Schwaninger H, Walter-Vracevic M and Philipp G are responsible for the logistics of electronic and mechanical development of the NextGen-O2k.

9. Acknowledgement

We thank Lisa Tindle-Solomon for proofreading and Paolo Cocco for taking and editing the NextGen-O2k photos.

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