NextGenO2k-Manual: NADH-Module

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NextGenO2k: NADH-Module manual

Carolina Doerrier[1], Hannes Schwaninger[2], Michael Walter-Vracevic[2], Markus Haider[3], Wolfgang Niedenzu[1], Timea Komlódi[1], Philipp Gradl[2], Erich Gnaiger[1]

[1]Oroboros Instruments
High-Resolution Respirometry
Schoepfstrasse 18, A-6020 Innsbruck, Austria
Email: instruments@oroboros.at
www.oroboros.at

[2]WGT-Elektronik GmbH & Co KG
Rettenbergstraße 30a, A-6114 Kolsass, Austria

[3]Haider Technology Consulting
Fischnalerstraße 23, 6020 Innsbruck, Austria
Schoepfstrasse 18, 6020 Innsbruck, Austria
Email: info@shtech.at
www.shtech.at

NextGen-O2k

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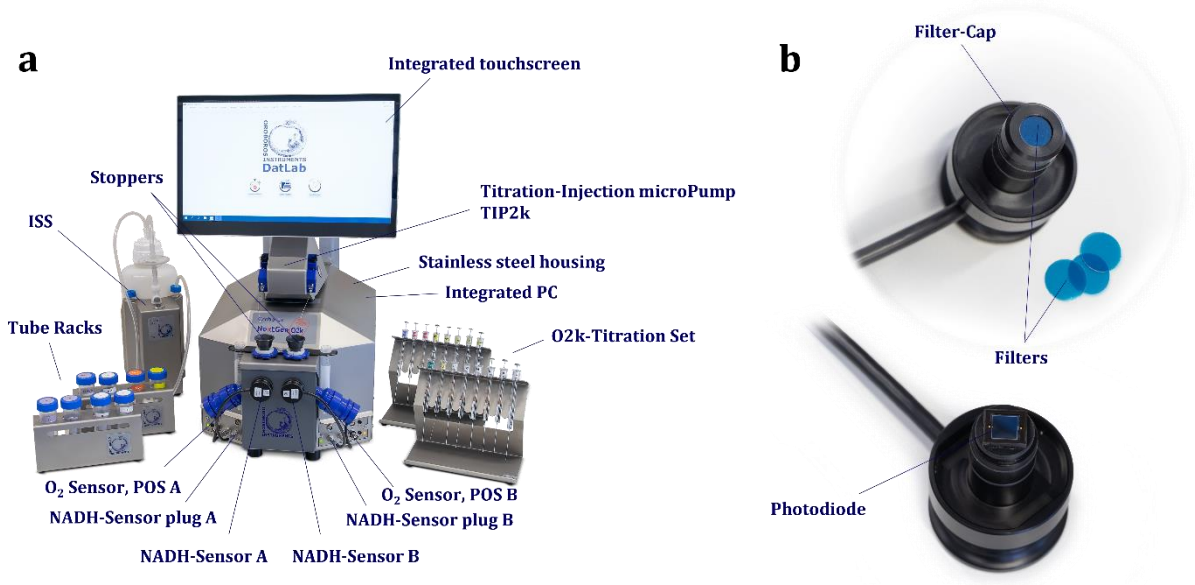
1. Introduction

Nicotinamide adenine dinucleotides 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 NADH/NAD^+-redox states affect the cellular redox homeostasis resulting in various pathological conditions (e.g. aging, cancer, neurodegenerative diseases, diabetes). Therefore, monitoring the nicotinamide adenine dinucleotide (NAD) 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, enables measurement of the NAD-redox state based on 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 (O_2) 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, designed for use with the NextGen-O2k, consists of an integrated ultraviolet (UV) LED light and NADH-Sensors. The UV light includes a built-in lens (IN-C33ATNU2, Inolux, US) and a 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. O_2 consumption and NAD(P)H fluorescence data are monitored real-time and acquired with the software DatLab 8 (Oroboros Instruments, Austria). The NextGen-O2ks 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 NADH-Module for the Oroboros NextGen-O2k instrument allows simultaneous measurement of O_2 consumption and NAD(P)H autofluorescence. **(a)** NextGen-O2k with NADH-Module consisting 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-sets R370 for NADH (12/Pkg)
2 Stirrer-Bar\black PEEK\15x6 mm
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 to allow exchange of the photodiode filters (e.g. number of filters, different filter type, damaged filter):

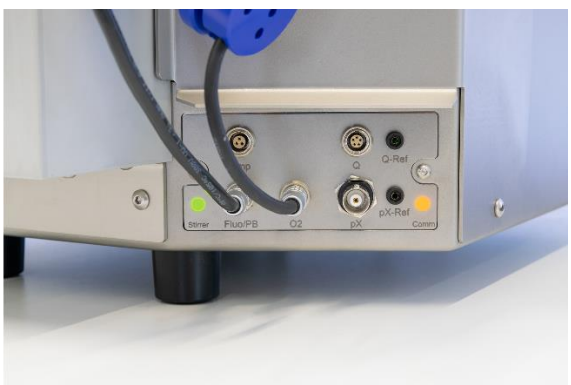
1. Pull the Filter-Cap from the sensor without any rotational movements.
2. Remove all filters and store them in the corresponding Filter-set case found in the NADH-Service box.
3. Insert and fit the new filters in the round window of the Filter-Cap.
4. Hold the sensor and the Filter-Cap in a vertical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

2.2. Assembly of the NADH-Sensor

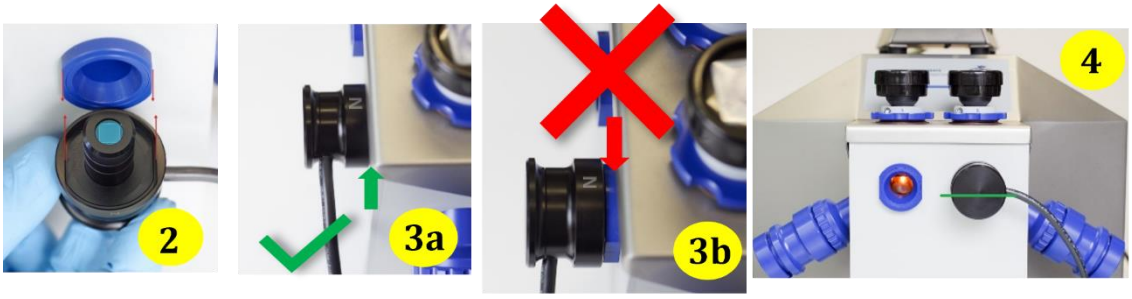
The NADH-Sensors are shipped in the NADH-Service box as an add-on module of the NextGen-O2k. NADH-Sensors are UV light intensity-calibrated with sensor-specific memory and direct input into DatLab 8.

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

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



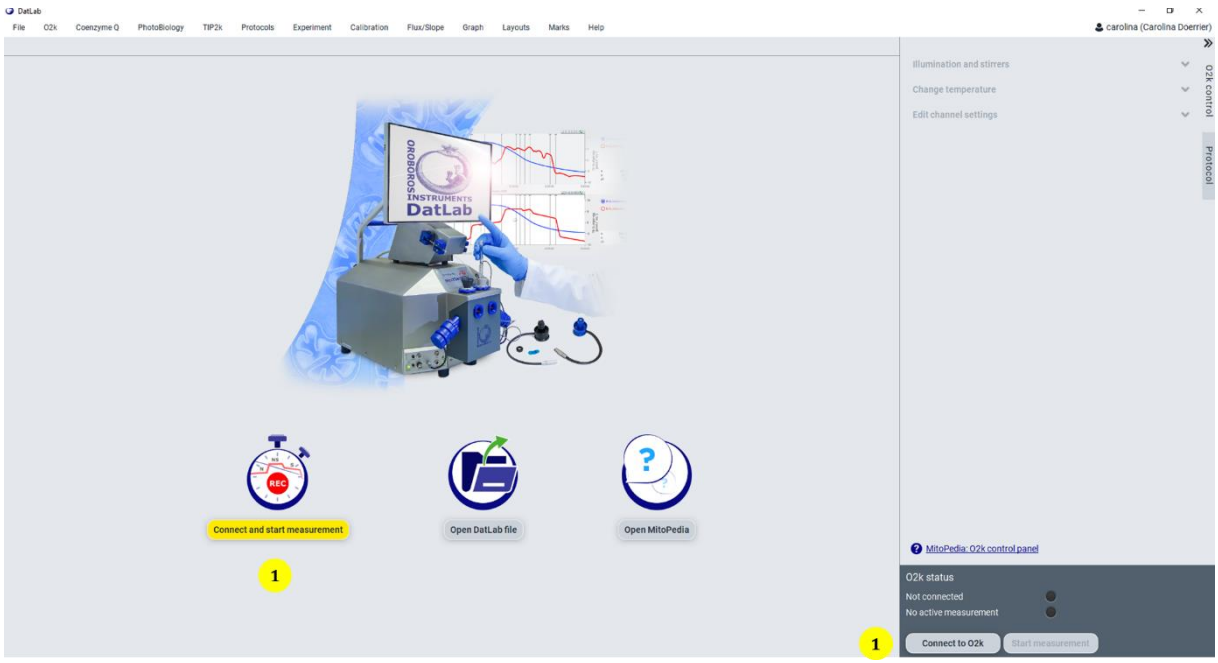
2. The blue frame of the chamber window and the NADH-Sensor are specially designed to only connect at a specific orientation, when the flat edges align (2). In this optimal position, the NADH-Sensor is carefully inserted in the window opening until the sensor is flush with the main unit, covering the chamber window entirely (3a,b). In this position, the cable routing is horizontal (4).



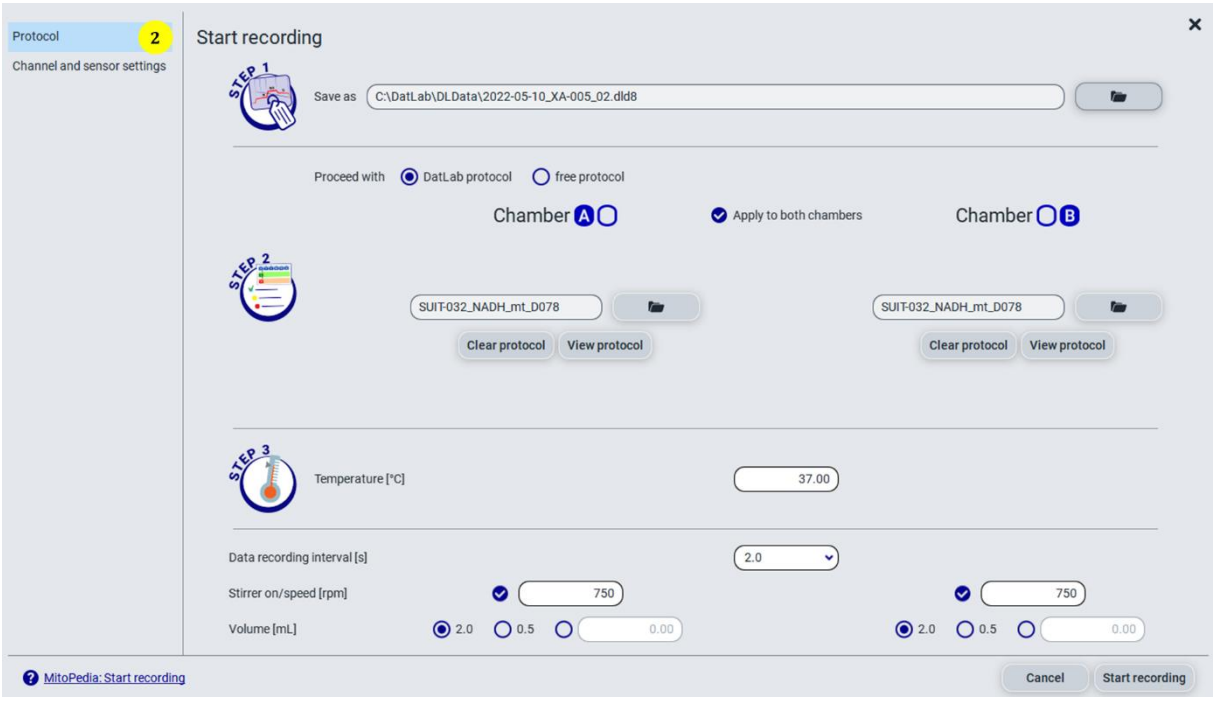
3. Connect DatLab 8 and start the NADH measurements.

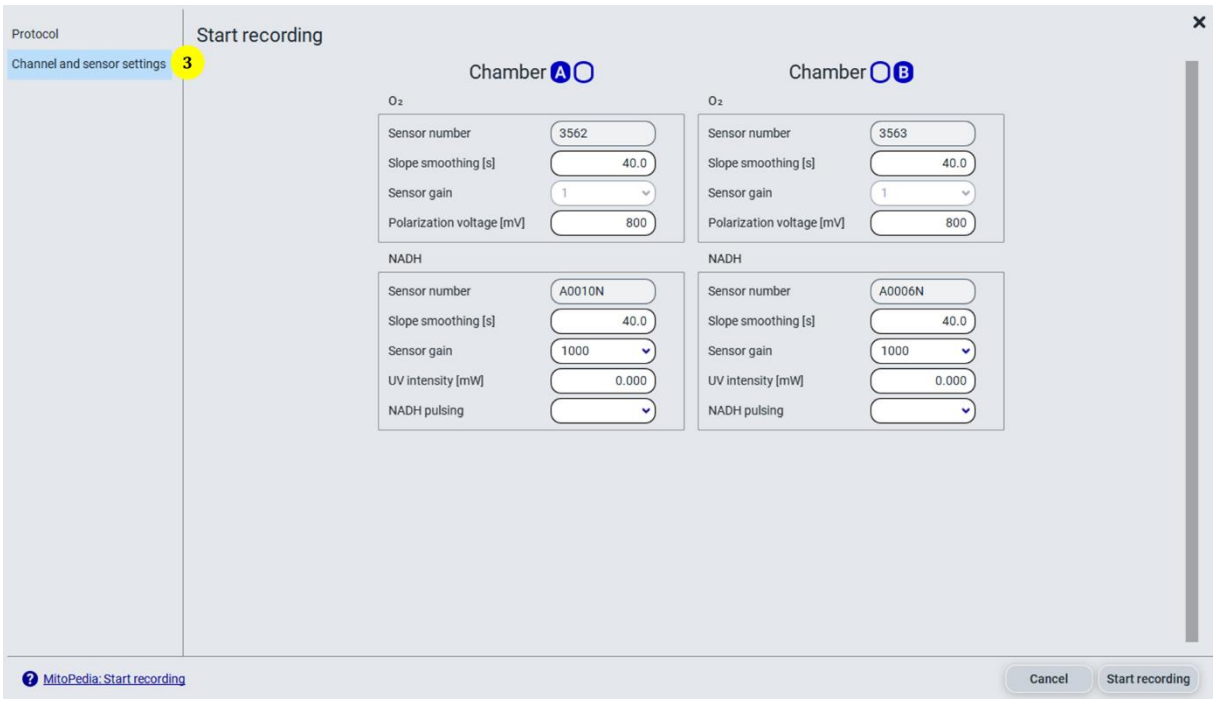
3. Operating instructions using DatLab 8

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



In the **Protocol** tab of the "Start recording" window (2), choose a directory to save the DatLab 8 file (.dld8), assign to each chamber either a DatLab protocol (DLP) or a free protocol, and set experimental temperature, data recording interval (default 2 s), stirrer speed (default 750 rpm) and chamber volume. In the **Channel and sensor settings** tab (3) edit/set the optimal settings for the NADH measurement (e.g. NADH sensor gain, UV light intensity, NADH pulsing mode). In a measurement using a DLP, pre-defined settings will be automatically loaded.





Oxygen, O_2

Sensor number: Enter the O_2 sensor number, which can be found on the side of the polarographic O_2 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).
Slope smoothing [s]: For biological experiments slope smoothing should be 40 s.
Sensor gain: The gain should be 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.

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.
Slope smoothing [s]: For biological experiments slope smoothing should be 40 s.
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) to set the frequency of the UV light pulse 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.

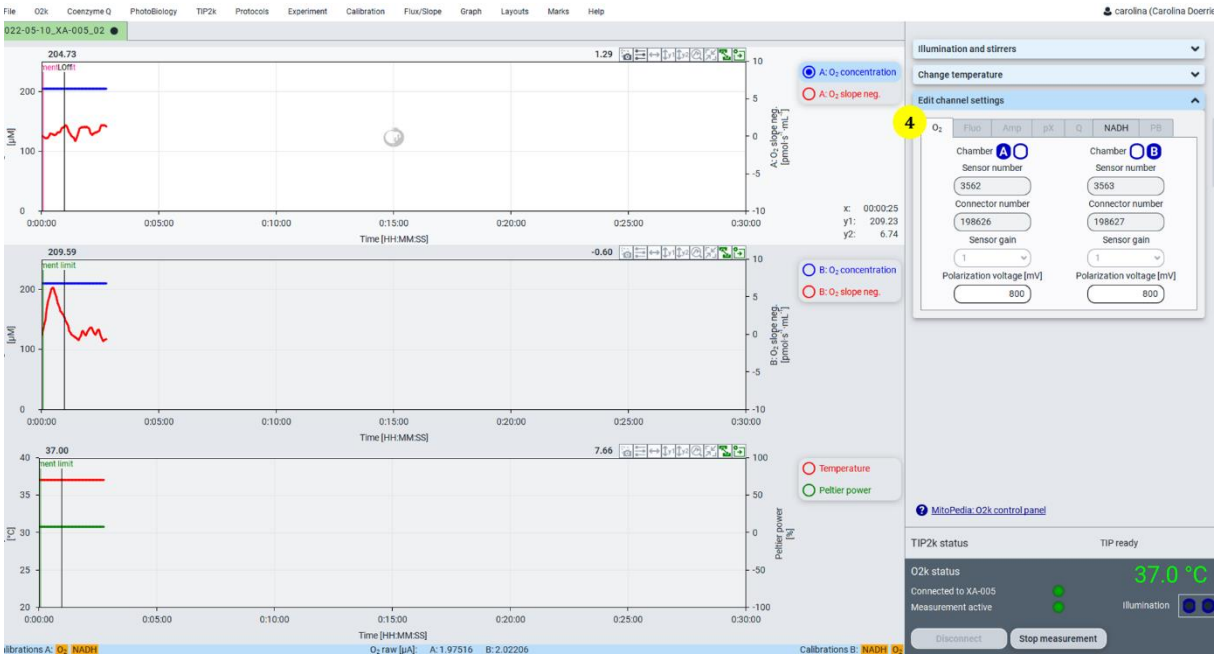
Time [s]	2	4	6	8	10	12	14	16	18	20	22	24
Pulsing set to 0 UV LED (on/off)*	■	■	■	■	■	■	■	■	■	■	■	■
Pulsing set to 1 UV LED (on/off)*	■		■		■		■		■		■	
Pulsing set to 2 UV LED (on/off)*	■			■			■			■		
Pulsing set to 3 UV LED (on/off)*	■				■				■			
Pulsing set to 4 UV LED (on/off)*	■					■					■	
Pulsing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

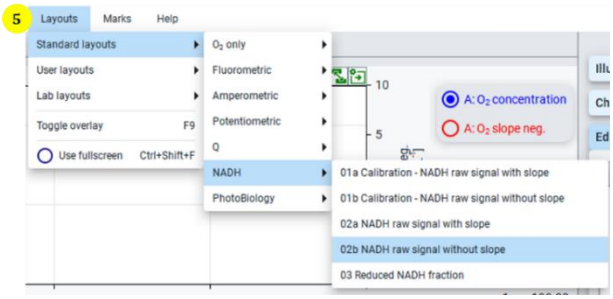
Click on **Start recording**.

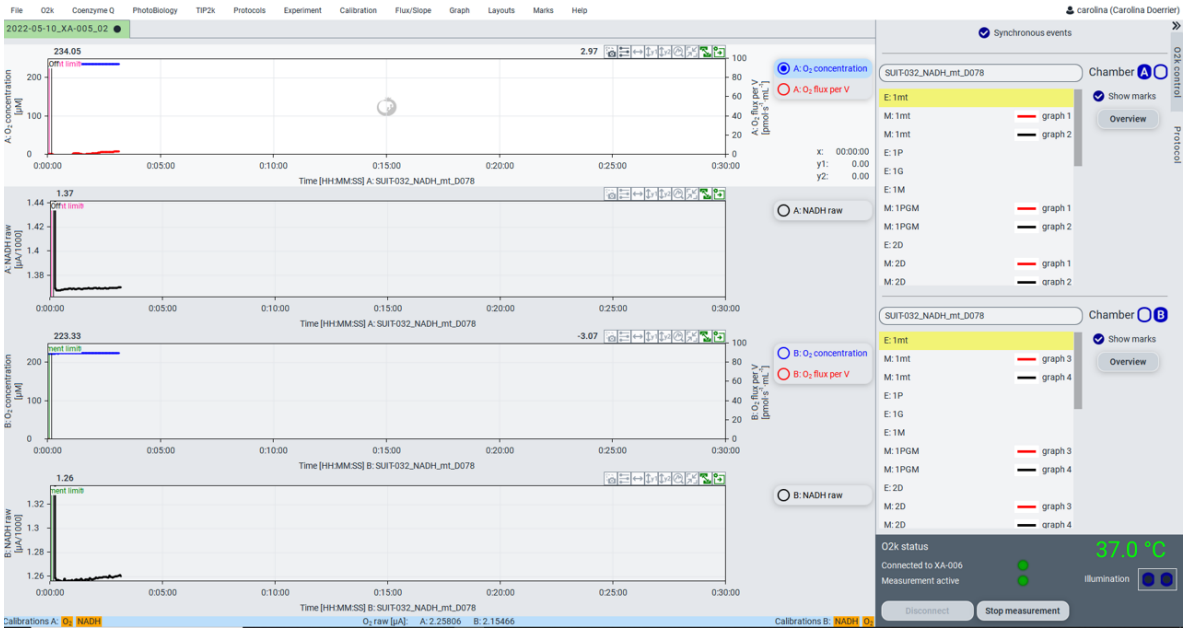
O_2 and NADH settings can be modified in the side menu **O2k control****Edit channel settings** (4) .

Illumination of both chambers is automatically switched-off after 60 s.



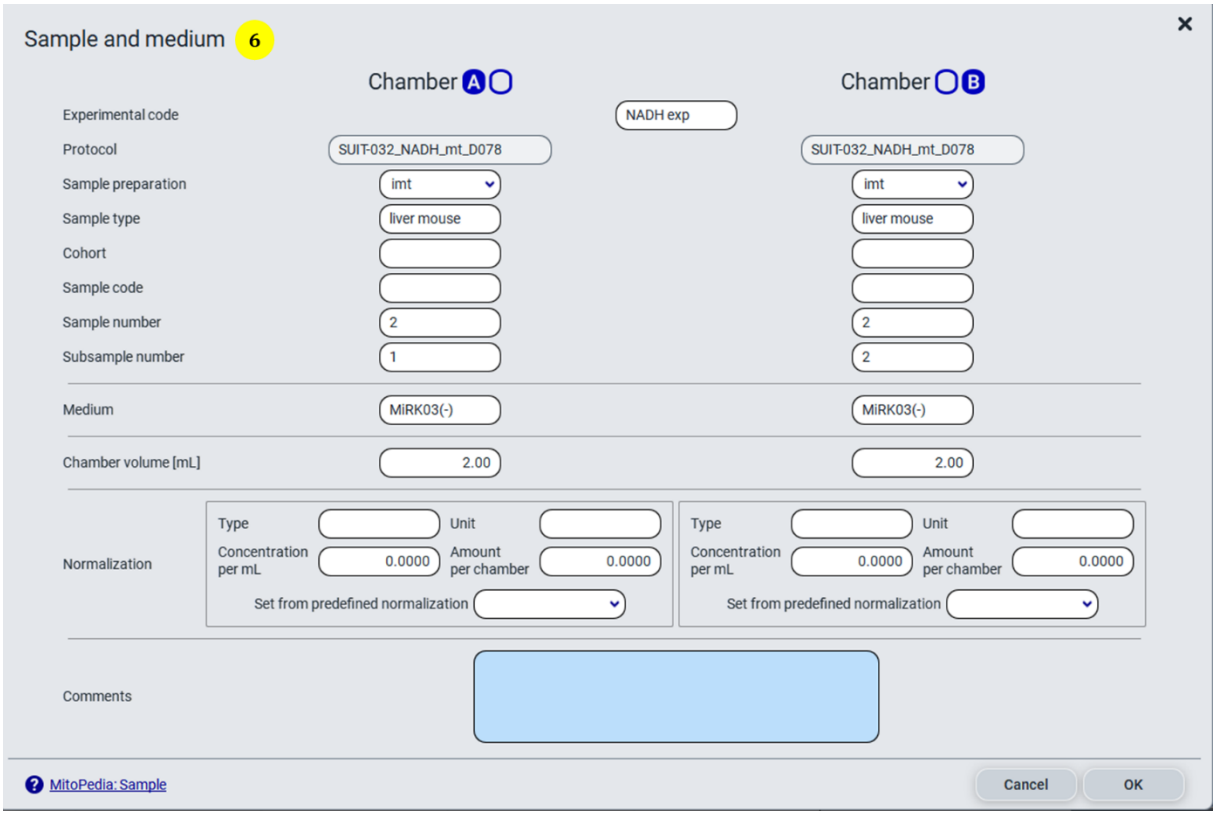
To display standardized graphs, plots and scaling of axis for NADH, in the menu **Layouts** (5), select **Standard layouts****NADH****02b NADH raw signal without slope**: Graphs 1 and 3 show the O_2 concentration (blue line; left axis) and O_2 flux per volume (red line; right axis) for chamber A (left) and B (right), respectively. Graphs 2 and 4 show the fluorometric raw signal (black line; left axis) expressed as [nA].



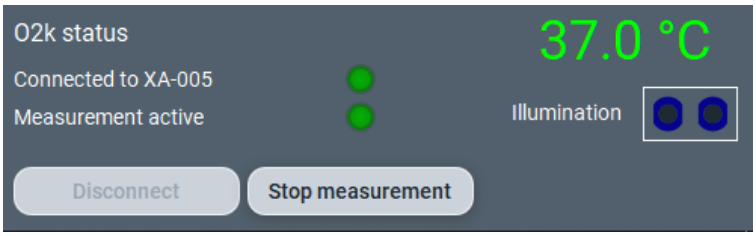


Click on **Experiment****Sample and medium** menu or press **F3** and edit the fields displayed in the window (6). This information will remain for quality control purposes.

Proceed with the desired experiment.



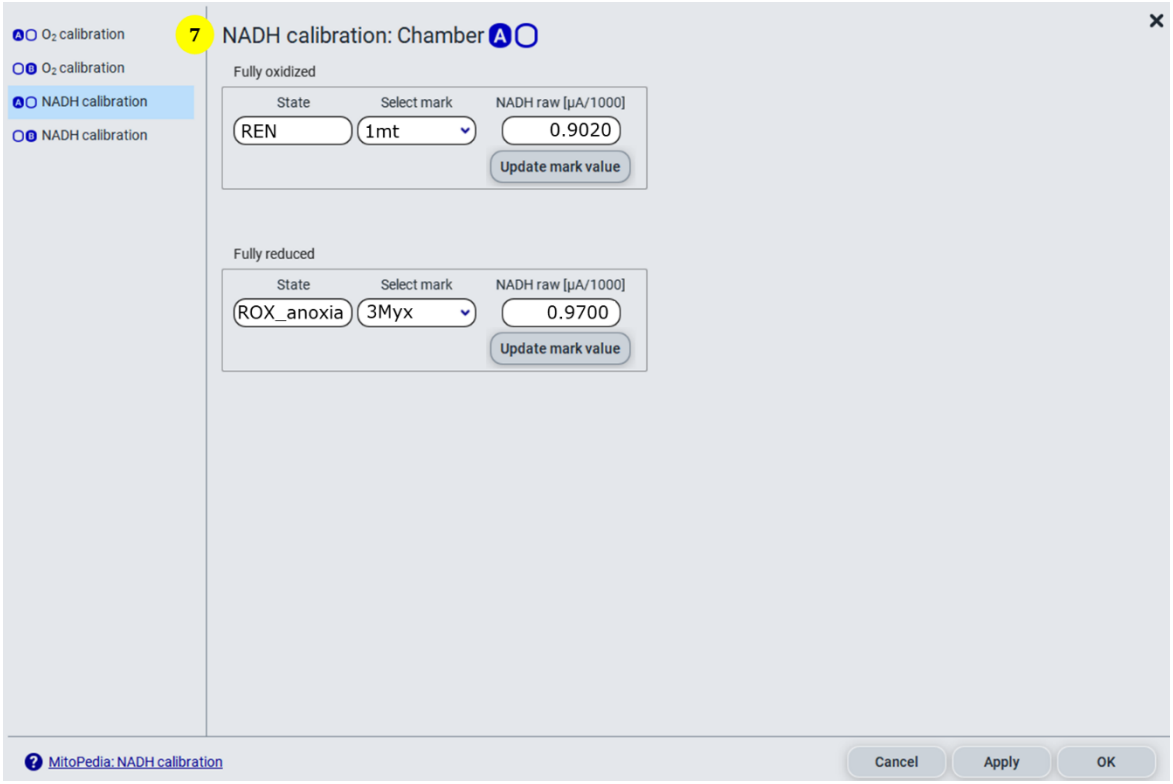
To stop the simultaneous measurement of O_2 consumption and NAD(P)H autofluorescence, click on **Stop measurement**. The dld8. file will be automatically saved and the UV light will switch-off.



4. Analysis: reduced NADH-fraction

For evaluation of NAD-redox state, we express NAD-redox ratios as the reduced NADH-fraction. Reduced NADH-fraction is calibrated for minimum and maximum NADH fluorescence values obtained at fully oxidized and fully reduced NAD-pool. Fully oxidized NAD (taken as 0) can be obtained (*1*) in the presence of isolated mitochondria (in the absence of exogenous substrates), or (*2*) after depletion of endogenous substrates. Fully reduced NAD (taken as 1) can be obtained after inhibition of the electron transfer system.

DatLab 8 allows the calibration of fully oxidized and fully reduced NAD. Click on **Calibration****Calibration** menu in the upper menu or press **F5**, and select the tab **A or B NADH calibration** (7). Select the mark corresponding to the fully oxidized and fully reduced NAD and click on **Update mark value**. Click on **Apply** and repeat for chamber B. To plot the reduced NADH-fraction, in the upper menu under **Layouts**, select **Standard layouts****NADH****03 Reduced NADH fraction**.



5. Demo experiment

5.1. Materials

- Isolated mitochondria (mt): isolated mitochondria from mouse liver. Final concentration: 0.1007 mg/mL.
- Respiration medium: Mitochondrial respiration medium MiRK03 without BSA, MiRK03(-) (see reference: https://wiki.oroboros.at/index.php/MiRK03).
- NADH: MW: 709.41 g·mol^{-1} (VWR/424237L). Stock solutions of 0.22 mM freshly prepared each 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 (H_2) obtained from Oxia (oxygen regime controller from HyperOxia to HypOxia in the O2k) can be used to decrease the O_2 concentration in the O2k chambers.
- DatLab 8 software.

5.2. General settings

- Temperature [° C]: 37
- Data recording interval [s]: 2
- Illumination: off
- Stirrer: on

- Stirrer speed [rpm]: 750
- Chamber volume [mL]: 2

5.3 O_2 channel settings

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

5.4. NADH channel settings

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

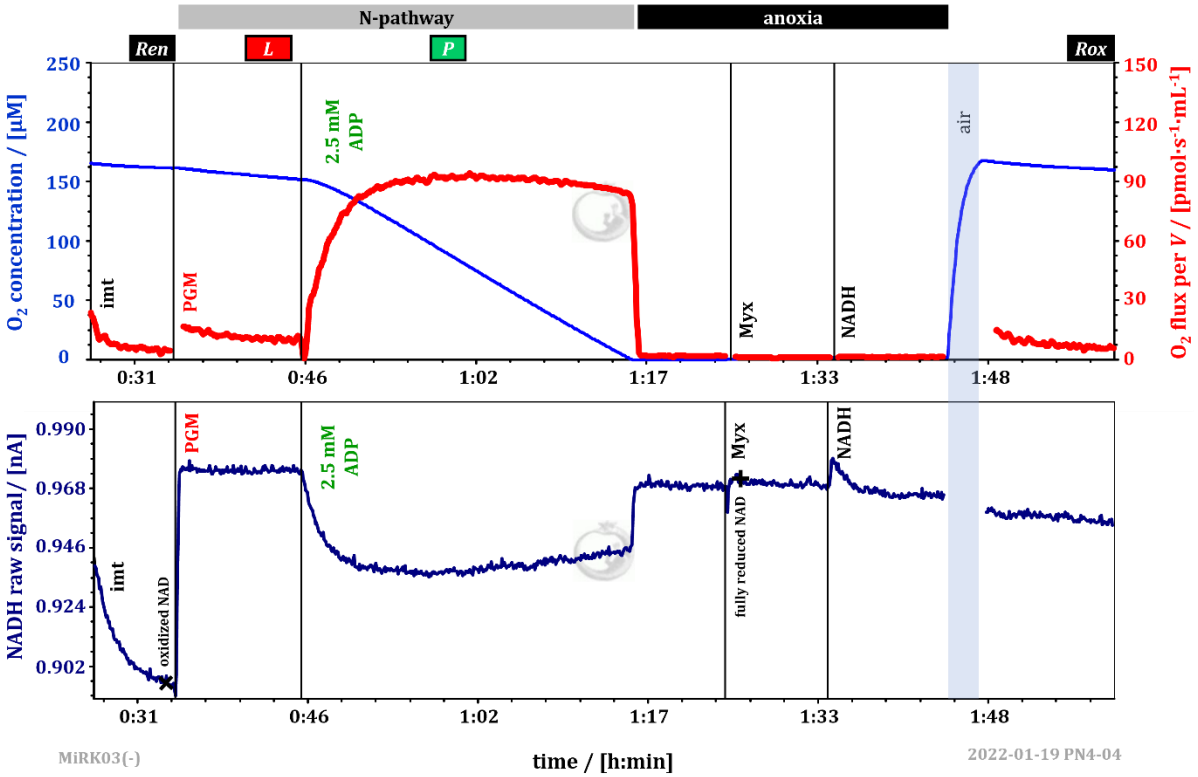
5.5. DL-Protocol

- 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
Myx: myxothiazol, 0.5 µM
NADH: reduced nicotinamide adenine dinucleotide, 0.22 µM

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

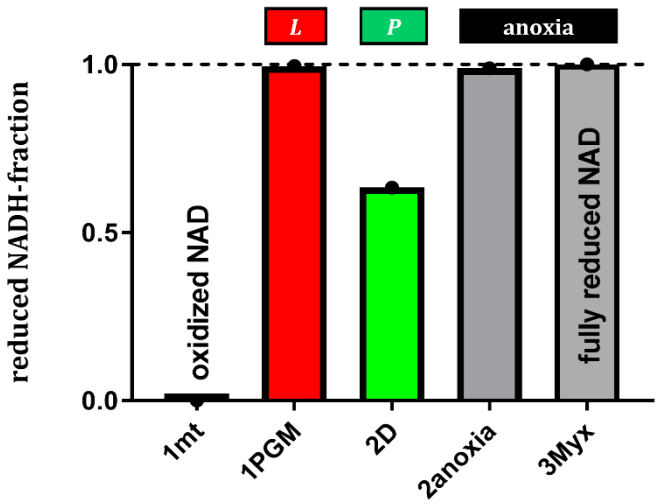
Simultaneous measurement of O_2 consumption and NADH autofluorescence starts after the addition of isolated mitochondria imt. Once O_2 fluxes and NADH autofluorescence signal stabilized, NADH-linked substrates were added (pyruvate, glutamate and malate, PGM) to support NADH-pathway in the LEAK state. In the LEAK state, we observed an increase in the NADH fluorescence which was attributed to a large reduction of mitochondrial NAD-pool. 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. Afterwards, NAD-pool was reduced during aerobic-anoxic transition. Next, Complex III inhibitor myxothiazol Myx was added under anoxia to measure the maximal reduction of the NAD-pool. Finally, reoxygenation allowed *Rox* measurement for baseline correction of O_2 fluxes.



Simultaneous measurement of O_2 flux and NADH autofluorescence using mitochondria isolated from mouse liver. SUIT-032 NADH mt D078. 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: 2022-01-19 PN4-04.

5.6. Reduced NADH-fraction

Reduced NADH-fraction values indicate that in the LEAK state (NADH-pathway using pyruvate, glutamate and malate as fuel substrates) 99 % of the NAD-pool was reduced, while in OXPHOS state the reduced NAD was 63 %.



Reduced NADH fraction in mitochondria isolated from mouse liver. Approximate calibration of oxidized NAD: isolated mitochondria (1mt). Calibration of fully reduced NAD: CIII inhibitor myxothiazol added under anoxia (3Myx).

6. References

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7. 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.

8. Acknowledgement

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

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