



Instruction Manual for the ibidi OPAL – Optical O₂ Measurement System



In collaboration with



Table of contents

Safety Considerations.....	2
System Requirements.....	5
Specifications	6
Preface.....	7
Notices.....	7
Limited Warranty.....	8
Installation Requirements.....	9
Installation and Connecting Cables	10
Maintenance and Operation	11
Waste Treatment	12
1 Safety Instructions	13
2 Testing and Measurement Basics	14
2.1 Operating Principle.....	14
2.2 Measurement Determination of the O ₂ content.....	14
3 Description of the OPAL components.....	17
3.1 ibidi OPAL Controller.....	17
3.1.1 Front Side	17
3.1.2 Back Side	18
3.2 LED Light Source.....	19
3.3 OPAL Detector Unit.....	20
3.4 OPAL-Software.....	21
3.4.1 Main Window "Optical Measurement of Oxygen"	21
3.4.2 Window "Save Measurement Data"	28
3.4.3 "Log File" Window	30
3.4.4 "Viewer" Window.....	31
3.4.5 "Calibration" Window	32
4 CPOx-Beads.....	39
4.1 Specifications of the CPOx-Beads.....	39
4.2 Applications with CPOx-Beads, Placement within Samples	39
5 Installation of the OPAL measurement system	40
6 Preparation for Measurement.....	46
6.1 Switching on of the LED Light Source	46
6.2 Focusing CPOx-Beads.....	47
6.3 Tuning the Detector Sensitivity and Illumination Intensity.....	48
6.4 Adjustment of the Iris Diaphragm and Identification of the Center Position of the Image	50
6.5 Setting the Zero Position of the Phasing (Zero Point Referencing)	51
7 Calibration	53

Safety Considerations

To ensure operation safety, the ibidi heating system must be operated correctly and maintained according to a regular schedule. Carefully read to fully understand all safety precautions in this manual before operating the instrument. Please take a moment to understand what the signal words **WARNING!**, **CAUTION** and **NOTE** mean in this manual.

Safety symbols

WARNING!

A **WARNING!** indicates a potentially hazardous situation which, if not avoided, could result in serious injury or even death.

CAUTION

A **CAUTION** indicates a potentially hazardous situation which, if not avoided, may result in minor or moderate injury. It may also be used alert against damaging the equipment or the instrument.

Do not proceed beyond a **WARNING!** or **CAUTION** notice until you understand the hazardous conditions and have taken the appropriate steps.

NOTE

A **NOTE** provides additional information to help the operator achieve optimal instrument and assay performance.



Read manual label. This label indicates that you have to read the manual before using the instrument. This label is positioned at the backside of the temperature controller.



This symbol indicates important notes.

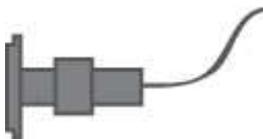
Nomenclature



ibidi OPAL Controller



OPAL Detector Unit



LED Light Source



Filter Cube



CPOx-Beads

WARNING! Do only operate the ibidi OPAL System with the delivered cables and plugs. If not doing so you risk electric shock and fire.

CAUTION Ensure that the power plug is well accessible. The ibidi OPAL System has to be installed in a way that it does not hinder the access to the power plug.

CAUTION Operate the ibidi OPAL System only on a power socket with protective conductor to ground the housing of the OPAL Controller.

CAUTION The weight of the ibidi OPAL Controller is approx. 1.5 kg. Provide a secure stand for the controller, where it cannot be accidentally dropped. Moving the OPAL System poses a risk of personal injury or damage to the instrument.

CAUTION The manual opening of the OPAL Controller is not allowed. Manual opening pose a risk of personal injury or damage to the controller. Contact ibidi service personnel if you need to open the controller.

CAUTION Only use correct replacement fuses. The correct values of the replacement fuses are indicated on the backside of the OPAL Controller. Using wrong replacement fuses can lead to overheating of the OPAL Controller which can cause fires.

CAUTION Only ibidi staff is allowed for servicing and opening of the OPAL Controller. Turn off the power switch and unplug the power cord before servicing the system, unless otherwise noted. Connect the OPAL Controller only to the delivered power plug. Do not use extension cords. Have an electrician immediately replace any damaged cords, plugs, or cables. Not doing so poses a risk of personal injury or damage to the system.

CAUTION Do only use the OPAL System in dry rooms.

CAUTION Turn off the power switch in the back of the chassis, when the OPAL System is not in use.

System Requirements

Compatible operation systems:

- Windows XP
- Windows 7
- Windows 8 (not with Windows 8 RT)

Specifications

OPAL – Optical O₂ Measurement System	
Power Consumption	
Supply voltage	100 - 240V 1.5A 50 - 60Hz
Output voltage	12V 5.0A
Max. power input	< 90VA
Max. power output	60VA
Surrounding Conditions	
Storage Temperature	-5 – 50°C
Operating Temperature	15 - 40°C (Indoor use only)
Humidity	80 % relative humidity up to 31 °C, 30 % relative humidity up to 40 °C
Operating altitude	max. 2000 m
Temperature Control Range	
	Ambient temperature to +55°C
Lifetime range	
	1 – 1000 µsec
Net Dimensions (h×w×d)	
Controller	90 x 170 x 230 mm ³
Weight	
Controller	1.5 kg
Filter set	
Emission	607 / 70 nm
Extinction	531 / 40 nm
Dichroic Mirror	555 nm

Preface

This manual is your guide for using the ibidi OPAL System for measuring oxygen concentration in cell culture experiments on an optical microscope. It instructs first-time users on how to use the instrument, and serves as a reference for experienced users.

Before using the ibidi OPAL – Optical O₂ Measurement System, please read this instruction manual carefully, and make sure that the contents are fully understood. This manual should be easily accessible to the operator at all times during instrument operation. When not using the instrument, keep this manual in a safe place. If this manual becomes lost, order a replacement from ibidi GmbH.

Notices

1. ibidi shall not be held liable, either directly or indirectly, for any consequential damage incurred as a result of product use.
2. Prohibitions on the use of ibidi software
 - Copying software for other than backup
 - Transfer or licensing of the right to use software to a third party
 - Disclosure of confidential information regarding software
 - Modification of software
 - Use of software on multiple workstations, network terminals, or by other methods
3. The contents of this manual are subject to change without notice for product improvement.
4. This manual is considered complete and accurate at publication.
5. This manual does not guarantee the validity of any patent rights or other rights.
6. If an ibidi software program has failed causing an error or improper operation, this may be caused by a conflict from another program operating on the notebook (PC). In this case, take corrective action by uninstalling the conflicting product(s).
7. ibidi is a registered trademark of ibidi GmbH in Germany and other countries.

Limited Warranty

Products sold by ibidi, unless otherwise specified, are warranted for a period of one year from the date of shipment to be free of defects in materials and workmanship. If any defects in the product are found during this warranty period, ibidi will repair or replace the defective part(s) or product free of charge.

THIS WARRANTY DOES NOT APPLY TO DEFECTS RESULTING FROM THE FOLLOWING:

1. IMPROPER OR INADEQUATE INSTALLATION.
2. IMPROPER OR INADEQUATE OPERATION, MAINTENANCE, ADJUSTMENT OR CALIBRATION.
3. UNAUTHORIZED MODIFICATION OR MISUSE.
4. USE OF UNAUTHORIZED CELL CULTURE WARE.
5. USE OF CONSUMABLES, DISPOSABLES AND PARTS NOT SUPPLIED BY AN AUTHORIZED IBIDI DISTRIBUTOR.
6. CORROSION DUE TO THE USE OF IMPROPER SOLVENTS, SAMPLES, OR DUE TO SURROUNDING GASES.
7. ACCIDENTS BEYOND IBIDI'S CONTROL, INCLUDING NATURAL DISASTERS.

This warranty does not cover consumables like cell culture dishes, reagents and the like.

The warranty for all parts supplied and repairs provided under this warranty expires on the warranty expiration date of the original product. For inquiries concerning repair service, contact ibidi after confirming the model name and serial number of your ibidi system.

Installation Requirements

To ensure operation safety, observe the following conditions:

1. Do only operate the ibidi OPAL System with the delivered power cable.
2. Only connect the power cable of the ibidi OPAL System to an electrical socket containing a protective conductor terminal.
3. Ensure that the power plug of the power supply is well accessible. The OPAL System has to be installed in a way that it does not hinder the access to the external power supply and its power plug.
4. Operate the OPAL System in an atmospheric pressure range of 800 – 1060 hPa.
5. Do not operate the OPAL System under conditions which pose a risk of explosion, implosion or the risk of the release of gases.
6. Avoid strong magnetic fields and sources of high frequency. The OPAL system may not function properly when near a strong magnetic field or high frequency source.
7. Avoid vibrations from vacuum pumps, centrifuges, electric motors, processing equipment and machine tools.
8. Avoid dust and corrosive gas. Do not install the OPAL System where it may be exposed to dust, especially in locations exposed to outside air or ventilation outlets.
9. Do not install the OPAL System in a location where it may be exposed to direct sunlight.
10. Install the OPAL System in a horizontal and stable position. This includes a table, bench or desk upon which the instrument is installed.
11. Ensure that no air conditioner blows air directly onto the OPAL System. This may prevent stable conditions.
12. Install the OPAL System in a location that allows easy access for maintenance.

NOTE: The above conditions do not guarantee optimal performance of the OPAL System.

Installation and Connecting Cables

Connecting the ibidi OPAL System to the power supply

Confirm that the power switch of the OPAL System is off (power switch is at the backside, right of the OPAL controller).

WARNING! Do only operate the ibidi OPAL System with the delivered cables and plugs. If not doing so you risk electric shock and fire.

CAUTION Ensure that the power plug of the external power supply is well accessible. The OPAL System has to be installed in a way that it does not hinder the access to its power plug.

Connect the OPAL System to the Notebook by using the delivered network cable.

Switch on the OPAL System and the Notebook.

Maintenance and Operation

Pay attention to the OPAL System operating environment and always keep it clean so that the instrument can be used in a stabilized condition over a long period. Do not place anything heavy on the OPAL Controller.

Operating the ibidi OPAL System

Use the instrument only in the intended application described in this manual.

CAUTION Unintended use which is not in compliance with this manual can lead to a failure of the instrument protective systems. This failure may cause personal injury or damage.

Cleaning the ibidi OPAL System

Switch off the OPAL System and remove the power plug from the electrical socket. Only use dry cloth or cloth wetted with water (ultrapure) for cleaning of the instrument.

CAUTION Do not use ethanol or other types of organic solvents to clean the instrument as they may remove the instrument paint.

Transporting the ibidi OPAL System

Switch off the OPAL Controller and remove the power plug from the electrical socket. Carry the instrument carefully and avoid mechanical shocks.

CAUTION The weight of the OPAL Controller is approx. 2.0 kg. Dropping the instrument while moving it, may cause personal injury or damage.

Functional Disorder

In case of a functional disorder switch off the OPAL Controller and wait for five minutes, then switch on the instrument again. If there is still a functional disorder switch off the device, unplug the power cable and contact the ibidi service.

Repairing the ibidi OPAL System

Do not try to repair the instrument by yourself. Do contact the ibidi service for repairing the instrument.

CAUTION The manual opening of the instrument is not allowed. Manual opening pose a risk of personal injury or damage to the instrument. Contact ibidi service personnel if you need to open the instrument.

Waste Treatment

Waste treatment is your own responsibility. You must hand it to a company specialized in waste recovery. Do not dispose the ibidi OPAL System in a litter bin or at a public waste disposable site. For detailed information please contact the ibidi service.

1 Safety Instructions

→ Operate the OPAL system only under the conditions and for the purposes in accordance with its designated use. The intended use of the instrument is to measure the oxygen content optically in combination with a fluorescence microscope in industrial establishments, universities and research laboratories. During all kinds of operation, the legal accident prevention regulations for electrical units and optical emitters must be observed. In Germany these regulations are specified by the "Berufsgenossenschaft" (employer's liability insurance association).

→ Appropriately qualified and trained personnel must do all handling of the system. The operation instructions of this manual must be complied to. In schools and training facilities, trained personnel must supervise the operation of the instrument.

→ **WARNING:** Do not look into the ray of the LED Light Source.

A green (530 nm) high power light emitting diode with a peak output of 300 mW is used as excitation light source. The radiation of this LED Light Source may injure eyes. Before connecting the LED Light Source to the OPAL Controller and before switching on, make sure that no light can reach the eye directly.

→ **WARNING:** Make sure that no excitation light of the LED Light Source is guided directly to the eyepieces of the microscope.

Usually, fluorescence microscopes are constructed in such a way that no light arising from the fluorescence lamp can reach the eyepieces directly. However, since there are countless types of microscope systems, it cannot be completely excluded that there are single types without this protective function. It might be that special accessories or custom modifications removed this protective function. Therefore, verify if there are settings, which could guide the light directly to the eyepieces. If there should be such potentially harmful settings, be careful and do not select them when the LED Light Source is switched on.

→ **WARNING:** If the housing, connection cables or power supply is damaged, the OPAL System must not be used any more. In this case, unplug the power plug and contact the ibidi service.

→ **WARNING:** If any liquid gets into the housing of the OPAL Controller, the system must not be used any more. In this case, unplug the power plug and contact the ibidi service.

→ **WARNING:** The instrument must not be operated outdoors.

→ **WARNING:** The optical oxygen sensor (CPOx-Beads) must be treated like chemicals. In particular they are not intended for consumption. Keep away from children.

2 Testing and Measurement Basics

2.1 Operating Principle

In the presence of molecular oxygen (O_2) the phosphorescence is quenched dynamically. Dynamic quenching means that energy is transferred from the dye molecule to the oxygen molecule once they come into contact with each other. The energy transfer is specific to O_2 molecules due to their exceptional electronic structure. The O_2 probes of ibidi (CPOx-Beads) may be used for both, O_2 measurements in the gas phase and in aqueous solutions. Other substance, e.g. CO_2 , N_2 , CH_4 , water, H^+ and other ions, ingredients of culture media like carbohydrate and proteins, will not interfere to the quenching process. (There is cross sensitivity to NO, which has an electronic structure similar to O_2 .) The more oxygen molecules are present the more collisions it has with the sensor dye thus transferring energy. With increasing ambient O_2 concentration, both, phosphorescence intensity and phosphorescence decay time decrease. In practice, the measurement of decay times is less prone to errors. In contrast to intensities, decay times are independent from the intensity of excitation light, the sensor concentration and are not interfered by absorption or scattering within the sample.

2.2 Measurement Determination of the O_2 content

The OPAL – Optical O_2 Measurement System determines phosphorescence decay times with a two-frequency phase modulation technique, which suppresses the interference of back ground fluorescence. The light of the excitation source is modulated with two different sinusoidal frequencies. The CPOx-Beads emit a phosphorescence signal, which also modulates with two sinus frequencies. However, the signal is phase shifted, since the phosphorescence emission occurs time delayed in dependence of the ambient oxygen concentration. From the measured phase shifts at the two frequencies, the OPAL system evaluates the amount of fluorescence background signal, which unavoidably arises from biological samples, and subsequently calculates the phosphorescence decay time of the CPOx-Beads.

The absorption spectra of CPOx-Beads, which are stained with Ru- or Pt-sensor dye, allow excitation wavelengths from the violet to green spectral range. The emission occurs in the orange (Ru, E_{max} ca. 590 nm) and in the red (Pt, E_{max} ca. 650 nm) spectral range, respectively. In the case of biological samples, it is favorable to use green light for excitation to avoid photochemical side reactions in culture media. (For instance, riboflavin, which is an

ingredient of culture media, generates singlet oxygen, if it is exposed to blue light. An unintended generation of singlet oxygen may falsify the measurement.)

The O₂ concentration is calculated from the decay time by use of a calibration curve. In the ideal case, dynamic phosphorescence quenching by O₂ is described by the Stern Volmer equation

$$\frac{\tau}{\tau_0} = \frac{1}{1 + K_{SV} \times [O_2]}$$

where [O₂] is the current O₂ concentration, τ the corresponding phosphorescence decay time, K_{SV} the Stern Volmer constant and τ_0 the phosphorescence decay time in an ambience without any O₂. The temperature dependence is measured by use of temperature factors for τ und τ_0 (not shown in the equation).

In a solid polymer matrix, single sensor dye molecules are unequally accessible to penetrating oxygen molecules. Therefore, the phosphorescence decay times of individual sensor dye molecules slightly differ. This leads to deviations of the Stern Volmer equation. In principle, an extension of many terms containing different Stern Volmer constants would be necessary. However, experimental data show that, in practice, an extension by only one single term with K_{SV2} = 0 is sufficient.

$$\frac{\tau}{\tau_0} = \frac{f}{1 + K_{SV} \times [O_2]} + (1 - f)$$

The above equation describes a separation of the sensor dye molecules into two groups: One fraction f is assumed to be equally accessible for O₂ molecules. The other fraction f – 1 is completely inaccessible for O₂ molecules. For this reason, f takes a value between 0 and 1.

The required values of τ_0 , K_{SV} and f can be evaluated by a three point calibration. To calibrate the OPAL System, decay times τ_{100} , τ_{50} , τ_0 at 100, 50 and 0% air saturation, and the respective temperature at the moment of measuring are determined and stored in the software. Usually, even a two-point calibration of τ_{100} and τ_0 is sufficient. In this case, for f (which is related to τ_{50}) an experiential value for the used sensor type is utilized.

K_{SV} and τ_0 depend on the temperature. With increasing temperature, the phosphorescence decay time decreases. To allow for a relatively easy two or three-point calibration procedure, a simplifying linear temperature dependence of τ_0 und τ_{100} is assumed. The corresponding temperature factors can be changed in the software.

Units

The OPAL System displays the measured O₂ content as % air saturation (short: % air). This unit has the advantage that it makes sense for both the gas phase and dissolved O₂. 100% air means that the gas phase (at standard pressure) contains the oxygen content of air (21% by volume). In aqueous solution 100% air means that the O₂ content of water is in thermodynamic equilibrium with water vapor saturated air. To convert % air into mg/L or into mmol/L can be done by use of solubility tables, e.g. Bruce B. Benson, Daniel Krause: The concentration and isotopic fractionation of gases dissolved in freshwater in equilibrium with the atmosphere. 1. Oxygen, Limnol. Oceanogr. 25 (1980) 662-671.

Conversion of % air saturation into other units:

In order to calculate the O₂ concentration at a certain temperature, multiply the displayed value with the corresponding factor shown in the table below.

	µmol/L	mg/L	vol. %
22 °C	2.73	0.874	0.21
37 °C	2.10	0.673	0.21

3 Description of the OPAL components

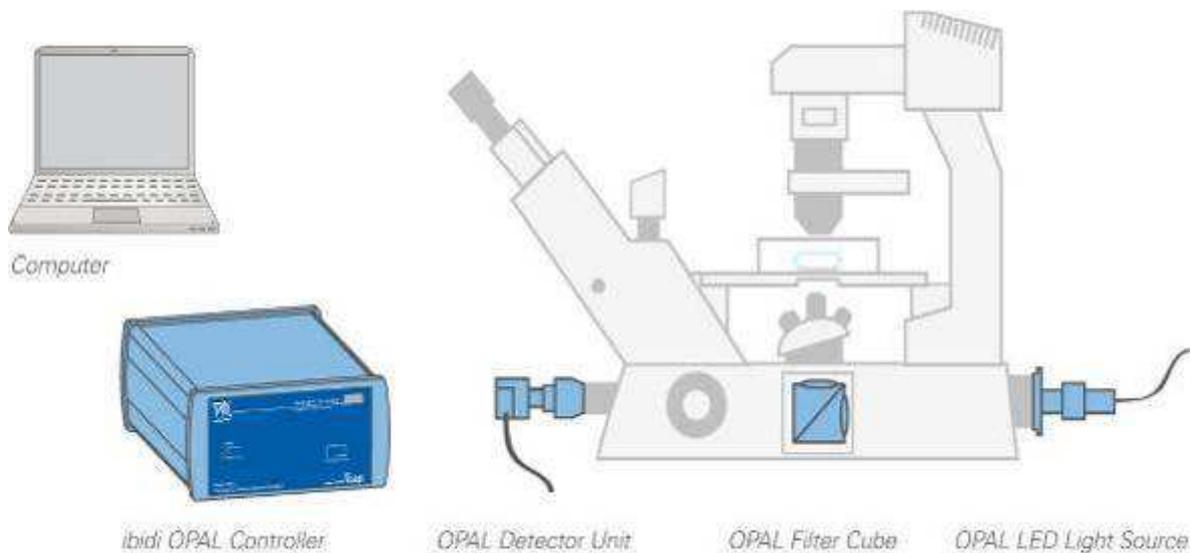


Fig. 3.1: Components of the ibidi OPAL – Optical O₂ Measurement System

3.1 ibidi OPAL Controller

The ibidi OPAL Controller is the electronic device, which drives all components and records and evaluates the measurement values. It includes the driver for the LED Light Source, a power outlet for the Detector Unit, a module for temperature measurements, and the electronics for signal acquisition and processing.

3.1.1 Front Side

The following elements are located at the front side (Fig. 3.2).

➔ LED array:

LED #1 (at the top): operating indicator.

LED #2: on, if the light source driver is activated by the software.

LED #3: on, if the connection to the software is cut off (by unplugging the USB cable or by closing the software).

LED #4: on, if the Detector Unit is activated by the software.

- Connector for temperature sensor. The OPAL Controller is provided with a 2-pin connector for a temperature sensor (type K; NiCr-Ni).



Fig. 3.2: Front side of the ibidi OPAL Controller for connection of the temperature sensor.

3.1.2 Back Side

The following elements are located at the back side (Fig. 3.3).

- 3 pin connector "Light Source" for the LED Light Source (comprising sample and reference LED).
- BNC socket "Oscillator Out" (sinus A+B). Optional; can be used to control an external laser or LED driver or for servicing. In the normal mode this connector keeps free.
- 5 pin connector "Detector 1 Control" for the Detector Unit (power outlet and control voltage to align sensitivity).
- SMB socket "Signal 1 In" to be connected with the signal line of Detector Unit.
- 5 pin connector "Detector 2 Control". Optional. Used only in the special case of connecting a second Detector Unit for optical referencing. In the normal mode this connector is free.
- SMB socket "Signal 2 In". Optional. Used only in the special case of connecting a second Detector Unit optical referencing. In the normal mode this connector is free.
- Mini USB to connect a PC or Laptop.
- Power connector for the 12 V power supply.

→ On/off switch.

The opening on the top left can be used for a common Laptop cable lock.



Fig. 3.3: Back side of the OPAL Controller.

3.2 LED Light Source

The LED Light Source (figure 3.4) contains two identical green high-power LEDs, fixed on a common heat sink. The sample LED is intended to illuminate the sample. On the side of the sample LED, the adapter to connect the light source with the lamp port of a fluorescence microscope is mounted. Depending on the microscope type, different adapter types are required. On the other side of the heat sink, the reference LED is located. In front of the reference LED there is a neutral density filter and an adapter to connect an optical fiber. The optical fiber is intended to guide light from the reference LED directly to the Detector Unit. This allows for the determination of the zero position of the phase shifts.



Fig. 3.4: LED Light Source.

3.3 OPAL Detector Unit

The OPAL Detector Unit (Fig. 3.5) comprises a detector module, a beam splitter cube and a tube with iris aperture. As detector module a Hamamatsu H10723-20 is used. Inside the beam splitter cube there is a dichroic mirror, which guides the sensor signal to active surface of the detector. Face to face with the detector there is an adapter to connect the reference light guide. The tube contains a lens and an iris diaphragm to exclude the signals of close-by CPOx-beads.

Note:

The sensitivity of the Detector Unit is controlled by an integrated high-voltage module, which is controlled by the OPAL-Software. Once high-voltage is applied, the Detector Unit can easily be damaged by too high light intensity. Be sure that no intense extraneous light (e.g. arising from the transmission light source of the microscope) can reach the Detector Unit, when high-voltage is applied. If the OPAL Detector Unit is not used for measurements, it is recommended to close the iris diaphragm at the detector tube for safety (position 0, see fig. 6.2, on page 51).



Fig. 3.5 OPAL Detector Unit

The Detector Unit is equipped with a C-mount for an easy installation on camera ports of common microscopes. If there is not a free camera port available, the OPAL Detector Unit can be alternatively connected to the microscope by use of an ocular adapter (fig. 3.6). This option is ideal for taking measurements and camera use in parallel. However, an intensity loss of more than 50% has to be accepted.



Fig. 3.6: OPAL Detector Unit with ocular adapter.

3.4 OPAL-Software

3.4.1 Main Window “Optical Measurement of Oxygen”

After starting the OPAL-Software, the main window opens. The control elements are described in the following:

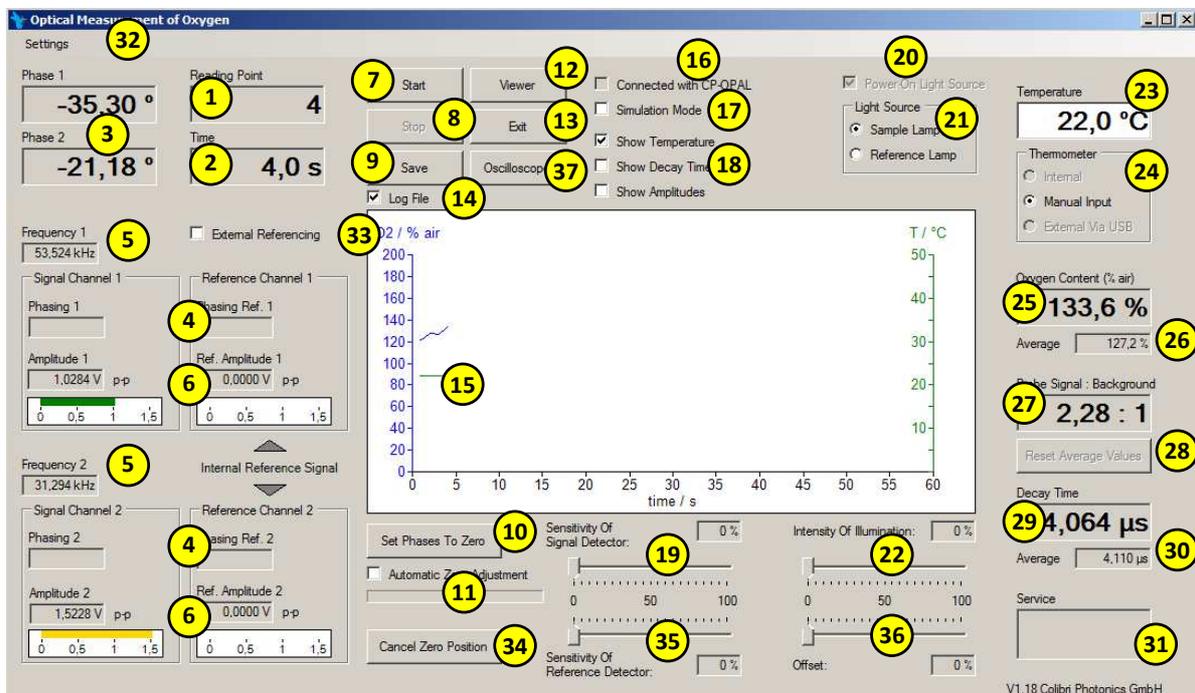


Fig. 3.7.: User interface in “expert mode”. All displays and controls are shown.

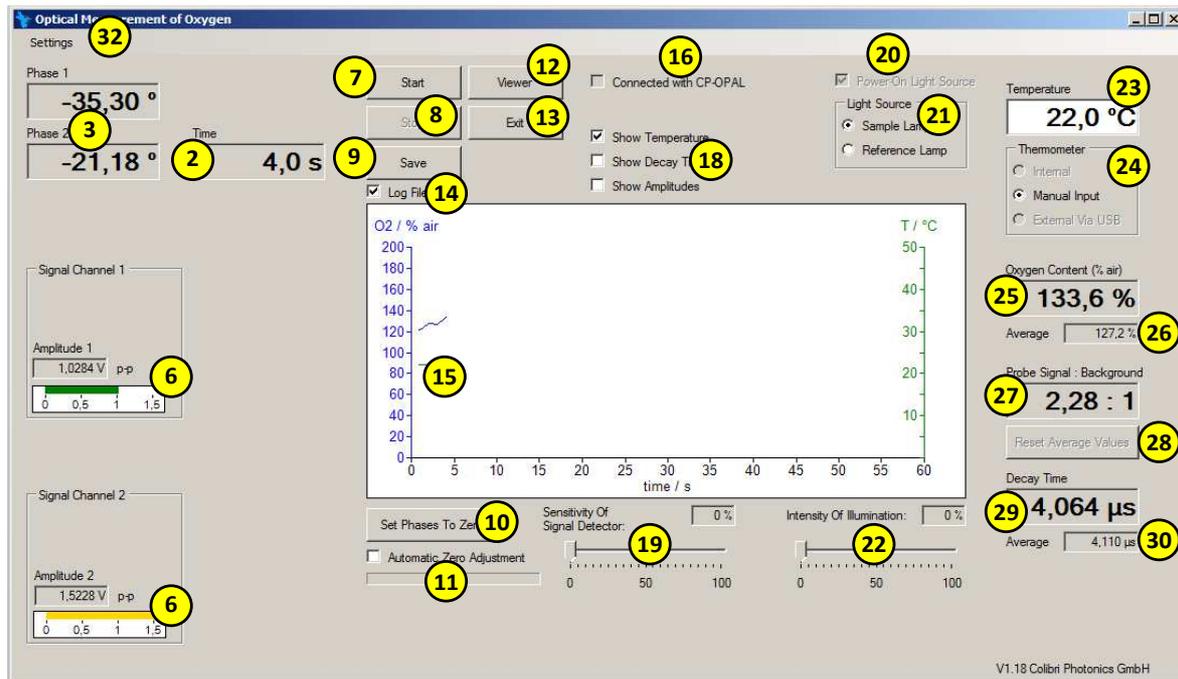


Fig. 3.8.: User interface in the “standard mode”. Only the most important displays and controls are shown.

(1) Measurement point: Shows the number of measurement points recorded since the start of a measurement.

(2) Time: Shows the elapsed time since the start of a measurement. An update is made at every new measurement point.

(3) Phase 1 and Phase 2: Show the measured phase shifts (relative to the zero value reference) at the respective sinus frequencies 1 and 2 (5). Phase 1 and Phase 2, respectively, are the differences between the phase signal and reference channel (4) at the respective frequency (5). Additionally, phase offsets, which are determined by the zero value reference (10), are subtracted to eliminate non-optical phase shifts caused by the electronics of the light source and the detector. When a measurement is running, the phase at the current measurement point is shown. When the measurement is stopped, the phase is updated every second.

(4) Shows the measured electronic phase angles of the signal and reference channels. (Only required for service purposes and for control purposes with external referencing (33)).

(5) Frequency 1 and Frequency 2: Display of the set modulation frequencies. The modulation frequencies are set depending on the sensor type used.

(6) Signal strength: In the text box and at the bar graph the respective amplitudes of the sinus signals at the modulation frequencies 1 and 2 are shown. Please note that only AC signal fractions at both modulation frequencies are displayed. Possible DC signals or signals with other frequencies are not displayed. (During normal operation, the amplitude displays of the reference channels show the electric sinus signal amplitudes of the oscillator. In the "External Referencing" mode (33), the sinus signal strengths of the "Signal 2 In" connector, e.g. from a second Detector Unit will be displayed.)

(7) Start: Click to start a measurement.

(8) Stop: Click to stop a measurement.

(9) Save: Click to open the window "Save Measurement Data" to save the data of the last measurement.

(10) Set Phases To Zero: Sets the phases to the zero value reference. Clicking defines the currently measured phase shifts as zero values. This is done to eliminate electrical phase shifts arising from light source or detector. If there is a signal with constant phasing, 0.0° should be displayed in (3) after clicking (10).

(11) Automatic Zero Adjustment: If the checkbox is checked unlike an automatic zero point reference is performed after clicking the start button (7). Different than clicking button (10), the automatic zero adjusting function assumes control over the alignment of the detector sensitivity (19) and toggles between the sample and the reference lamp automatically.

(12) Viewer: Click to open a window, which allows viewing of curves with saved measurements.

(13) Exit: Click to close the OPAL-software.

(14) Log File: If checkbox is checked and a measurement is started, the data of every measurement point is saved to a file just after its acquisition. In the case of an unexpected

interruption, all data before the shutdown will be saved. The properties of the log file can be preset by selecting "Log File" within the menu strip "settings" (32).

(15) Curve Box: Click the start button (7) to show the measurement data in curve form.

(16) Connection indicator: This checkbox is checked, if the software has found an OPAL Controller unit ready for operation.

(17) For service and demonstration: If checked, after clicking the start button, measurement values will be generated at random automatically, regardless if a OPAL Controller is connected or not.

(18) Selection of the data to be shown as curves in the picture box (15). Depending, which of the boxes are checked, temperature, decay time and signal strength (= amplitude) are displayed.

Notes:

- The O₂ content is always displayed as a curve, as long as a relevant reference value can be determined.
- In a save file or a log file all data are written, regardless of which boxes (18) are checked.

(19) Sensitivity Of Signal Detector: The slider controls the control voltage for the Detector Unit. An increasing control voltage (by pulling the slider to the right) causes an increase of the high voltage at the detector tube.

Note:

- If control voltage is applied, the Detector Unit can be damaged by excessive light. Verify before increasing the control voltage that no bright extraneous light reaches the detector (e.g. transmission light of the microscope, ceiling light, sun light).
- If the OPAL Controller reports a signal strength that is too high, the track bar is highlighted red and the warning "Overload" is displayed. In this case, decrease the detector sensitivity quickly and prevent possible incidence of excessive light.

Overload may also occur without a warning! If the light intensity is excessive, the voltage output of the photomultiplier decreases again. Thus, a heavy overload, which occurs suddenly (e.g. switching on the transmission light of the microscope by accident), might not be recognized by the software and the photo multiplier could be damaged even when no warning is displayed.

- ➔ The displayed percent scale indicates the relative value of control voltage and not the actual detector sensitivity. At values around "40 %", the sensitivity abruptly increases. Therefore it is important to move the slider slowly.
- ➔ To prevent damage by accidentally light, it is recommended aligning the control voltage only during a measurement. Otherwise the slider should be set to zero.
- ➔ If the checkbox "Automatic Zero Adjustment" (11) is checked, after clicking the start button (7), the control voltage will increase step by step automatically. To cancel this procedure click the stop button (12). The control voltage is then set to zero.

(20) Power on light source: Check this box to switch on the LED Light Source. Uncheck this box switch to off the LED Light Source.

WARNING:

The radiation of the LED Light Source may injure eyes. Before connecting the Light Source to the OPAL Controller and before switching on, make sure that no light can reach the eye directly.

(21) Select light source: Click on the respective radio button to select the light source. The Light Source contains two high power LEDs. One is used as sample lamp to excite the phosphorescence of the CPOx-Beads during the measurement. The second is used for the reference lamp and is used to determine the zero position of the phasing (zero point referencing).

(22) Intensity of Illumination: The track bar aligns the AC voltage of the oscillator as well as the AC current and the light intensity of the LED's of the Light Source.

WARNING:

The radiation of the LED Light Source may injure eyes. Before increasing the intensity of the LED Light Source, make sure that no light can reach the eye directly.

(23) Temperature: Shows the temperature, which is used to evaluate the O₂ content from the measurement data. It is measured by a temperature sensor or is entered manually (24).

(24) Selection of Thermometer: Use the radio buttons to select the temperature sensor connected to the OPAL Controller (“internal”), a manually entered value (“Manual Input”) or an external thermometer (“External via USB”) as source for the temperature value. “External via USB” only is active, if a suitable USB thermometer is connected to the computer. Please contact ibidi if you wish to use an external temperature sensor.

Note:

Once switched to “Manual Input” the last measured temperature is taken as a fixed value. To change the value, click the temperature field (23) and enter a new value.

(25) Oxygen Content: Displays the last measured O₂ concentration in % air. 100% air means that the gas phase (at standard pressure) contains the oxygen content of air, which is 21% by volume. In aqueous solution 100% air means that the O₂ content of water is in thermodynamic equilibrium with water vapor saturated air. Conversion of % air into mg/L or into mmol/L can be done by use of solubility tables (page 17).

(26) Averaged Oxygen Content: Displays the average value of all measured O₂ concentrations since the start of a measurement or since the last click on “Reset Average Values” button (28).

(27) Probe Signal: Background Ratio: The OPAL Controller unit discriminates signal fractions arising from the phosphorescent probe and from fluorescence background (which nearly unavoidably arises from cells or culture media). For meaningful results the ratio should be at least 1.5:1.

(28) Reset Average Values: Click to reset the average values of oxygen content and decay time, displayed in the textboxes (26) and (30), respectively restarts the averaging using the subsequent measurement values.

(29) Decay Time: Displays the measured phosphorescence decay time of the probe.

(30) Averaged Decay Time: Displays the average value of all measured decay times since the start of a measurement or since the last click on “Reset Average Values” button (28).

(31) *Only Required For Service*: Displays internal raw data of the software.

(32) Settings: Click on “Settings” on the menu bar to open the sub menus for *Language*, **Thermometer-Com Port**, **Log File**, **Calibration** and **User Interface**.

- Language: The language of the software can be switched to English or German.
- Thermometer-Com Port: For external Thermometer. Please contact ibidi if you wish to use an external temperature sensor.
- Log File: Opens a window to change presets of the log file.
- Calibration: Opens a window to display and change calibration values, modulation frequencies, measurement interval, thermo sensor calibration etc.
- User Interface: In the “Advanced Mode” all displays and control elements are shown; in the user mode, only the most important values are shown.

(33) External Referencing. In the “Normal Mode”, the instrument is referenced internally and the “External Referencing” is not checked. For special applications it is also possible to reference the instrument externally, e.g. optically by the use of a second photo detector, which is connected to the “Signal 2 In” input. This box is checked only during this special application.

Note:

- In the “Normal Mode”, the amplitude display (6) of the reference channels shows the electric sinus signals amplitudes of the oscillator. In the “External Referencing” mode, the sinus signal strengths applied to the “Signal 2 In” input, e.g. from a second Detector Unit, are shown.
- If you try to perform “normal” measurements with an accidentally checked “external Referencing” box, no or very small amplitudes will be displayed at the reference channels. As a result the measured phases will become instable, which in turn will lead to errors.

(34) Cancel Zero Position. Clicking cancels (i.e. sets to zero) the phase offset, which was previously set by clicking “Set Phases To Zero” (10) or using the “Automatic Zero Adjustment” (11).

Note:

In the “Normal Mode”, an improper phasing is corrected by repeating the zero point referencing. In special cases of External Referencing (33) phase offsets may be troublesome and resetting them may be helpful.

(35) Sensitivity Of Reference Detector: The track bar aligns the control voltage of an optional second Detector Unit, which is only connected for the special external referencing operation mode (33) to the sockets “Detector 2 Control” and “Signal 2 In”. The function principle is the same as (19). Please note the instructions specified under (19).

(36) Offset: The track bar aligns the DC offset of the LED Light Source. Depending on the characteristic of the LED, a DC offset may be necessary to avoid a deformation of the sinusoidal modulation.

(37) Oscilloscope: Clicking on this button opens a new window, which shows the curves of the signals applied to the signal inputs 1 and 2. The oscilloscope window can be used to check the quality of the input signals. Possible disruptions, e.g. deformations or interferences with external light, are visualized.

Note:

If the oscilloscope window is open, the automatic overload control is only active for the visualized input channel.

3.4.2 Window “Save Measurement Data”

Clicking the save button (9) opens the window “Save Measurement Data”.

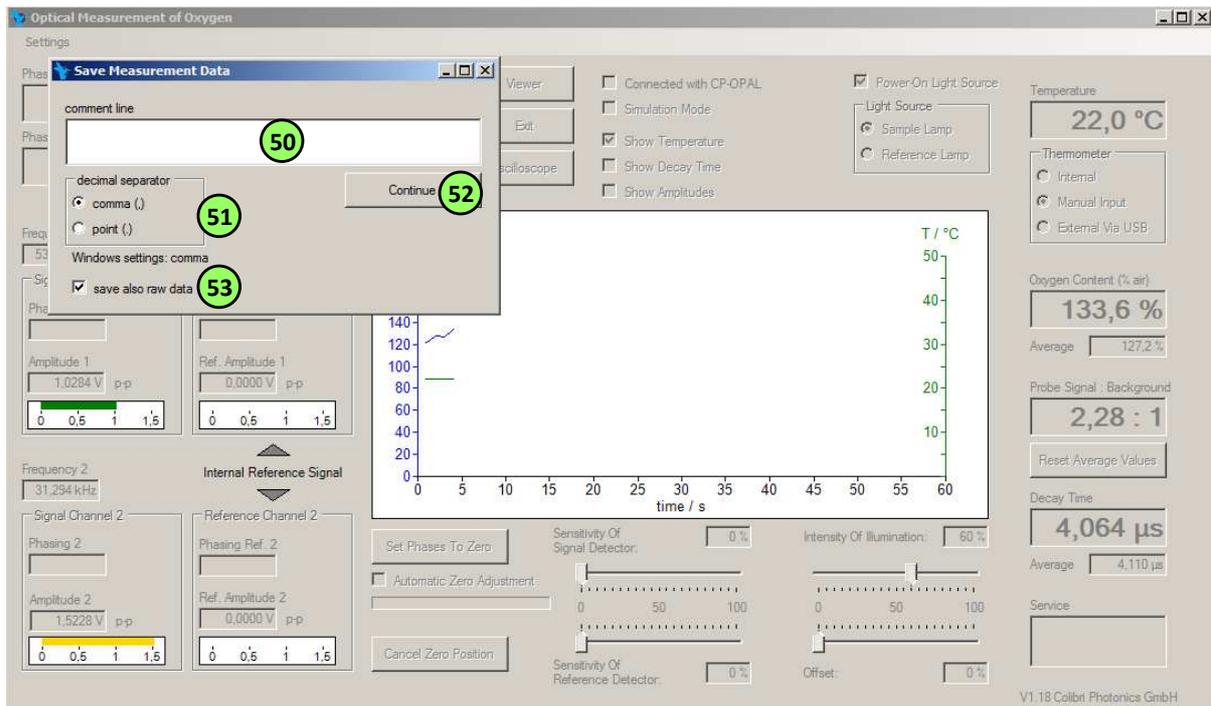


Fig. 3.9: .. Open window "Save Measurement Data"

Measurement data is saved in the form of an ASCII text file. A column separator tab (ASCII code CHR 9) is used. For further evaluation the data files can be imported to common spreadsheet processing programs (e.g. Excel, Origin).

50 In this field, any comment can be entered. The comment will appear in the header of the saved file.

51 Decimal Separator: The radio buttons allow selection of a comma or decimal point as a separator for the numerical data. By default, the separator of the current Windows setting is used. Selecting between the comma or point, depends on the settings of the spreadsheet processing program to be used and the settings of the computer that the spreadsheet processing program is installed on.

52 Continue: After clicking the Windows dialog field "save as" opens. Choose a filename and save the measurements at a desired destination.

Note:

The language of the Windows "save as" dialog depends on the computer's Windows settings. The OPAL-software cannot change languages of standard windows dialogs.

(53) If checked, all raw data is added to the file as additional columns.

3.4.3 “Log File” Window

Click Settings (32) on the menu bar to select the sub menu for the “Log File”. A corresponding window will open to set the properties of the log file. When the system is taking measurements all of the values are automatically written and saved to the log file. If a measurement is unexpectedly interrupted, the values evaluated will be saved up until the moment of the interruption.

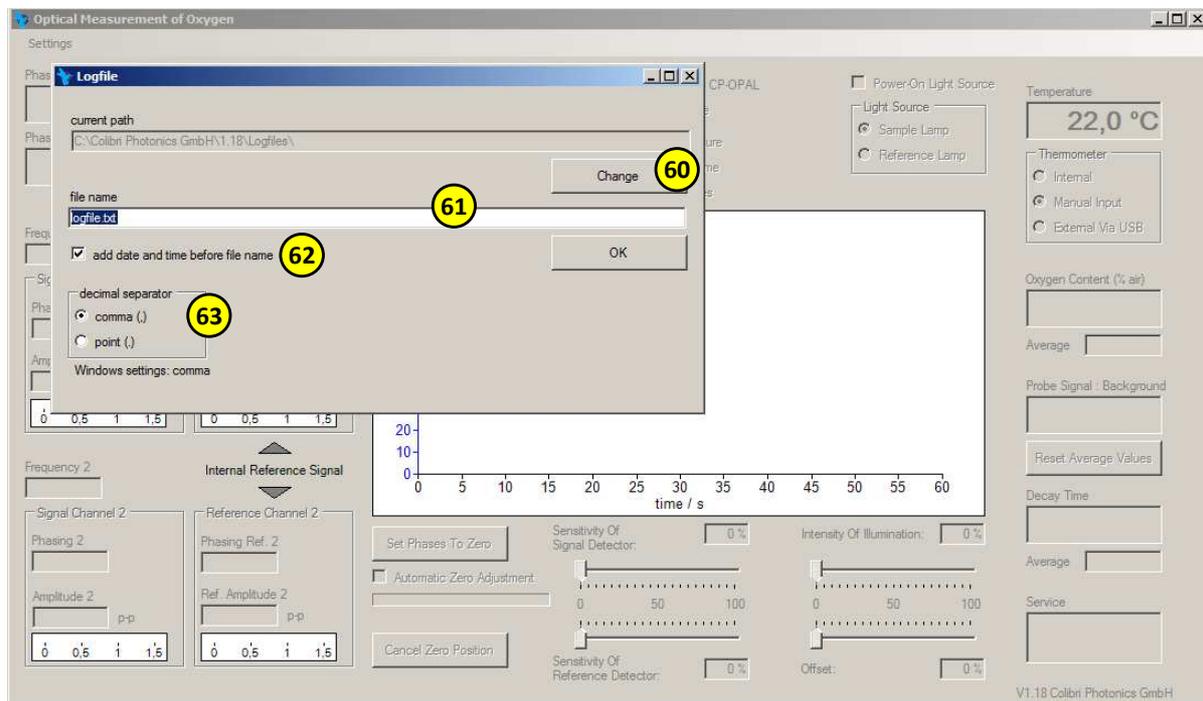


Fig. 3.10.: Open window “Logfile”

The log file is structured as a standard measurement data file: The measurement data are saved as ASCII column text files. A column separator tab is also used (ASCII code: CHR 9). For further evaluation the data files can be imported to common spreadsheet processing programs (e.g. Excel, Origin).

(60) Click “Change” to open the Windows dialog “change folder”. Select the folder in which the log file should be saved. The corresponding path is shown in the line above the “Change” button.

Note:

The language of the Windows “change folder” dialog depends on the computer’s Windows settings. The OPAL-software cannot change languages of standard windows dialogs.

(61) File Name. Enter the desired name of the log file and confirm it by clicking the OK button.

(62) Add Date and Time: If checked, the current date and time will be added to the name of the log file. Every log file will automatically receive a distinct name to prevent any unintended overwriting of old log files.

(63) Decimal Separator: The radio buttons allow for the selection of a comma or decimal point separator for the numerical data. By default, the separator of the current Windows settings is used. The decimal separator selection depends on the settings of the spreadsheet processing program that will be used and the settings of the computer, on which the spreadsheet processing program is installed.

3.4.4 “Viewer” Window

Clicking the “Viewer” **(12)** button opens a window to display curves from saved measurement data.

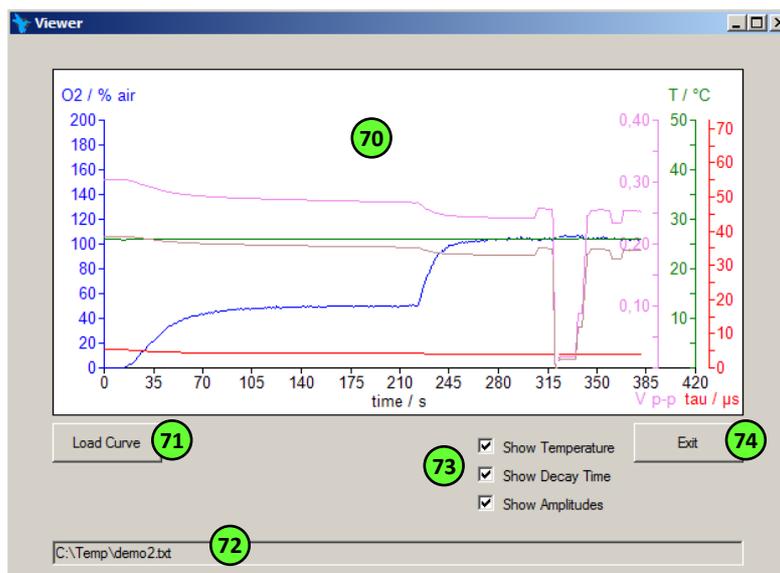


Fig. 3.11.: User interface “Viewer”

(70) The picture box displays the measurement data as a curve graph after opening the corresponding file.

(71) Load Curve: Click to open the Windows dialog "open". Select the data file to be displayed as a curve graph.

Note:

The language of the Windows "open" dialog depends on the computer's Windows settings. The OPAL-software cannot change languages of standard windows dialogs.

(72) Shows the name and path of the opened measurement data file.

(73) Checking temperature, decay time, and/or signal strength (= amplitudes) selects the data shown as curves in the picture box (70).

Note:

The O₂ content is always displayed as a curve, if the file contains corresponding data.

(74) Click "Exit" to close the window.

3.4.5 "Calibration" Window

Click Settings (32) on the menu bar to select the sub menu for the "Calibration". A corresponding window will open to set the calibration values and to change some instrument settings.

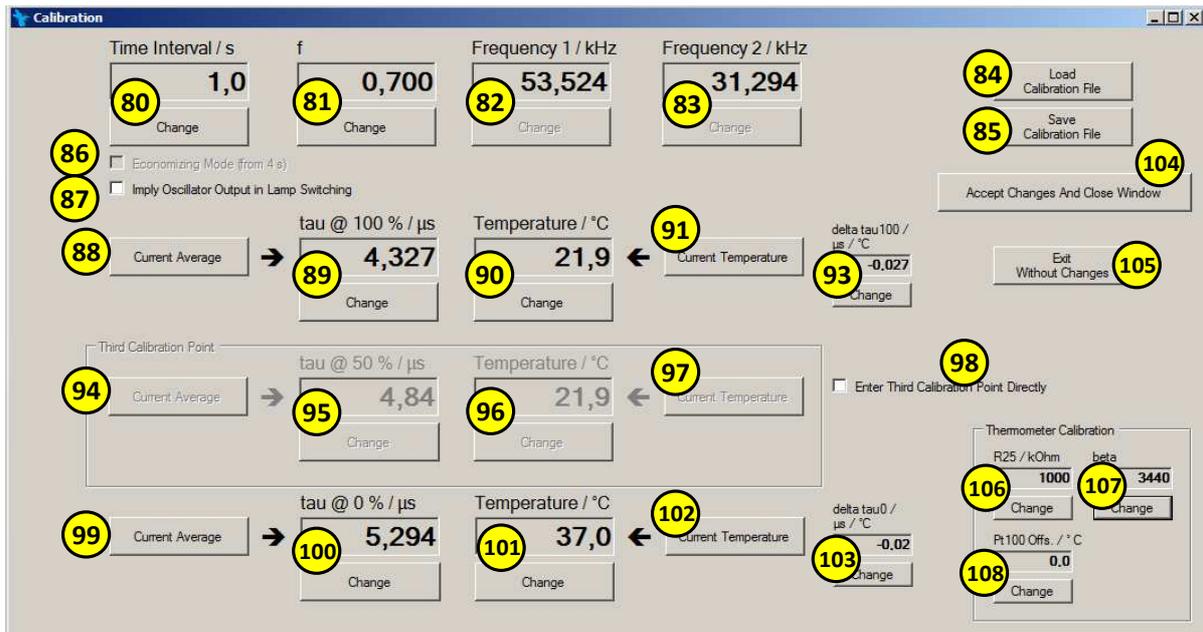


Fig. 3.12.: User interface "Calibration"

(80) Time interval: The time interval between two measurement points. Click the change button to enter a new time interval in the text box (range=0.1 to 3600 s). Confirm the input by pushing Enter on the keyboard.

Notes:

- ➔ The set time interval is only active if a measurement is running, otherwise the values for phases and amplitudes are refreshed every second.
- ➔ The average depth is automatically adjusted to the time interval listed. Larger time intervals result in a reduction of noise.

(81) f value: Describes the deviation from the ideal Stern Volmer behavior of the phosphorescence quenching. Simplified, f stands for the fraction of sensor dye molecules, which are accessible within the polymer matrix for O₂ molecules (see also 3.3). Click the change button to enter a new f value into the text box. Confirm the input by pushing Enter on the keyboard.

Notes:

- Entering a value for f is equivalent to entering a third calibration point.

- The range of f is between 0 and 1 by definition.
- Manual input of values for f may mathematically lead to meaningless values for $\tau @ 50\%$ air saturation (e.g. negative values or values of $\tau @ 50\%$, which are outside of the interval between $\tau @ 0\%$ and $\tau @ 100\%$). This indicates a wrong f value, wrong values for $\tau @ 0\%$ or $\tau @ 100\%$, wrong temperatures or wrong temperature factors.

(82) Frequency 1: Shows the modulation frequency at No. 1. Click the change button to enter a new value into the text box. Confirm the input by pushing Enter on the keyboard.

(83) Frequency 2: Shows the modulation frequency at No. 2. Click the change button to enter a new value into the text box. Confirm the input by pushing Enter on the keyboard.

(84) Load Calibration File: Click to open the Windows dialog box "open". Select the file containing the calibration data and settings to be loaded.

(85) Save Calibration File: Click to open the Windows dialog box "save as". Select the name and the path of the calibration file to be saved.

Notes:

- The calibration file contains values for the time interval (80), f value (81), modulation frequencies (82, 83), decay time $\tau @ 100\%$ (89), decay time $\tau @ 0\%$ (100), corresponding temperatures (90, 101) and corresponding temperature factors (93, 103).
- Furthermore, the current values are saved for the intensity of illumination (22), phase offsets determined by the zero point referencing (10), language, DC offset of the illumination intensity (36), user interface and thermometer calibration (106, 107, 108).
- The saved values, are values, which are currently displayed in their corresponding text boxes, regardless if they have been confirmed by clicking the button "Accept Changes and Close Window" (104) or not.
- The language of the Windows dialogs depends on the computer's Windows settings. The OPAL-software cannot change languages of standard windows dialogs.

(86) Economizing mode: If checked, the light source will be switched on before the record of a measurement point. This can only be checked at measurement time intervals $\geq 4\text{s}$ (80).

(87) Imply oscillator output into lamp switching: If checked, the oscillator output (fig. 3.2) of the OPAL Controller is switched on and off in time with the light source. This may be helpful in a special operation mode, when an external light source driver is used.

(88) Current Average: Click to activate the text box (89) and transfer the current average of the decay time (30) of the last measurement into the text box. Confirm the input by pushing Enter on the keyboard.

→ Click the button “Current Temperature” (91) to the right of the temperature text box related to tau @ 100 %. The current temperature is transferred to the text box and becomes active. Confirm the input by pushing Enter on the keyboard.

Notes:

- By clicking “Current Temperature”, the temperature measured at the moment of clicking, will be transferred. To perform an accurate calibration, the transferred temperature must be taken at the time the curve is recorded.
- Decay time and corresponding temperature can be manually entered. To do so, click one of the “Change” buttons (89, 90). The corresponding text box will become active and the values can be entered by using the keyboard. Confirm the input by pushing Enter on the keyboard.
- To exit an active text box press the Esc key on the keyboard. The input procedure will be cancelled and the former value will be displayed.
- Click the “Exit Without Changes” (105) to close the calibration window without making any changes.

(89) tau @ 100 %: Displays the decay time at 100 % air saturation. Click the corresponding change button to activate the text box and a new value can be entered manually. Alternatively the current average value can be transferred to the text box by clicking (88). Confirm the input by pushing Enter on the keyboard.

(90) Temperature: Displays the temperature, at which tau @ 100 % was determined. Click the corresponding change button to activate the text box and a new value can be entered

manually. Alternatively the current temperature can be transferred to the text box by clicking (91). Confirm the input by pushing Enter on the keyboard.

(91) Current Temperature: Click to transfer the current temperature into the text box (90).

(93) delta tau100: Temperature factor of the decay time at 100 % air saturation (89). Click the corresponding change button to activate the text box and a new value can be entered manually. Confirm the input by pushing Enter on the keyboard.

Notes:

- Buttons and text boxes (94 - 97) will be inactive, when (98) is not checked.
- tau @ 50% is the 3rd calibration point. Before entering the 3rd calibration point, correct values for tau @ 100% and tau @ 0% (and the corresponding temperatures) must be determined and entered correctly. Reentering of tau @ 100% or tau @ 0% (or of the corresponding temperatures) will lead to an automatic recalculation of tau @ 50%.
- The decay time tau @ 50% must be between the values of tau @ 100% and tau @ 0%.
- Entering a new value for tau @ 50% will change f (81) automatically. Vice versa, the input of a new f value automatically changes the corresponding value of tau @ 50%.
- A manual input of tau @ 50% may lead mathematically to forbidden values of f (outside of the interval $0 \leq f \leq 1$). If this happens, the calibration values of tau @ 100% and/or of tau @ 0% are likely incorrect (e.g. the values were measured during overload of the detector). Alternatively the corresponding temperatures (90, 96, 101) or the temperature factors (93, 103) are incorrect.

(94) Current Average: Click to activate the text box (95) and transfer the current average of the decay time (30) of the last measurement to the text box. Confirm the input by pushing Enter on the keyboard.

(95) tau @ 50%: Displays the decay time at 50% air saturation. Click the corresponding change button to activate the text box and a new value can be entered manually. Alternatively the current average value can be transferred to the text box by clicking (94). Confirm the input by pushing Enter on the keyboard.

(96) Displays the temperature, at which tau @ 50% was determined. Click the corresponding change button to activate the text box and a new value can be entered manually. Alternatively the current temperature can be transferred to the text box by clicking (97). Confirm the input by pushing Enter on the keyboard.

(97) Current Temperature: Click to transfer the current temperature into the text box (96).

(98) Enter Third Calibration Point Directly: Check to activate the text boxes for tau @ 50% (95), for the corresponding temperature (96), and the corresponding buttons to transfer average values and current temperature (94, 97). Please consider the notes from (94 - 97) specified above.

(99) Current Average: Click to activate the text box (100) and transfer the current average of the decay time (30) of the last measurement to the text box. Confirm the input by pushing Enter on the keyboard.

(100) tau @ 0%: Displays the decay time at 0% air saturation. Click the corresponding change button to activate the text box and a new value can be entered manually. Alternatively the current average value can be transferred to the text box by clicking (99). Confirm the input by pushing Enter on the keyboard.

(101) Temperature: Displays the temperature, at which tau @ 0% was determined. Click the corresponding change buttons to activate the text box and a new value can be entered manually. Alternatively the current temperature can be transferred to the text box by clicking (102). Confirm the input by pushing Enter on the keyboard.

(102) Current Temperature: Click to transfer the current temperature into the text box (101).

(103) delta tau0: Temperature factor of the decay time at 0% air saturation (100). Click the corresponding change button to activate the text box and a new value can be entered manually. Confirm the input by pushing Enter on the keyboard.

(104) Accept Changes and Close Window: Click to activate all changes performed within the calibration window. The changes will become active after the button is clicked.

Note:

After loading a calibration file, it is required to click button (104) to activate the new data.

(105) Exit Without Changes: Click to exit the calibration window without making any changes.

(106, 107) Calibration of the thermocouple: "R25" specifies the value of the reference resistor at 25°C. The reference resistor is located inside the OPAL Controller near the temperature sensor socket. "beta" specifies the slope. Default values are respectively 1000 and 3440.

(108) Calibration of the Pt100: The offset indicates the deviation from the set point. Positive values will decrease the displayed temperature.

4 CPOx-Beads

4.1 Specifications of the CPOx-Beads

CPOx-Beads are optical O₂ sensors in the form of microspheres. They are optimized to be used with the OPAL Controller. The standard diameter is 50 µm. CPOx-Beads consist of a biocompatible latex matrix, which contains a sensor dye and photo stabilizers. The sensor dye changes the phosphorescence decay time in dependence with ambient oxygen content. The photo stabilizers prevent early bleaching. Standard sensor dyes are a Ru-phenanthroline derivative (orange emission) or a Pt-porphyrine derivative (red emission).

4.2 Applications with CPOx-Beads, Placement within Samples

CPOx-Beads can be used to measure the O₂ content within the gas phases and within aqueous solutions. The beads can be placed in a sample by interspersing them with a microspatula or by pipetting suspensions of CPOx-Beads. The calibration curves calculated in the gas phase, of water and in various culture media are quite similar. Proteins, carbohydrates and pH value do not show any significant interference. If necessary, CPOx-Beads may be fixed using a *suitable* oxygen-permeable adhesive. Polydimethylsiloxan (PDMS) has proved to be suitable.

5 Installation of the OPAL measurement system

The OPAL oxygen measurement system is compatible with various types of fluorescence microscope makes and models. The following models have been tested successfully:

- Leica DM 6000 CS
- Olympus IX71, IX81, CKX41
- Nikon Eclipse Ti
- Zeiss Axio Vert.A1 FL, Axiovert 40 CFL, AxioStar plus FL, Axio Observer

Please confirm makes and models not listed above.

Note:

The fluorescence microscopes listed above are available with various options. Attached parts may change the geometry. Please confirm before purchasing an OPAL – Optical O₂ Measurement System to clarify, if custom adapters are necessary.

Below the installation of an OPAL – Optical O₂ Measurement System for a **Zeiss Axio Vert.A1** is explained:

Step 1: Installation of the LED Light Source

The LED Light Source (fig. 5.1) is linked with the fluorescence lamp port of the fluorescence microscope. For this purpose, the LED Light Source is equipped with a suitable adapter. Insert and fix the adapter according to the instructions of the microscope manufacturer. If all lamp ports of the microscope are occupied, dismount one of the lamps according to the manufacturer's instructions. Typically, the connecting adapter of the LED Light Source is inserted into the open lamp port and fixed by a hex screw. In fig. 5.1 (right) the LED light source was inserted into the free lamp port of a Zeiss lamp port switch.

Afterwards connect the 3 pin round plug to the "Light Source" socket at the OPAL Controller.



Fig. 5.1, Left: LED Light Source with connection adapter for Zeiss microscope.
Right: LED Light Source fixed in the lamp port of a Zeiss Axio Vert.A1.

Notes:

- ➔ Lamp port switches and LED Light Sources with light guide connection adapters are available on request.
- ➔ Depending on the microscope type, it may be necessary to align the x y position of the LED Light Source. XY translation mounts are available on request.

Step 2: Installation of the OPAL Detector Unit

The tube of the OPAL Detector Unit (fig. 5.2) is equipped with an inner c-mount thread to be attached to the c-mount camera port of the microscope. An already present camera that occupies the port must first be taken off the microscope. Refer to the instructions of the camera's manufacturer for removing from the microscope. If necessary, e.g. due to limited space, the detector module can be screwed off during the installation procedure. If the detector is taken off, be sure that the active area is neither touched nor exposed to bright light.

Next connect the 5 pin round plug (supply and control voltage) to the socket "Detector 1 Control" to the OPAL Controller. If necessary, use the enclosed extension cable.

Connect the BNC-connector (signal) to the SMB socket "Signal 1 In" to the OPAL Controller using the enclosed adapter cable.



Fig. 5.2, left: OPAL Detector Unit. The detector module is visible in the front.

Right: OPAL Detector Unit attached to the c-mount camera adapter of a Zeiss Axio Vert.A1.

Notes:

Camera ports which allow the installation of two cameras (installation of the OPAL Detector Unit and another camera) at one c-mount adapter, are available on request. Through the beam splitter on the trinocular microscope, the light can be either guided to the camera or to the OPAL Detector Unit.

Step 3: Installation of the filter cube

Place the filter cube (fig. 5.3) in the corresponding filter cube holder of the microscope. Refer to the instructions of the microscope's manufacturer. Next, move the filter into the light path. Typically this is achieved by shifting the filter slide or by turning the filter carousel to a certain position.

Notes:

- For various types of O₂ sensors (CPOx-Beads) different filter sets may be required.
- Depending on the microscope model, different filter cube types are required. Every filter cube can be equipped with the necessary filter set.



Fig. 5.3 Left: Zeiss filter cube "P&C".
Right: Fluorescence filter cube inserted into the Filter carousel (red arrow) of a Zeiss Axio Vert.A1.

Step 4: Connection of the OPAL Controller unit

Put the OPAL Controller unit beside your microscope.

Back side:



- Connect the 3 pin plug of the LED Light Source to the plug socket "Light Source".
- Connect the 5 pin plug of the OPAL Detector Unit to the plug socket "Detector 1 Control". Connect the BNC plug of the OPAL Detector Unit to the BNC socket "Signal 1 In". If necessary, use the enclosed extension cables.
- Connect the power supply to the 12 V DC connector.
- Connect the OPAL Controller unit to a desktop computer or laptop by use of the enclosed USB cable.
- The connector "Oscillator Out" is intended to control custom external light sources and for service purposes. During normal operation it is kept free.
- The connectors "Detector 2 Control" and "Signal 2 In" are intended for a special optical referencing mode. During normal operation they are kept free.

Front side:



Connect the enclosed temperature sensor to the plug socket "Temperature Sensor". The OPAL Controller is equipped with a socket to connect a temperature sensor (2 pin socket).

The completed cabling is shown in fig. 5.5.



Fig. 5.5., Cable connection of the OPAL Controller to the LED Light Source, the OPAL Detector Unit, computer via USB and power supply.

Step 5: Installation of the Reference Light Guide

The reference light guide (fig. 5.6) guides light from the LED Light Source directly to the OPAL Detector Unit. Remove the protecting caps from the SMA fiber optic connectors. Do not touch the front surfaces of the connectors. Insert the connectors into the corresponding sockets at the beam splitter cube of the OPAL Detector Unit and at the LED Light Source.



Fig.5.6.: Left: Reference light guide.

Right: OPAL Detector Unit and LED Light Source, connected to each other with the reference light guide.

Step 6: Installation of the Software

- ➔ The software requires Microsoft .NET Framework 4. If .NET Framework 4 is not installed on the computer, it will have to be installed. A web installer for downloading and installing .NET Framework 4 can be found on the Microsoft homepage and is available on the enclosed software CD. Please note that the web installer requires connection to the internet.
- ➔ Verify that the OPAL Controller is connected to your computer via a USB cable. Insert the software CD and switch on the OPAL Controller unit. The required USB drivers are installed from the CD.
- ➔ Start the installation of the OPAL Software by clicking “setup.msi” on the CD.
- ➔ Start the OPAL Software by clicking on the Colibri icon on the desktop screen or from the start menu. If the OPAL Software finds an OPAL Controller unit, the connection indicator (16) will be checked.

6 Preparation for Measurement

Before addressing adjustment tasks with the microscope, set the track bar of the detector sensitivity (19) to zero. The high voltage of the detector unit is now switched off. Be sure to avoid that major light intensities (e.g. arising from the transmitting light halogen lamp) reach the active surface of the detector.

Follow the steps listed below:

- Switching on of the LED Light Source.
- Focus CPOx-Beads.
- Tune the illumination intensity and detector sensitivity.
- Adjust the iris diaphragm and identify the center position of the image.
- Set the zero position of the phasing (zero point reference)
- Calibrate the CPOx-beads

6.1 Switching on of the LED Light Source

- ➔ Verify that the filter cube containing the appropriate filters for the CPOx-Beads is inserted into the microscope. Place the filter cube in the adequate position by moving the filter slide or the filter carousel.
- ➔ Place a red or orange fluorescent alignment strip (fluorescent safety strip) on a glass microscope slide. Position the 10x objective. Select for the correct fluorescent filter cube so the alignment strip can be viewed through the eyepieces. If necessary focus on the alignment strip under transmitted light and then switch back to the fluorescent light source.
- ➔ Select the correct filter cube for the green light of the LED Light Source.
- ➔ Select "Sample Lamp" (21) in the software and move the bar of the "Sensitivity of Detector" (19) to approximately a 30% intensity. Power on the LED Light Source by checking the check box (20). A bright green light will appear (fig. 6.1) through the objective on the microscope. Through the eyepieces the bright orange fluorescence of the alignment strip will be visible.

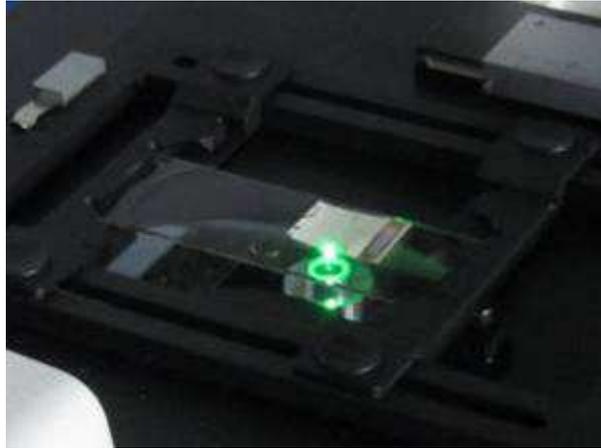


Fig. 6.1.: Green excitation light arising from the LED Light Source.

- ➔ The fluorescent region within the field of view may require centering. In this case an optional XY translation mount has to be installed with the LED Light Source. To center the fluorescence turn the x and y screws until the light is centered.

6.2 Focusing CPOx-Beads

- ➔ Place a sample containing CPOx-Beads on the microscope stage. Select the 10x objective and select the correct light to view the sample through the eyepieces using transmitted light
- ➔ Focus on one CPOx bead and shift it to the center of the image by moving the microscope stage as needed. If possible, use a sample area containing only one single bead within the image field. A microscope reticle with a cross-line is recommended to center the CPOx-Bead.
- ➔ Select for "Sample Lamp" in the "Light Source" field (21). Move the bar of the "Intensity of Illumination" (22) to approximately 50%. Check the "Power On Light Source" (20) to switch on the lamp. In the focus of the objective a bright green light spot will appear. Switch off all other light sources except for the LED Light Source (e.g. the halogen lamp behind the condenser). The centered CPOx-Bead, will appear as an orange or red shining sphere through the eyepieces.

Note:

- CPOx-Beads, located inside a scattering sample, will not appear completely in focus. In this case try to focus the image as much as possible.
- If the phosphorescence of the CPOx-Beads cannot or is barely recognized through the eyepieces, the signal intensity is probably too low for O₂ measurement. In this case, increase the illumination intensity by moving the corresponding bar. If bright phosphorescence is still not visible at full

illumination intensity, check the light path. Further filters or apertures may need to be removed. If the excitation light path is exceptionally long (e.g. caused by additional microscope components), an additional lens in front of the LED Light Source may increase the intensity.

6.3 Tuning the Detector Sensitivity and Illumination Intensity

- Verify through the eyepieces that the CPOx-Bead is focused and centered. At about 50% illumination intensity (22) the phosphorescence of the CPOx-Bead should be clearly visible.
- Verify that the bar of the “Sensitivity Of Detector” (19) is set to 0%. Ensure that no extraneous bright light (e.g. bright room light) can reach the OPAL Detector Unit.
- Adjust the transmitted light path of your microscope so the fluorescence signal of the sample is guided to the OPAL Detector Unit.
- Completely open the iris diaphragm of the OPAL Detector Unit tube (position 4 at the division scale = all open, 0 = all closed; fig 6.2)
- Increase the high voltage of the detector (= detector sensitivity) by moving the bar for the detector sensitivity (19) slowly, step by step to the right. Watch for Amplitude 1 and Amplitude 2 (6) to display and for the corresponding bar graphs. As the detector sensitivity increases, amplitude values (given in V peak to peak, V_{p-p}) will be displayed. Move the bar slowly to the right until an approximate amplitude value of 0.5 V is shown. Important: If at 60% or more detector sensitivity no amplitudes appear, set the bar back to zero and verify the optical pathways and the electrical connections.

Notes:

- O_2 measurements work well in an amplitude range from 0.1 to 1.5 V_{p-p} (green bar). The Ideal range is from 0.2 to 1.2 V_{p-p} . (dark green bars).
- The detector module of the OPAL Detector Unit will change with delay due to the high voltage. It is important to move the bar slowly as indicated above.
- The percent scale indicates the level of the control voltage and does not display the actual detector sensitivity. There is no linear relation of sensitivity to the control voltage. At values more than “40%”, the sensitivity increases abruptly. Therefore, it is important to move the bar slowly.

- Too high detector sensitivity leads to decreasing amplitudes. This is an overload of the detector. It is absolutely necessary to avoid an overload. Overload can lead to wrong measurement results and the detector may be damaged by the high light intensities. To verify an overload: Decrease the detector sensitivity by one step (position the mouse pointer to the far left of the bar (19) and click). Watch the amplitude displays (6) after clicking. If the displayed amplitude increases in spite of reducing the detector sensitivity, the detector is overloaded. If this occurs quickly reduce the detector sensitivity further.
 - An overload may also occur, if the phosphorescence increases due to a decrease of the oxygen content or if the sample is moved and more CPOx-Beads get into the focus.
 - The detector sensitivity may be aligned when a measurement is running. In contrast to the illumination intensity, changing the detector sensitivity will not interfere with the phasing.
- ➔ If the signal intensity is too high, i.e., amplitude levels of $0.5 V_{p-p}$ or more are already achieved at detector sensitivities below 70%, reduce the illumination intensity, by moving the corresponding bar (22) to the left. Subsequently increase the detector sensitivity, until amplitude levels of about $0.5 V_{p-p}$ are shown again. If necessary, repeat this procedure.
- ➔ If the signal intensity is too low, i.e., amplitude levels of $0.5 V_{p-p}$ cannot be achieved or are only achieved at much more than 70 % detector sensitivity; increase the illumination intensity, by moving the corresponding bar (22) to the right.

Note the determined positions of the bars of the detector sensitivity and the illumination sensitivity for future measurements.

Notes:

- The illumination intensity is well aligned, if at 70 % detector sensitivity amplitudes between 0.2 und $1 V_{p-p}$ are achieved.
- In case there is a high signal, it is recommended to reduce first the illumination intensity then the detector sensitivity. Generally, working with a lower illumination intensity preserves samples and probes. Furthermore, it reduces the risk to induce interfering photochemical side reactions. Due to the electrical characteristics of the lamp, the illumination intensity should not be below 5%.
- When the signal intensity is too low, it is recommended to increase the detector sensitivity. At more than 80%, the noise (dark count rate) will strongly increase and the detector will become susceptible to damage towards extraneous light (e.g. if the transmitted light of the microscope is switched on accidentally).

- The illumination intensity affects the phasing and therefore the measuring result. The bar must not be moved when a measurement is running. After changing the illumination intensity, a new zero point reference must be carried out.

6.4 Adjustment of the Iris Diaphragm and Identification of the Center Position of the Image

- ➔ Close the aperture of the iris diaphragm at the detector tube by one step (position 4 at the division scale = all open, 0 = all closed; fig 6.2). Watch the amplitude displays (6) and the corresponding bar graphs.



Fig. 6.2.: Iris diaphragm at the detector tube.

- ➔ Close the aperture of the iris diaphragm step by step until the signal intensity (= amplitudes) is decreased.
- ➔ Move the x and y position of the microscope stage to change the position of the centered CPOx-Bead. Align the x and y position so that the maximum amplitude is achieved. (Small changes in the positioning may result in large changes in the amplitudes. Be careful to move the stage carefully and slowly).
- ➔ If the x y alignment leads to no change in the signal level, close the iris diaphragm further one step and repeat the x y alignment.
- ➔ If the x y alignment continues below the previous one, reopen the iris diaphragm by one step. Switch the transmitted light path of the microscope to the eyepieces to observe the CPOx-Bead. Keep in mind the exact position of the CPOx-Bead (e.g. by using the cross line reticle in one of the eyepieces). Use this position for all further measurements.

Notes:

- When the x y alignment is performed, the picture seen with the eyepieces may not be consistent with the picture shown by the camera. The deviation at the center of the picture is very small.

- During ideal adjustment, the whole signal of one single 50 µm CPOx-Bead pass the step 1 aperture of the iris diaphragm.
- Closing the iris diaphragm usually decreases the amount of back ground noise. The probe signal to background ratio (27) usually improves (= increase) by reducing the aperture.
- A well aligned iris aperture and a well determined center position will allow the inspection of 3D samples containing CPOx-Beads located in various z levels. CPOx-Beads that are not in focus will be masked by the confocal effect of the iris diaphragm.

6.5 Setting the Zero Position of the Phasing (Zero Point Referencing)

The OPAL Controller evaluates the O₂-dependent phosphorescence decay time of the CPOx-Beads by accurately measuring the phase shifts between signal and excitation light. Additional phase shifts arise from electronic components. These phase shifts must be quantified and eliminated mathematically. This is done by the zero point referencing. During the zero point referencing procedure, excitation light from the LED Light Source is directly guided to the detector. The phasing measured in this process is defined as zero. The zero point measurement can be performed manually and automatically.

Manual Zero Point Referencing

- ➔ Position the bar for the detector sensitivity (19) to the zero position.
- ➔ Set the bar for the illumination intensity (22) to a value > 5%, e.g. to 50%.
- ➔ Check reference lamp to set the LED Light Source (21).
- ➔ Turn on the LED Light Source by checking "Power On Light Source" (20).
- ➔ Increase the high voltage of the detector (= detector sensitivity) by moving the bar for the detector sensitivity (19) slowly step by step to the right. Watch Amplitude 1 and Amplitude 2 (6) to display and for the corresponding bar graphs. As the detector sensitivity increases, amplitude values (given in V peak to peak) will be displayed. Move the bar slowly to the right until an approximate amplitude value of 0.5 V is shown. Important: If at 60% or more detector sensitivity no amplitudes appear, set the bar back to zero and verify the optical pathways and the electrical connections.
- ➔ Wait approximately 5 seconds until the phasing (3) has adjusted to a constant level.
- ➔ Click "Set Phases to Zero" (10). The phasing display (3) should now show values around zero. The OPAL Controller is now zero point referenced.

Note:

Depending on the modulation frequencies, deviations and oscillations around $\pm 0.05^\circ$ are considered normal. Repeat the procedure, if the phasing deviates from zero.

Automatic Zero Point Referencing

If the checkbox Automatic Zero Adjusting (11) is checked, an automatic zero point referencing is performed after clicking the start button (7). The detector sensitivity will be increased automatically step by step. If there are no amplitudes at 60 % detector sensitivity, the bar will automatically set back to zero and an error message will appear. If this occurs verify the optical pathways and the electrical connections.

7 Calibration

Normally the CPOx-Beads can be calibrated a batch at a time or using the calibration values included in the delivery. If CPOx-Beads in dry form are stored at room temperature in a dark place, the calibration values won't change. A verification of the calibration or recalibration may be necessary after a certain time or treatment.

To calibrate, the CPOx-Beads must be placed in environments with 100%* and 0% air saturation (in the case of the three point calibration additionally in an environments with 50% air saturation). The calibration can be done within the gas phase and within aqueous solution.

- ➔ Put a few CPOx-Beads either dry or in aqueous solution into the μ -Dish or a similar vessel.
- ➔ Prepare a measurement according to the chapter Preparation of a measurement: Focus one CPOx-Bead or better a group of CPOx-beads. Open the iris diaphragm as wide as the signal arising from the group of CPOx-Beads. Determine suitable values for illumination intensity and detector sensitivity.

Notes:

- It is advantageous to focus on an entire group of CPOx-Beads. In doing so differences between individual beads are averaged. Be sure that the iris diaphragm is opened wide enough to collect the signal from the entire group of CPOx-Beads.
 - If some CPOx-beads are focused simultaneously, there is a risk to overload the detector. Be sure that the intensity of illumination and the sensitivity of the detector are adequately regulated.
 - Keep the illumination intensity at a moderate level: If many CPOx-Beads are intensely illuminated in small volume, it may cause a back reaction of the singlet O_2 , which is generated during the quenching process and the diffusion of triplet O_2 into the CPOx-Beads is not fast enough. This can lead to a temporary depletion of triplet O_2 inside the CPOx-Beads and incorrect measurement values (→ error sources).
- ➔ Use a cover plate with small holes to insert the temperature sensor and, if necessary, to insert a small tube to introduce gas.

(*) 100% O₂ saturation is meant to be either O₂ concentration in the air at standard conditions for temperature and pressure (20.9 % O₂) or the maximum of O₂ concentration in your investigated system (e.g. blood).

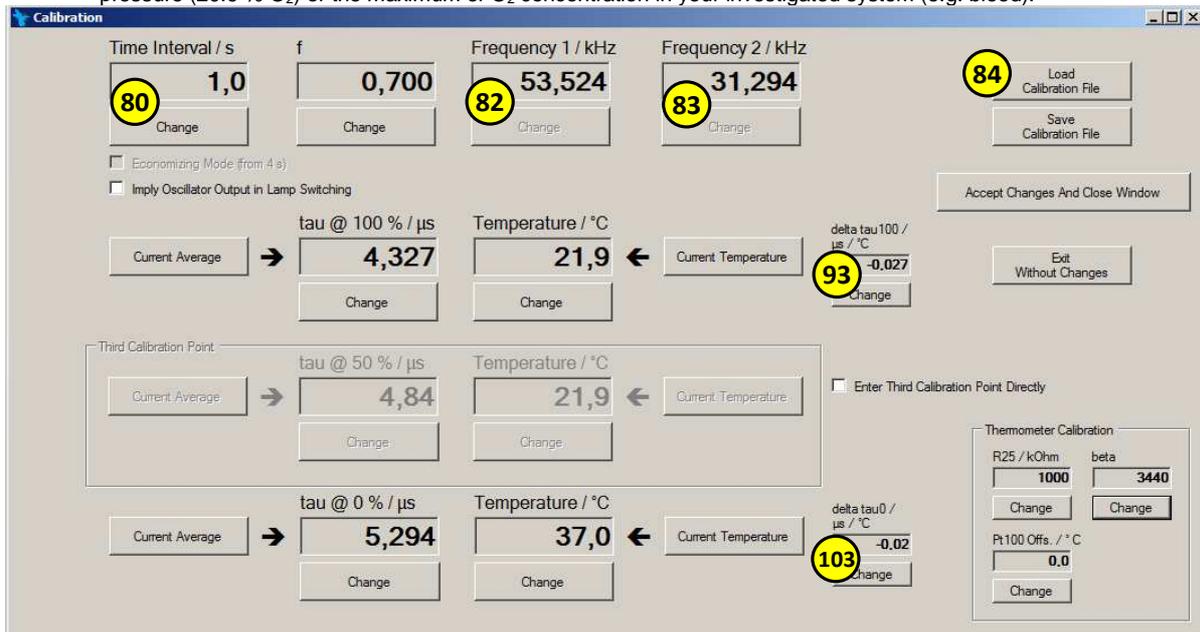


Fig. 7.1.: Open window "Save Measurement Data"

➔ Depending on the type of CPOx-Beads to be calibrated the temperature factors (93, 103) and modulation frequencies (82, 83) have to be set. For calibration, a time interval (80) of 1s is suggested. It is recommended to load a calibration file by clicking on the "Load Calibration File" button (84).

Notes:

- The modulation frequencies have to be in accordance with the phosphorescence decay times of the CPOx-Beads within the measurement range. The following calculation is used to determine the modulation frequencies:

$$F = \frac{1}{2\pi\tau}, \quad F_1 = 1.313 * F, \quad F_2 = 0.692 * F$$

- The exact values of the factors are irrelevant. But do not use integer ratios to avoid beating waves.
- The maximum modulation frequency is 80 kHz.
- For τ choose a value in the middle of the measurement range, e.g. τ_{50}

Calibration of the value at 100% air (1st calibration point)

Calibration in the gas phase	Calibration in aqueous solution
<p>Use an ibidi Heating System working with an ibidi Gas Incubation System. Set the O₂ concentration to 21% and wait for the value settle on a constant concentration.</p> <p>Alternatively the air in a well aired room can be used for 100% calibration. Please note that N₂ or CO₂ sources near the sample, e.g. a vessel with dry ice, can interfere with the result.</p> <p>Air can also be taken from a gas cylinder or from a compressed-air line.</p>	<p>Cover the CPOx-beads with water, buffer or medium. Carefully pass air into the liquid (e.g. using a pump and small tube.) At the end of the tube air bubbles should arise. Alternatively, to pass air into the sample, water can be used that was well shaken in advance to achieve air saturation. Verify that the temperature sensor measures the temperature of the liquid.</p>

- ➔ Start the measurement by clicking on the start button (7) in the main window of the software.

Notes:

It is important that a Zero Point Referencing was carried out successfully before. The Zero Point Referencing can also be completed automatically during the start process, by checking the control box Automatic Zero Adjusting (11).

- ➔ Wait until the temperature and displayed O₂ content have adjusted to a constant level (horizontal measurement curves; before a calibration it is likely that the displayed O₂ content differs from 100%). Afterwards click on "Reset Average Values" (28) and wait until a number of measurement values have been recorded to achieve a usable average value (usually at a 1s time interval with a 1 min or less average).

Notes:

- While the measurement curve for the average is recorded, the temperature must remain constant.
- To show the temperature curve, check the related box (18).
- It is recommended to calibrate at the same temperature that will be used later during measurement

- ➔ Stop the measurement by clicking on the stop button (8) in the main window of the software.

- ➔ Open the calibration window by selecting “Calibration” (32) in the “setting” menu on the menu strip.



Fig. 7.2.: 1st calibration point at air with 20.9% O₂ (Δ 100%)

- ➔ Click the button “Current Average” (88) to the left of the text box of “tau @ 100%”. The average value of the decay time, which was measured prior to this, is transferred to the text box. Confirm the value by pressing the enter button at the keyboard. It will now become active.
- ➔ Click the button “Current Temperature” (91) to the right of the temperature text box related to tau @ 100%. The current temperature is transferred to the text box. Confirm the value by pressing the enter button at the keyboard. It will now become active.

Notes:

- By clicking “Current Temperature”, the temperature, which is measured at the moment of the click, will be transferred. To perform an accurate calibration, the transferred temperature must be the temperature, while the measurement curve was recorded.
- Decay time and corresponding temperature can also be entered manually. To do so, click one of the “Change” buttons (89, 90). The corresponding text box will become active and the values can be entered by using the keyboard. Confirm the value by pressing the enter key on the keyboard.
- You can exit an active text box by pressing the Esc key on the keyboard. The input procedure will be cancelled and the former value will be shown again.
- Click the button “Exit Without Changes” (105) to close the calibration window without taking over any of the changes.

- By clicking the button “Accept Changes And Close Window” (104) the software transfers the calibration value tau @ 100% air saturation, the corresponding temperature, and all further changes performed within the calibration window.

Calibration of the value at 0% air (2nd calibration point)

Calibration in the gas phase	Calibration in aqueous solution
<p>Use an ibidi Heating System working with an ibidi Gas Incubation System. Set the O₂ concentration to 0% and wait for the value settle on a constant concentration.</p> <p>Alternatively pass nitrogen or argon into the calibration vessel containing the CPOx-Beads by using a small tube. Tube diameters < 1 mm are ideal. Cover the vessel with a cap with small holes to insert the gas tube and the temperature sensor. Keep the gas flow moderate, since turbulences will feed ambient O₂ rather than displace it.</p>	<p>Cover the CPOx-beads with water, buffer or medium. Cover the vessel with a cap with small holes for inserting the gas tube and the temperature sensor. Carefully pass nitrogen or argon into the liquid. At the end of the tube small air bubbles should arise. Be sure that the temperature sensor measures the current temperature of the liquid.</p> <p>Alternatively a spatula tip of sodium sulfite (Na₂SO₃) can be added to the water. The subsequent chemical reaction will eliminate all dissolved O₂. Be sure that the temperature sensor measures the current temperature of the liquid.</p>

- Start the measurement by clicking the start button (7) in the main window of the software.

Notes:

It is important that a Zero Point Referencing was carried out successfully before. The Zero Point Referencing can also be completed automatically during the start process, by checking the control box Automatic Zero Adjusting (11).

- Wait until the temperature and displayed O₂ content have adjusted to a constant level (horizontal measurement curves; before a calibration it is likely that the displayed O₂ content differs from 100%). Afterwards click on “Reset Average Values” (28) and wait until a number of measurement values have been recorded to achieve a usable average value (usually at a 1s time interval with a 1 min or less average).

Notes:

- Keep the temperature constant while the measurement curve for the averaging is recorded.
- To show the temperature curve, check the related box (18).

- It is recommended to calibrate at the same temperature that will be used later during measurement
- ➔ Stop the measurement by clicking on the stop button (8) in the main window of the software.
- ➔ Open the calibration window by selecting “Calibration” (32) in the “setting” menu at the menu bar.

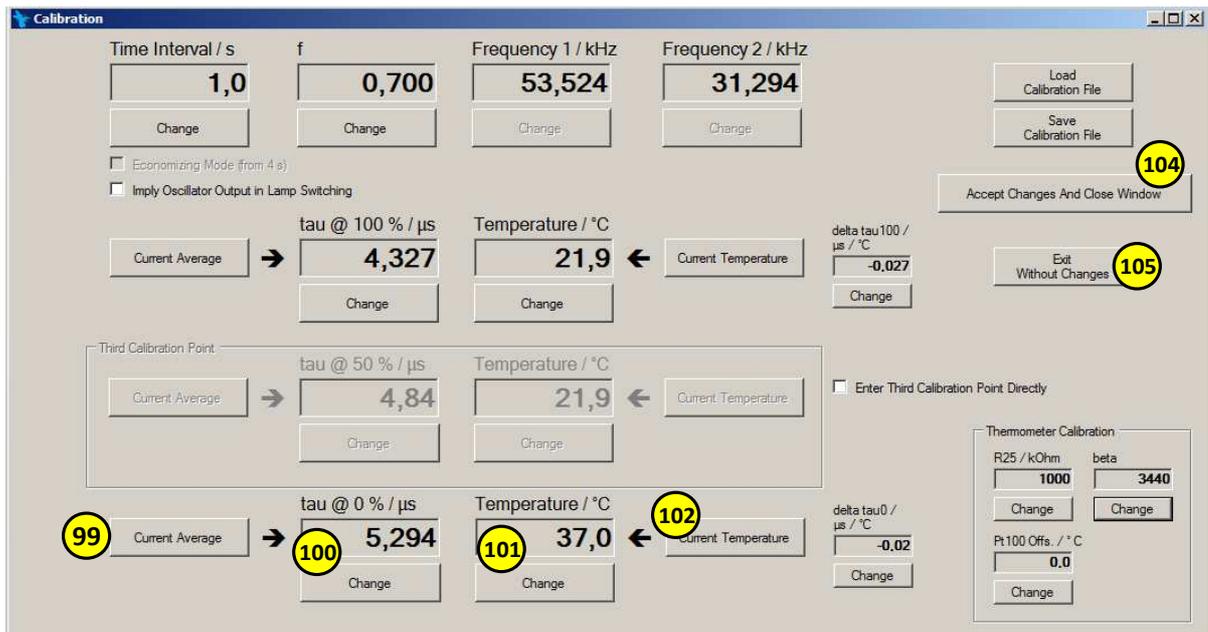


Fig. 7.3.: 2nd calibration point at air with 0% O₂ (Δ 0%)

- ➔ Click the button “Current Average” (99) to the left of the text box of “tau @ 0%”. The average value of the decay time, which was measured prior to this, is transferred to the text box. Confirm the value by pressing the enter button at the keyboard. It will now become active.
- ➔ Click the button “Current Temperature” (102) to the right of the temperature text box related to tau @ 0%. The current temperature is transferred to the text box. Confirm the value by pressing the enter button at the keyboard. It will now become active.

Notes:

- By clicking “Current Temperature”, the temperature, which is measured at the moment of the click, will be transferred. To perform an accurate calibration, the transferred temperature must be the temperature, while the measurement curve was recorded.
- Decay time and corresponding temperature can also be entered manually. To do so, click one of the “Change” buttons (100, 101). The corresponding text box will become active and the values can be entered using the keyboard. Confirm the value by pressing the enter key on the keyboard.

- You can exit an active text box by pressing the Esc button. The input procedure will be cancelled and the former values will be shown again.
 - Clicking the button “Exit Without Changes” (105) will close the calibration window without taking over any of the changes.
- ➔ Clicking the button “Accept Changes And Close Window” (104) to take on the calibration value tau @ 0 % air saturation, the corresponding temperature, and any further changes performed within the calibration window.