MULTI-COLOR-PAM-II

Manual

2.162/11.201825
1rst Edition, April 2021
MC_PAM-II-2024-7.docx

Heinz Walz GmbH • Eichenring 6 • 91090 Effeltrich • Germany
Phone +49 9133 77650 • Telefax +49 9133 5395
E-mail info@walz.com • Internet www.walz.com

Contents

1 Safety Instruction

1.1 General Safety Instructions

- Read safety instructions and the operating instructions prior to operation of the device.
- Pay attention to all safety warnings.
- Keep device away from water or high moisture areas.
- Keep the device away from dust, sand and dirt.
- Do not put the device near sources of heat.
- Ensure sufficient ventilation.
- Connect the device only to the power source indicated in the operating instructions or on the device. If the device is not in use, remove the mains plug from the socket.
- Ensure that neither liquids nor foreign bodies get inside the device.
- The device should only be repaired by qualified personnel.

1.2 Special Safety Instructions

- The MULTI-COLOR-PAM-II is a highly sensitive instrument which should be only used for research purposes, as specified in this manual. Follow the instructions of this manual in order to avoid potential harm to the user and damage to the instrument.
- The MULTI-COLOR-PAM-II can emit very strong light! In order to avoid harm to your eyes, never look directly into the emitter unit or Perspex rod connected to the emitter unit.

2 Introduction

- The “ST-Kinetics and Multi-Wavelength Chlorophyll Fluorometer MULTI-COLOR-PAM-II” has been designed for highly sensitive assessment of photosynthesis parameters in dilute suspensions of algae, cyanobacteria and isolated chloroplasts.
- The instrument stands in the tradition of the PAM chlorophyll fluorometers PHYTO-PAM-II, DUAL-PAM-100 and PAM-2500 and as well of the MULTI-COLOR-PAM-I. The MULTI-COLOR-PAM-II combines the major virtues of all these instruments in one compact device to which is added non-modulated STK analysis.
- The MULTI-COLOR-PAM-II is outstanding by virtue of its broad portfolio of applications. With respect to the PAM-applications different measuring and actinic light colors can be chosen allowing a matching between excitation wavelength and the antenna properties of the organism of interest. The broad range of frequencies, up to 200 kHz for the PAM-applications and up to 40 MHz for STK-applications, gives the user the choice to probe processes as slow as min to hours, down to electron transport chain reactions in the 10 µs/1 s range, down to the donor side of PS II and Car-triplets in the sub-µs/µs range.
- Its operating software, PamWin-4, is based on the PamWin-3 program introduced to control the PAM-2500. Major software extensions allow for control of all the features of the MULTI-COLOR-PAM-II, as well as the analysis of a variety of experiments.

- The MULTI-COLOR-PAM-II is optimized for the study of dilute suspensions of unicellular algae, cyanobacteria and isolated chloroplasts. In addition, the measuring system can be configured to study leaf photosynthesis.

2.1 Intention of this Manual

The MULTI-COLOR-PAM-II fluorometer displays a high degree of flexibility in measuring and analyzing fluorescence. Despite its high complexity, first measurements can be started using automated routines for standard measurements. Actually, the "intelligent" central control of all functions by the PamWin-4 software prevents operational mistakes harming the instrument. To make full use of the analytical opportunities offered by the MULTI-COLOR-PAM-II, however, it is necessary to become acquainted with the handling of the hardware and the software, and to understand the theoretical basis of chlorophyll fluorescence analysis. The present manual provides the information needed to professionally operate the MULTI-COLOR-PAM-II and also includes links to pertinent publications. The most important chapters of the manual are briefly introduced next:

- Chapter 3, page 7 (Components and Setup) explains hardware setup and software installation required to get started.
- Chapter 4, page 31 (SP-Analysis) introduces standard quenching analysis of chlorophyll fluorescence by the PamWin-4 software for the MULTI-COLOR-PAM-II.
- Chapter 5, page 92 (Fast Acquisition) deals with rise and decay kinetics of chlorophyll fluorescence as tools to analyze PS II and electron transport chain function.

- Chapter 6, page 129 (Script File Window) is devoted to programming the MULTI-COLOR-PAM-II using the script file option of PamWin-4.
- Chapter 7, page 145 (non-modulated STK-measurements) introduces the new functions and applications related to non-modulated flash analysis.
- Chapter 8, page 158 (View Mode) explains how to evaluate saturation pulse and fast kinetics data using PamWin-4.
- Chapter 9, page 166 (Definitions and Equations) provides some background on how fluorescence signals can be used to evaluate photosynthesis analysis by fluorescence signals.
- Sections 9.6 and 9.7 (page 180), and Chapter 10 (page 185) compile pertinent references.
- Chapter 13, page 200 (Appendix) presents a practical introduction into script file writing and automatic operation of the fluorometer.

This manual is complemented by information provided in the online help texts of PamWin-4 (see section 4.1, page 31).

3 Components and Setup

3.1 Extent of Delivery, Basic System

MCP-II-C Power-and-Control-Unit
MINI-PAM/L charger and power cable
USB cable type A-A
ST-101 Stand for mounting ED 101US/MD
PHYTO-T transport box

ED-101US/MD Optical Unit for Suspensions
US-K0 10 x 10 mm Quartz Glass Cuvette

Manual and USB-stick Software and Manuals

Selection of the following four heads:

MCP-II-D1 Detector Head
Long-pass filter RG 665
Short-pass filter SP 710

MCP-II-E Multi-Color Emitter Head

MCP-II-D2DST Combi-Detector Head
Long-pass filter RG 665
Short-pass filter SP 710

MCP-II-EDST STK flashlamp with detector

Basic system

3.1.1 Power-and-Control-Unit

The sockets and switches of the Power-and-Control-Unit are outlined and numbered in Fig. 1, their properties and functions are explained on subsequent pages.

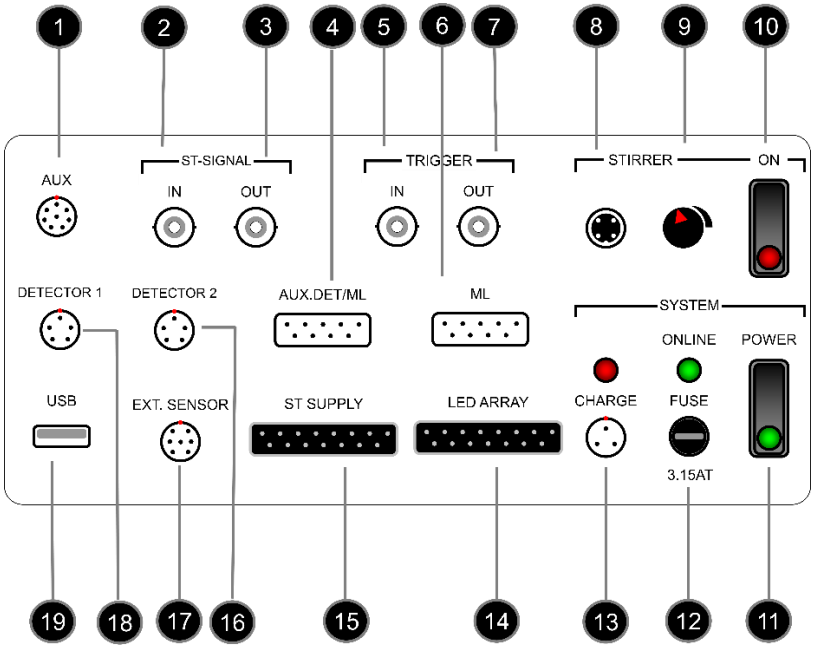
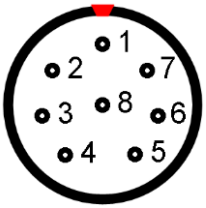


Fig. 1: Front panel of MULTI-COLOR-PAM-II Power-and-Control-Unit

1 AUX (Socket for auxiliary devices)



(1)	DGND (digital ground)	(5)	DGND (digital ground)
(2)	UBatt (battery voltage)	(6)	Trigger Out (5V)
(3)	GND (ground)	(7)	+5 V
(4)	DA out (digital output)	(8)	-5 V

2 ST SIGNAL IN BNC socket

Entry port for the ST signals coming from either the strong flash lamp (leaf configuration) or the combi-detector (suspension configuration).

3 ST SIGNAL OUT BNC socket

Provides 5 V signals to control the strong flash lamp in both suspension and leaf configuration.

4 Aux.Det/ML

Auxilliary ML (measuring light) for the non-modulated fast measurements with the strong flash lamp.

5 TRIGGER IN BNC socket

Provides 5 V signals to control external devices (e.g., an external flashlight or a laser).

6 Measuring Light ML (Fluorescence measuring light socket)

Socket to which the cable belonging to the LEDs emitting modulated light in the emitter head (MCP-II-E) should be connected.

7 TRIGGER OUT BNC socket
Provides 5 V signals to control external devices (e.g., an external flashlamp or a laser).

8 STIRRER socket
Socket to connect the ribbon cable of the stirrer PHYTO-MS.

9 Control dial for the stirrer speed

10 Stirrer switch
In the ON position, the stirrer is on **standby**, the red LED of the switch is off. To activate stirring, check the "Stirrer on" checkbox of the "Multi-Color" tab of PamWin-4. This will also turn on the LED of the stirrer switch.

11 Power switch
The green LED of the switch turns on when the device is turned on. At the same time the ONLINE LED starts to flash; it will start to burn continuously after connection between PamWin-4 and the MULTI-COLOR-PAM-II has been established.

12 ONLINE LED and FUSE
Continuous light indicates connection between computer and MULTI-COLOR-PAM-II. The fuse holder contains a 3.15 Ampere slow-blow fuse.

13 CHARGE socket [also POWER SUPPLY socket] and LED
Socket to which the external power supply for the MINI-PAM/L charging unit should be connected. The red LED remains dark (does not light up) in the absence of external power supply.

14 LED ARRAY socket

Socket for cable that is connected to the actinic light LEDs in the emitter head (MCP-II-E).

15 ST SUPPLY socket

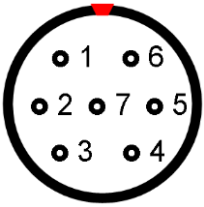
Socket for cable that is connected to the strong flashlamp LEDs in the ST emitter head (MCP-II-EDST).

16 DETECTOR 2

Socket to which the cable of the combi-detector (MCP-II-D2DST) should be connected.

17 EXT SENSOR (External Sensor Socket)

Socket for light sensors with spherical (US SQS/WB) or flat (US-MQS/WB) diffuser.



(1) +5 V
(2) Ground
(3) Analog input 1 (0 - 2.5 V)
(4) Analog input 2 (0 - 2.5 V)
(5) Analog input 3 (0 - 2.5 V)
(6) Trigger in (5 V)
(7) -5 V

18 DETECTOR 1

Socket to which the cable of the multi-color detector (MCP-II-D1) should be connected.

19 USB socket

Standard USB A socket for connection and communication with the computer.

3.1.2 MCP-II-E Multi-Color Emitter Head

Note: Pressure or strong force applied on the Perspex rod might destroy the LED array of the MCP-II-E Multi-Color Emitter Head located behind the rod

Light sources of the MULTI-COLOR-PAM-II consist of light emitting diodes (LEDs) which are densely arranged in a 10 x 10 mm area (Fig. 2, page 13). In total, the MCP-II-E head provides 6 spectrally different measuring light sources, and 5 spectrally different actinic light sources; the latter are complemented by white and far-red LEDs (for spectra see Fig. 3, page 14). To partially compensate for differences in brightness of the different types of light emission diodes, more LEDs are used when the intensity of the individual LEDs is low.

To collect LED radiation efficiently, the distance between LED array and Perspex rod is very short. Therefore, a considerable part of the LED emission enters the Perspex rod at low angles resulting in multiple reflections of radiation inside the Perspex rod creating a rather uniform light field at the opposite end of the rod.

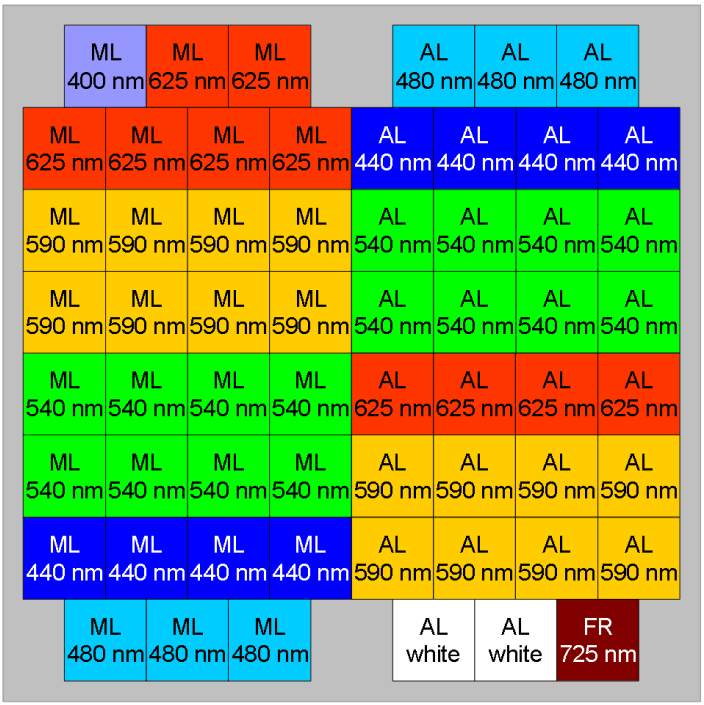


Fig. 2: Schematic top view of 1 x 1 cm LED board of MCP-II-E Multi-Color Emitter Head. AL, actinic light LED. ML, measuring light LED. FR: far-red light LED. Wavelengths indicate the emission peak (see Fig. 3, page 14).

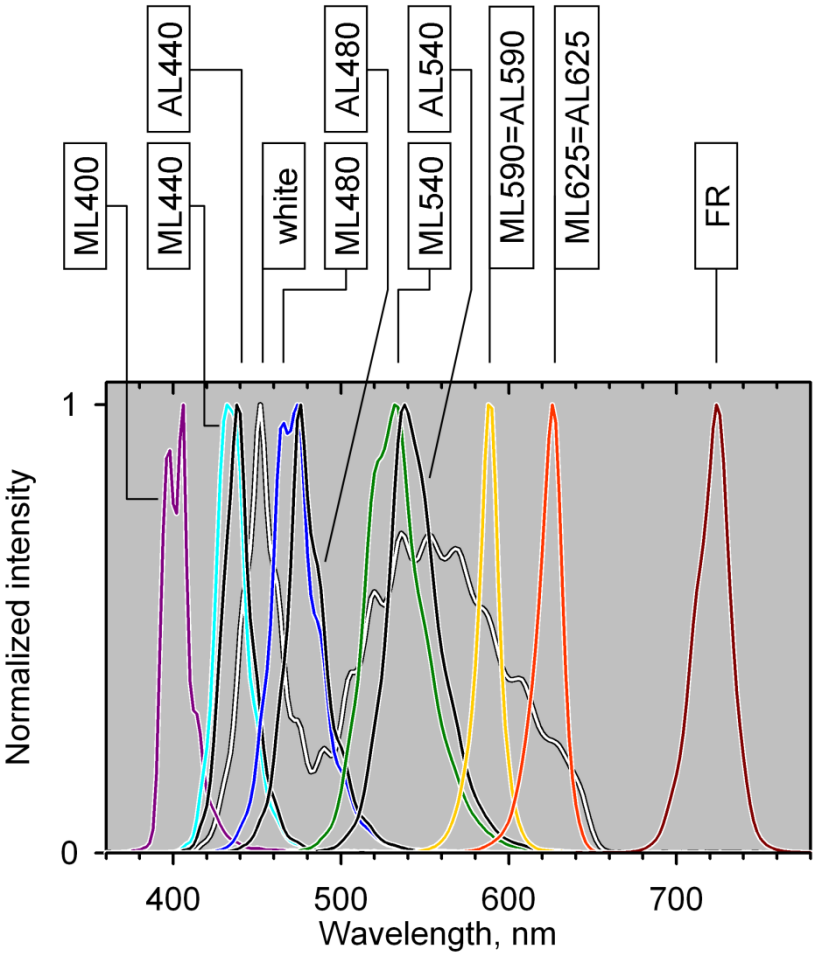


Fig. 3: Typical normalized emission spectra of MULTI-COLOR-PAM-II light sources. Spectra are not corrected for spectral response of the spectrometer. ML, modulated measuring light. AL, actinic light. FR, far-red light.

3.1.3 MCP-II-D1 Detector Head

A PIN photodiode with special pulse preamplifier for measuring fluorescence changes with maximum time resolution of 10 µs is used for signal detection in the MCP-II-D1 head.

The photodiode is protected from modulated and continuous excitation light by an RG 665 long pass filter which must **always** be inserted in the filter holder of the MCP-II-D1 detector. When the filter SP 710 is placed in the holder as well, fluorescence detection is additionally confined to wavelengths shorter than 710 nm (Fig. 4, page 15). The order of the two filters is not critical in this case.

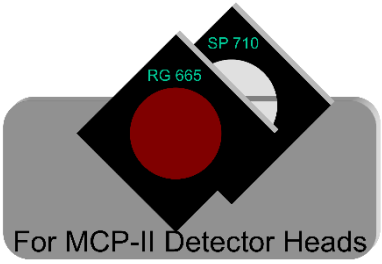


Fig. 4: Standard filters for MCP-II-D1 and MCP-II-D2DST Detector Heads

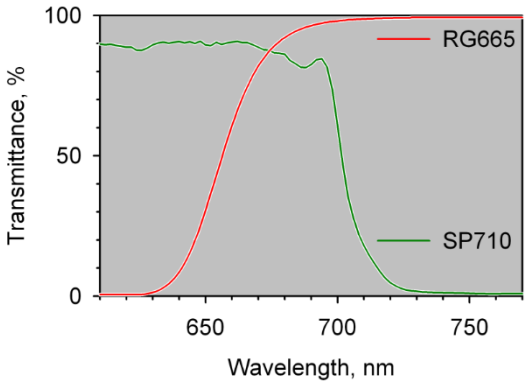


Fig. 5: Transmission spectra of RG 665 long pass and SP 710 short pass filters.

3.1.4 ED-101US/MD Optical Unit for Suspensions

In the optical unit ED-101US/MD (Fig. 6, page 16) a 10 x 10 mm cuvette forms the center surrounded by peripherally positioned ports for emitter and detector heads of the MULTI-COLOR-PAM-II. Perspex rods efficiently guide light from emitters to the sample, and from the sample to the detector. The figure shows a two head configuration, where two ports remain unused. Two

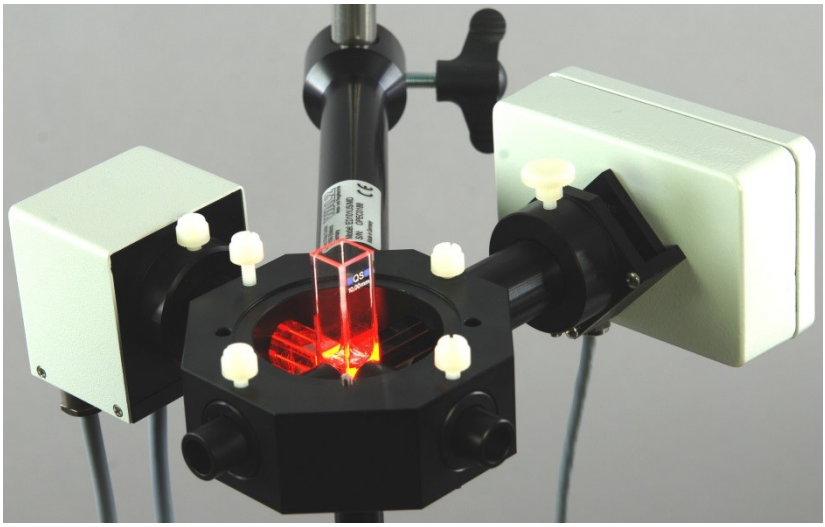


Fig. 6: ED-101US/MD Optical Unit with central quartz cuvette. Attached are the MCP-II-E Multi-Color Emitter Head, and the MCP-II-D1 Detector Head. To minimize background signals, the optical axes of the MCP-II-E and MCP-II-D1 heads are oriented at right angles to each other.

plugs are provided to close ports not occupied by emitter or detector heads. These plugs possess a light reflecting and a light trapping end. The reflecting end may be positioned opposite to the fluorescence detector to enhance the fluorescence signal;

the light-trapping part can be positioned opposite to the emitter to reduce effects of stray light on the signal.

The [two-part] standard cover of the unit has a pinhole through which chemicals can be added to the suspension using a syringe. To another port at the bottom of the ED-101US/MD unit the optional PHYTO-MS Magnetic Stirrer unit can be connected.

In the standard configuration, a sample volume of 1.25 mL just covers the area illuminated by the MCP-II-E emitter head. Larger cuvette volumes result in partial illumination of the sample and should be avoided, especially when the sample is being stirred.

3.1.5 Configurations

The figures below (Fig. 7, page 17 and Fig. 8, page 18) show the suspension and leaf configuration,

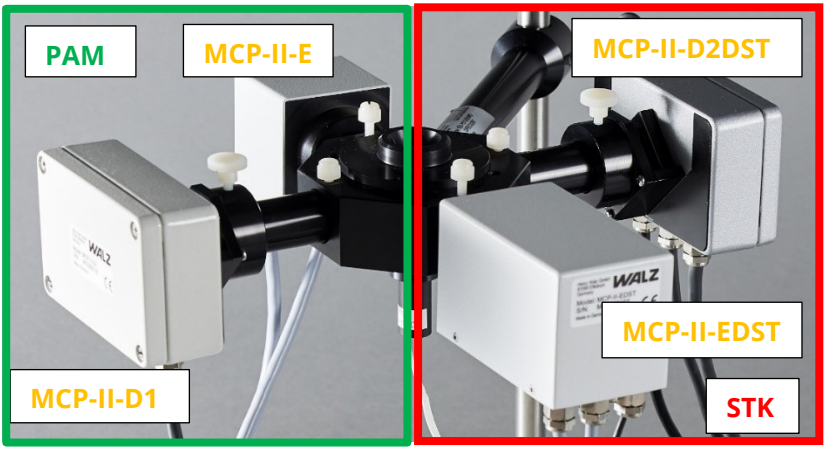


Fig. 7: the complete configuration of the MULTI-COLOR-PAM-II for suspension measurements. The PAM-units (in green) on the left-hand side of the photo and the STK-units (in red) on the right-hand side of the photo.

where the PAM and the STK-side can be used separately.

Fig. 8: the stand-alone configuration of the MCP-II-EDST (strong flash lamp) unit of the MULTI-COLOR-PAM-II for flash measurements on leaves. The bottom unit (also see the inset) is there to keep the leaf in place.

The two configurations are shown schematically below (Fig. 9, page 19), where the stand-alone configuration is connected to a gas chamber (3010-DUAL) to allow control of the gas conditions around the leaf. In addition, a pinhole is placed between the flashlamp and the sample to reduce the area from which fluorescence can be emitted and thereby the amount of fluorescence that is measured. Since, in the case of non-modulated fluorescence, the measured fluorescence intensity is a function of the light intensity, high flashlight intensities may lead to out-of-range fluorescence signals which may be brought on scale again by the use of optical pinholes.

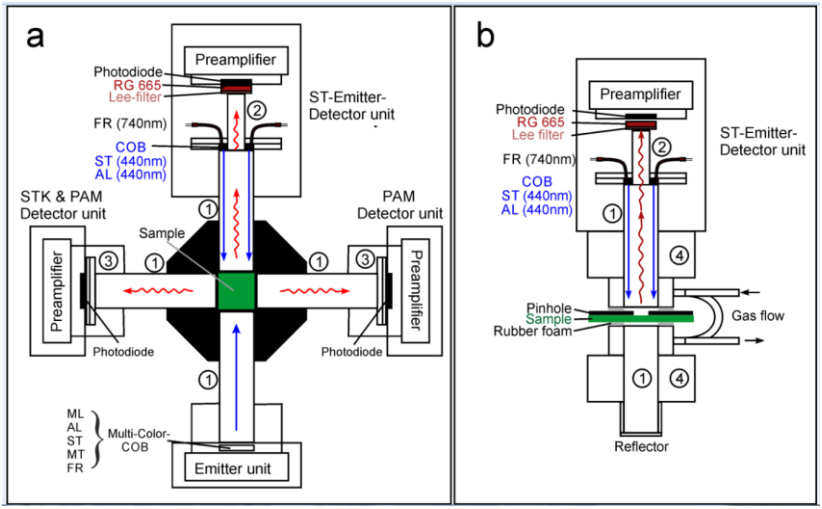


Fig. 9: schematic representations of the full configuration and the stand-alone configuration of the MCP-II-EDST head for leaf measurements.

Accessories

3.1.6 ED-101US/T Temperature Control Block for Cuvette

The Temperature Control Block is firmly screwed on the Optical Unit ED-101US/MD (Fig. 10, page 20). The temperature is controlled by an external water bath with pump circulator (not included). Efficient heat exchange is achieved by a spring-supported clamping mechanism providing close contact between cuvette and temperature block.

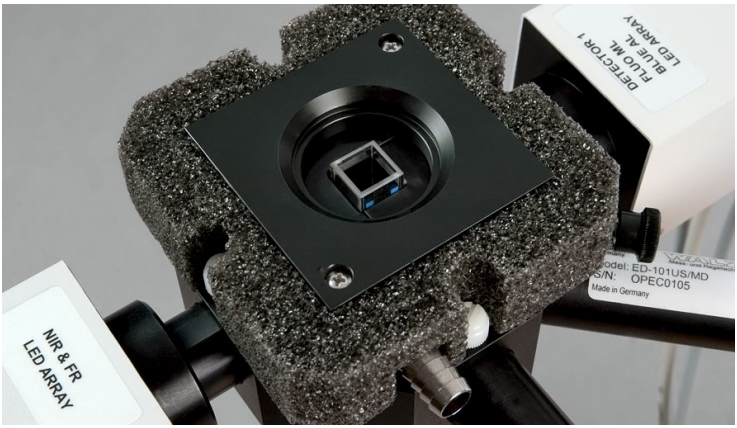


Fig. 10: ED-101US/T Temperature Control Block. The image shows how the temperature control block is screwed on the ED-101US/MD optical unit.

3.1.7 US-T Temperature Control Unit

The US-T unit is placed on top of the ED-101US/MD Optical Unit (Fig. 11, page 20). The device controls the temperature of the content of the cuvette making use of a Peltier-heat-transfer rod, which is immersed in the upper part of the sample suspension. Temperature is measured by a PT-100 sensor which is dipped in the cuvette. Temperatures of 15 K above and below ambient temperature can be achieved.

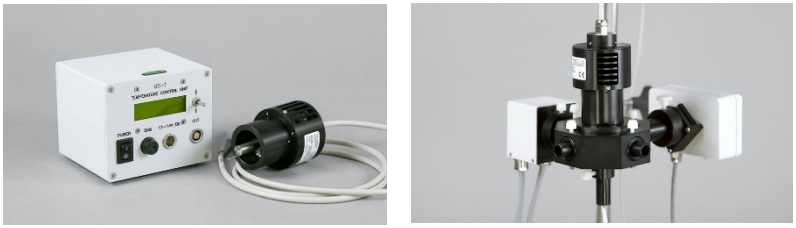


Fig. 11: US-T Temperature Control Unit. Left, the heat-transfer head with the Peltier element and the Control Unit; right, the connection between the head and the ED-101US/MD optical unit.

3.1.8 US-SQS/WB Spherical Micro Quantum Sensor

The US-SQS/WB device consists of a submersible spherical quantum sensor, US-SQS/L, plus a small amplifier unit, which is connected to the external sensor socket <EXT. SENSOR> of the central unit. The amplifier is tuned to yield correct PAR readings when a “Calibration factor for PAR” of 1.000 is selected in the PamWin-4 software. For stability of calibration, it is important to keep the diffuser (the whitish little ball) clean. Check calibration regularly by comparison with a standard quantum sensor. A calibration factor substantially greater than 1.000 indicates dirt-deposition on the diffuser. In this case, gently clean the diffuser using a cotton tip applicator moistened with some ethanol.

The 3.7 mm diffusing sphere of the US-SQS/L sensor picks up photosynthetically active radiation (PAR) with an angular response error of less than ± 5% under a -100° to 100° angle. Hence, the sensor is ideally suited to measure light conditions in the suspension cuvette where reflections and scattering result in randomization of the light direction. The determination of the incident light intensity with this sensor is essential for determination of the optical cross-section of PS II (Sigma(II)).

The US-SQS/WB has a hood/top to cover the cuvette inserted in the Optical Unit ED-101US/MD. This top has an inner part/cylinder to fit on top of another Walz instrument. To fit it on top of the ED-101US/MD unit this inner part has to be removed. Loosen/unscrew both screws on top of the hood with which the inner cylinder is connected to the top. When the two screws are loosened, the user is left with three parts. The inner cylinder is removed and the other two parts (a top disk and the outside cylinder) are screwed together again. To do that, the other two

holes (which are threaded) of the outer cylinder have to be used (turn top disk by 90° relative to the outer cylinder so that the two disk holes are aligned with the threaded holes of the hood and tighten the screws on top of the hood again).

3.1.9 PHYTO-MS Miniature Magnetic Stirrer

The stirrer has an inductive drive system, which makes its compact size possible, and which in turn allows its integration into the Optical Unit ED-101US/MD. The stirrer drives a miniature stir bar placed inside the cuvette in the optical unit. Stirrer action is software-controlled (**to turn the stirrer on, check <stirrer on> box in the Multi-Color tab**) and stirrer speed is adjusted manually by a control knob on the front panel of the MULTI-COLOR-PAM-II central unit.

3.1.10 MCP-BK Optical Unit for Leaf Measurements

For the MULTI-COLOR-PAM-I a leaf clip (MCP-BK) was developed that can still be used for e.g. the multi-color emitter (MCP-II-E) and detector (MCP-II-D1). The leaf clip is not only useful when working with leaves, but also for the measurement of seagrass and macro-algae. This leaf clip will be replaced in the near future by a clip that can accommodate three heads: the multi-color emitter and two detectors for fluorescence measurements at two different wavelengths.

The optional MCP-BK optical unit provides optical pathways defined by Perspex rods for efficient excitation and detection of fluorescence from solid flat samples such as leaves. The Perspex rod for fluorescence excitation points almost perpendicularly (12° angle of incidence) to the leaf and narrows from 10 x

10 mm at the emitter side to 6 x 6 mm at the measuring side (see Fig. 12, page 23). The Perspex rod for fluorescence detection has constant edge widths of 10 mm and collects fluorescence from a rather wide angle of incidence.

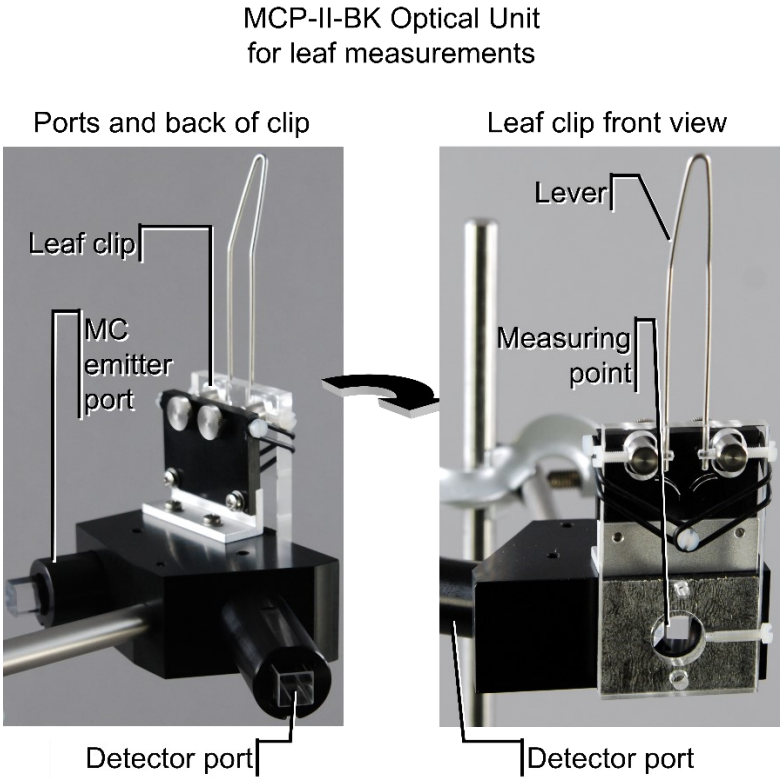


Fig. 12: MCP-BK Optical Unit. Left, image showing the ports for emitter and detector units. Right, view of leaf clip, rotated clockwise relative to left image.

Assembly

The MCP-BK Optical Unit is disassembled for shipping. To reassemble the clip, place the screw holes (denoted 1, see Fig.

13, page 24) of the MCP-BK Clip on top of the threads (2) of the MCP-BK Optics. On top of each screw hole, place one washer (3). Screw together MCP-BK Clip and MCP-BK Optics using the provided Phillips screws (3).

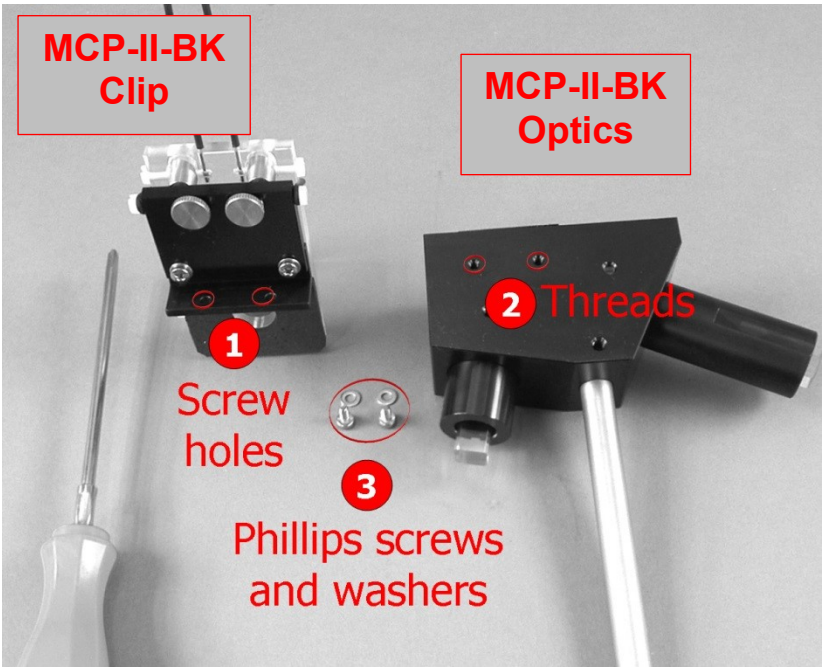


Fig. 13: Assembly of MCP-BK Optical Unit.

Field size and Illumination homogeneity

The MULTI-COLOR-PAM-II has been optimized for the measurement of dilute suspensions. For good and reproducible measurements of leaf/planar samples it is critical that illumination homogeneity is achieved. For the leaf chamber this was only possible by reducing the leaf/sample area that is measured

to 3.5 x 4.2 mm. To correct the measurement of the PAR-values for this area reduction, a correction factor of 1.620 (instead of 1.000) is required (see next section).

3.1.11 US-MQS/WB Mini Quantum Sensor

The US-MQS/WB combines the cosine response sensor, MQS-B, and a small amplifier unit specifically adjusted to this MQS-B sensor. When connected to the external sensor socket <EXT. SENSOR> of the central unit, the PamWin-4 software displays correct PAR readings for a “Calibration factor for PAR” of 1.620 (see previous section). The sensor fits to the opening of the measuring point or area of the MCP-II-BK Optical Unit.

Note: Do not force a plug into a [wrong] socket. When round sockets are red-dot marked, orient the plug in such a way that the red dot on the plug coincides with the red mark on the socket. Do not try to disconnect a plug by pulling at the cable. Disconnect round plugs by pulling at the rippled metal part.

3.2 Setup of Components

- Choose a smooth surface in a dry environment that is not directly exposed to sunlight or strong flickering light sources like fluorescent lamps.
- Assemble the stand with the wooden base plate, mount the ED-101US/MD Optical Unit for Suspensions or MCP-II-BK Optical Unit for Leaf Measurements on the stand, carefully attach the emitter and detector heads on

the optical unit. White plastic screws should be fastened finger-tight only.

- Insert the RG665 filter in the filter holder of the detector unit.*
- Without exerting force, optimize light paths by minimizing gaps in the light paths defined by the Perspex rods.
- Insert miniature magnetic stirrer unit (PHYTO-MS) in the bottom port.
- Connect peripheral devices to the central unit as shown in Fig. 14 (page 27).

*Standard, two filters are delivered with the MULTI-COLOR-PAM-II for each detector head: RG665 and SP710 (see Fig. 5 for transmission spectra). Inserting RG665, emitted light at wavelengths longer than 665 nm is detected. This means that both the PS II and PS I emission bands are measured. Inserting SP710 as well, reduces detection essentially to the PS II emission band. In leaves fluorescence is strongly reabsorbed at wavelengths shorter than 700 nm. According to Peterson et al. (2001) this means that fluorescence measured at wavelengths <700 nm is mainly emitted by PS IIs in chloroplasts near the leaf surface, whereas fluorescence detected at longer wavelengths (>710 nm) is dominated by fluorescence originating from deeper leaf layers. The strong reabsorption also means that the fluorescence intensity at wavelengths <700 nm is much weaker than the fluorescence detected at wavelengths longer than 710 nm (Pfündel 2021; Schreiber and Klughammer 2021). An additional 021 Gold Amber filter placed in front of the MCP-II-D2DST detector (no blue transmission) may further help to eliminate 440 nm light of the flashlamp from reaching the detector.

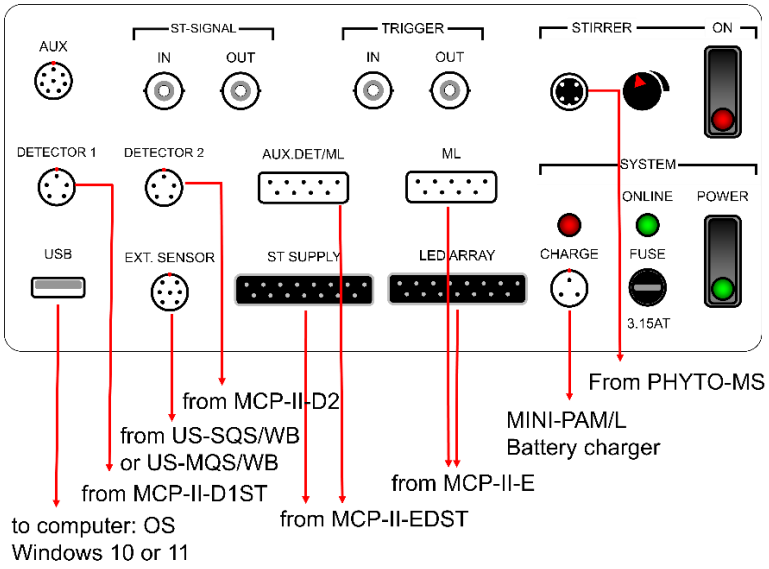


Fig. 14: Connections between central unit and peripheral components of the MULTI-COLOR-PAM-II.

3.3 PamWin-4 Software Installation

The MULTI-COLOR-PAM-II software PamWin-4 works, at the moment, under Windows 10/11.

a) The software needed for installation is delivered on a USB-stick placed in a little metal box. The USB-stick contains **only the setup file** (e.g. Pam-Win_4_02v_setup.exe) in the folder Software, and further a PDF of the actual manual.

Close all programs, double click on the setup file, and follow instructions. The setup routine will create in the C:\Program Files (x86) directory the folder “PamWin Folder” containing the PAM_WIN.exe program and accessory files to operate the MULTI-COLOR-PAM-II, the MULTI-COLOR-PAM-I and the PAM-2500. Further, an icon representing a shortcut, standard named Pam_Win_4, is placed on the computer desktop, and a program group called “PamWin_4” is created in the program list of the start menu. The PamWin_4 program group contains shortcuts to PAM_WIN.exe and to the uninstall command for PamWin_4.

b) Connect MULTI-COLOR-PAM-II to a computer via a USB-cable, connect the instrument to the mains (the MULTI-COLOR-PAM-II does not have a battery), switch the Power on and, with a minimum delay of about 5 s, start the PamWin-4 software by clicking on the “Pam_Win_4” icon. If the fluorometer is not switched on or connected, PamWin-4 will propose the offline mode of operation (VIEW-mode). To view and analyze data acquired by the MULTI-COLOR-PAM-II, select “MC-

PAM II" in the "Select Instrument" popup window (Fig. 15, page 29).

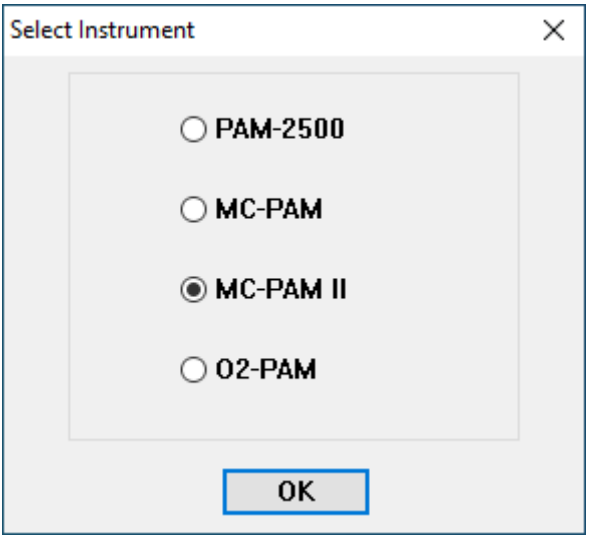
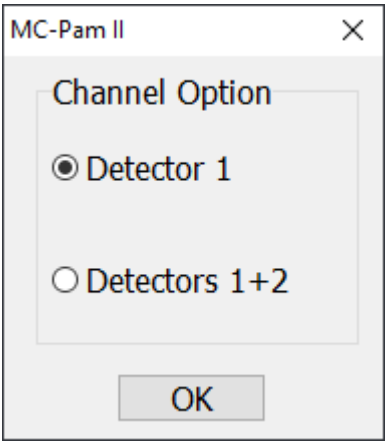


Fig. 15: Offline menu for PamWin-4 instruments

If the instrument is switched on and connected to the computer another popup window appears.



The user has to choose if the software should record data from one or two detectors (one or two channels)

3.4 Software Update.

The PamWin-4 software is continuously improved. Therefore, check regularly on the Walz website to see if a newer version of the software is available than that delivered with your instrument. PamWin-4 software for the MULTI-COLOR-PAM-II is available at:

https://www.walz.com/products/chl_p700/multi-color-pam/downloads.html

3.5 Firmware Update.

After a software update often a firmware update is needed. If there is a mismatch, the software will prompt the user to update the firmware. Firmware is the software residing on instrument RISC processors. The MULTI-COLOR-PAM-II contains 2 consecutively programmable processors: RISC and TINY, a tiny RISC processor. The Firmware update can be run by clicking the MC-PAM-Update file in the “PamWin Folder” and then selecting the file MC-PAM.bin.

Note: Stable power supply during firmware update is critical. Power failure can lead to a malfunction of the processors, which requires re-programming at Walz.

4 SP-Analysis

The PamWin-4 software offers two modes of online operation, i.e. in <MEASURE-mode>: <SP-Analysis> and <Fast Acquisition>. In <MEASURE-mode> only the data of the active record are accessible for analysis. For offline use there is <VIEW-mode>, which gives access to all data recorded, including the fast kinetics data of the saturation pulses, and provides various tools for data analysis and file management.

<SP-Analysis> mode can be used for classical saturation pulse analyses and routines for medium length to long experiments like dark-to-light induction (+ light-to-dark recovery) curves or light response curves. The emphasis of the <Fast Acquisition> mode is on the recording of kinetic changes controlled by fast trigger files (see section 5.2 and beyond). Fast trigger files allow rapid light source changes that one cannot achieve manually. With the exception of F_0 and F_M determinations and calculation of F_V/F_M, in <Fast Acquisition> mode fluorescence ratio parameters are not automatically determined.

4.1 Online Help (F1 or @)

All program levels of the PamWin-4 software offer online help texts with information on essentially all active user surface elements. To access the online Help-texts:

- Move mouse cursor on the window element of interest and wait until a small tag (“Tooltip”) appears.
- A “Tooltip” remains visible for 2.5 seconds. During this interval, pressing the **F1** or **@** key results in the display of the associated help text.

- In some cases, extensive help texts can be accessed via special <Help> buttons.

4.2 Peripheral Bars

The “General Settings” window is the first to appear after program start. The title, menu, side, and bottom bars shown in Fig. 16 are not related to the window “General Settings”; they are always present. Properties and functions of these peripheral panels will be described in this Chapter.

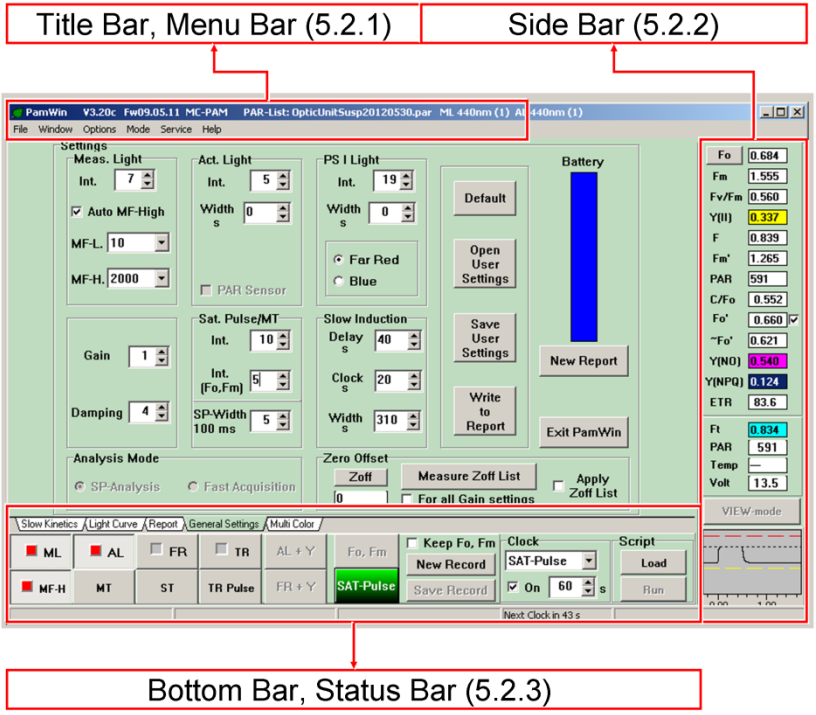
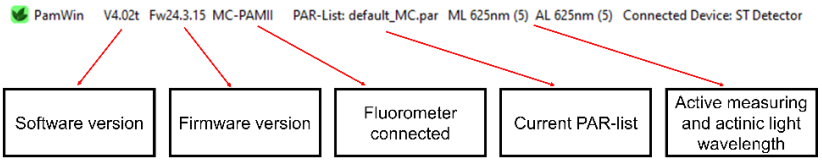


Fig. 16: Peripheral bars.

4.2.1 Title Bar, Menu Bar

Information provided by the title bar is sketched below:



The Control Unit of the MULTI-COLOR-PAM-II detects which devices/heads are connected, which is the last item on the title bar.

The menu bar of the “General Settings” window contains 6 drop-down menus:

File	Window	Options	Mode	Service	Help

The list of items in the File menu varies depending on the window chosen. For example, the <Print Graph> command is only available for the “Slow Kinetics” and “Light Curve” windows.

File Menu

New Report

File menu
New Report
Import Old Report
Load Script
Run Script
Printer Setup
Print Graph
Exit

This command creates a new Report and can also be executed via the <New Report> button in the Report window.

A Report can contain slow and fast fluorescence kinetics, and comments added by the user. The Report file name is automatically created using the format YYYYMMDD_# (Y: year, M: month, D: day, #: xth Report of that day).

For example, automatic naming would produce “20250317_3” for the third Report started on March 17, 2025. The Report “20250317_3” is stored in the directory named: C:\Program Files (x86)\PamWin Folder\Data_MCII\Report\20250317_3 which contains the Report data file 20250317_3.RPT3 together with fluorescence data and comments.

Modifications of a Report name are possible, but the name of the Report directory must be identical to that of the Report file.

Import Old Report

The command allows the import of data that have been saved in the past in the older Report file format *.RPT.

Load Script

This menu item is also accessible via the <Load> button in the bottom bar. The first time it is executed after program start, <Load Script> opens the PamWin-4 default directory for script files (C:\Program Files (x86)\PamWin Folder\Script Files) from which files can be imported by double-click on one of the file names in the list, or by single-click on the file name followed by the **Open** or <Enter> command. From there the user can navigate as well to other directories where script files may be stored. Once the script file is loaded the script file window should be closed by clicking on the ‘U-turn arrow’ icon or on <x> in the top right corner of Fig. 17**Fehler! Verweisquelle konnte nicht gefunden werden.**. Alternatively, the user can go to the script file directory by first clicking on <Load> and then clicking on (🗁) in the top left-hand side of Fig. 17. This way also a new script file can be started by closing the default script directory again by clicking on **Cancel** followed by entering the new file name. Clicking a second time on <Load Script>, opens the active script file in the window for ‘script file editing and script

file management' (Fig. 17). In that case it is also possible to start a new script by clicking:  which will clear the script window.

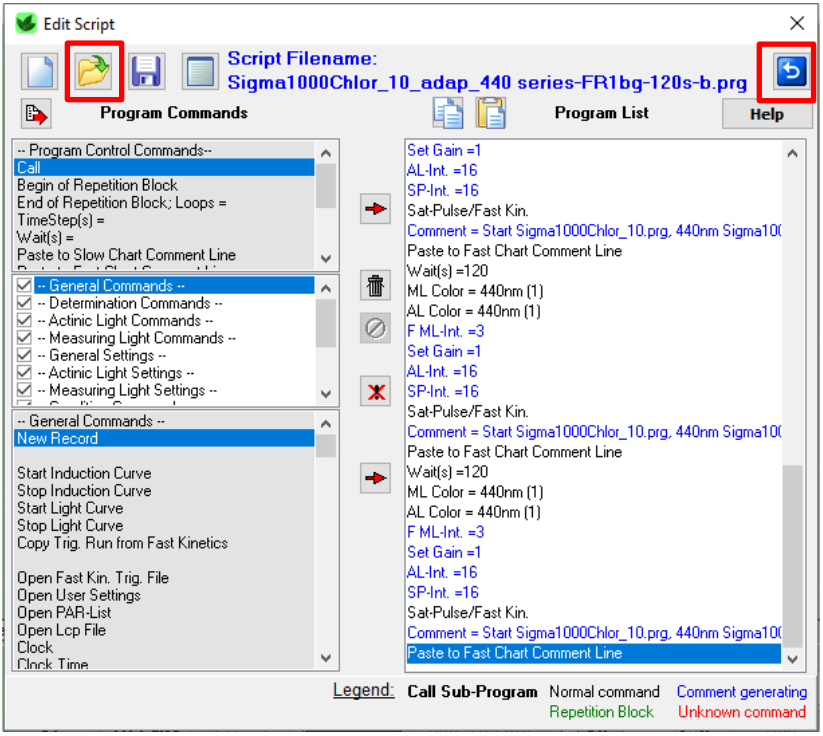


Fig. 17: Window for script file editing and script file management.

Run Script

Executes the presently loaded (active) script file. The command can also be triggered by the <Run> button in the Bottom-bar.

Printer Setup

Opens the Windows printer dialogue.

Print Graph (only Slow Kinetics and Light Curve windows)

Sends a black and white version of the current graph to the selected printer.

Print Report (only Report window)

Prints the current Report on the selected printer.

Exit

Saves current Report and kinetic data, writes current settings in a configuration file (INI File), and shuts down the PamWin-4 software. The same command can be triggered by the <Exit PamWin> button (General Settings, Fig. 16) and by the Close button: <x> in the upper right corner of all PamWin-4 windows.

Window Menu

The Window menu lists all windows currently available. The menu items vary depending on the acquisition mode selected (<SP-Analysis> or <Fast Acquisition>).

SP-Analysis	Fast Acquisition
Slow Kinetics	Fast Kinetics
Light Curve	Fast Settings
Report	Report
General Settings	General Settings
Multi Color	Multi Color
ST Kinetics	ST Kinetics
ST Settings	ST Settings

Only the first two tabs: Slow Kinetics and Light Curve respectively Fast Kinetics and Fast Settings are sensitive to the switch between SP-analysis and Fast Acquisition.

“General Settings”, “Report” and “Multi Color” are three PAM-specific windows shared between <SP-analysis> and <Fast Acquisition>.

The last two tabs are also insensitive to the switch, because they are STK-specific.

Options Menu

L[ight] Curve Fit Parameters

Options Menu
L Curve Fit Parameters
Light calibration
Temperature calibration
ETR Factor
AL Current/PAR Lists
FR Duty Cycle
FR Light Source
Trigger STK
STK Auto Zero
View Fast Kinetics of
White SP always
Blue PS I is also act. Light
S+H Damping
Kinetics auto save
No auto comment
White Chart Background

This menu item is an alternative way to get access to the "Light Curve Fit" window, which displays values of four parameters:

The **F_V/F_M x ETR factor** is the uppermost value, where F_V/F_M corresponds to the maximum quantum yield of charge separation of PS II reaction centers, and the ETR factor is the fraction of light absorbed by PS II in the sample (default value for leaves is normally 0.84/2=0.42), with the assumption that excitation energy is equally distributed between photosystems I and II. For a single sample, "F_V/F_M x ETR factor" is close to alpha, the initial slope of the light response curve, because

$$\text{alpha} = \frac{\text{ETR}}{\text{PAR}} = \frac{\text{Y(II)} \cdot \text{ETR factor} \cdot \text{PAR}}{\text{PAR}} = \text{Y(II)} \cdot \text{ETR factor}$$

and Y(II) approaches F_V/F_M at very low PAR.

Alpha, ETRmax and I_k are so-called cardinal points of a light response curve (see below). These parameters are obtained by fitting a theoretical equation to the measured data. PamWin-4 offers two different model equations for light response curves:

Model EP. Eilers PHC, Peeters JCH (1988) A model for the relationship between light intensity and the rate of photosynthesis in phytoplankton. Ecol Model 42: 199-215

Model Platt et al. Platt T, Gallegos CL, Harrison WG (1980) Photoinhibition of photosynthesis in natural assemblages of marine phytoplankton. J Mar Res 38: 687-701

The MULTI-COLOR-PAM-II allows the presentation of light-response curves on the basis of the quantum flux density of illumination, PAR (in units of µmol photons/(m^2·s)), or on the basis of PAR(II) which is the PAR absorbed by PS II in units of quanta/(PS II·s). When the independent variable is PAR, the units of the dependent variable, ETR, are µmol electrons/(m^2·s). For PAR(II) as the independent variable, the dependent variable ETR(II) has the units µmol electrons/(PS II·s)). The units of two of the three cardinal points change when the type of X and Y-axis data are changed.

The equation "PAR(II) = Sigma(II)·L·PAR" relates PAR to PAR(II). In the latter equation, Sigma(II) is the wavelength-dependent functional absorption cross-section of PS II, and L stands for the constant of Avogadro. Sigma(II) of a sample can be determined by analysis of the O-I_1 fluorescence rise kinetics in the fast acquisition mode (Section 5.3, page 112).

[1 µmol quanta m^{-2} s^{-1} corresponds to 10^{-6} x 6.022 x 10^{23} quanta m^{-2} s^{-1}, which is equivalent to 6.022 x 10^{-4} quanta nm^{-2} ms^{-1} (as 1 m^2 corresponds to 10^{18} nm^2)]

The three cardinal points of a light response curve are:

alpha Initial slope of light response curve which is related to the maximum efficiency of PS II electron transport. Unit: electrons/quanta

ETRmax (or ETR(II)max)

Maximum electron transport rate.
Unit: µmol electrons/(m^2·s) (or electrons/(PS II·s))

I_k (or I_k (II))

PAR (or PAR(II)) at the point of intersection between the ETRmax line and the extrapolated initial slope. Computationally, I_k is ETRmax/alpha. The I_k may be considered as the PAR (or PAR(II)) at which light-saturation of photosynthesis sets in.
Unit: µmol quanta/(m^2·s) (or quanta/(PS II·s))

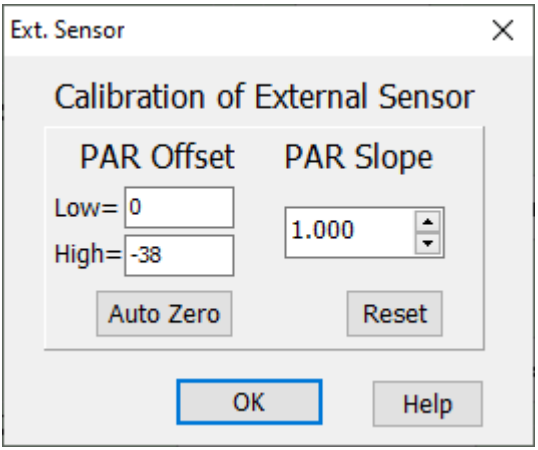


Fig. 18: Calibration of external sensor window

Light Calibration

For normal operation of the Walz quantum sensors (the spherical US-SQS/WB or the cosine-response type US-MQS/WB), the default calibration factor for PAR of 1.000 applies. For the spherical US-SQS/WB the factor 1.000 applies only to measurements made in water. In air the sensor is 1.7 times more sensitive and for air measurements this factor has to be entered.

The certificate of calibration that comes with the sensor gives the needed details. For the calibration factor of other sensors see the documentation that comes with these sensors. Valid factors are in the range of 0.200 to 5.000. For reasons of homogeneity of illumination, the measured area of the leaf clip MCP-BK of the MULTI-COLOR-PAM(-II) was reduced in size. Therefore, a calibration factor of 1.620 is required (see section 3.1.10).

Light Offset

The US-SQS/WB and the US-MQS/WB quantum sensors run within two ranges of signal amplification. For both ranges the **Auto Zero** function establishes automatically the correct offset values. Determination of light offset requires that the PAR sensor is kept in the dark. Also, offset values can be entered manually. However, in PamWin-4 this Options menu-item has been removed. It was integrated in the Light Calibration popup menu (Fig. 18).

Temperature Offset

<Temperature Offset> adjusts the offset value of an external temperature sensor (offset range is -30 to +30). Also, the Temperature Offset menu item of PamWin-4 was integrated in the Temperature Calibration popup menu in PamWin-4 and removed as an individual item from the Options menu.

ETR Factor

The ETR factor corresponds to the fraction of incident photons absorbed by PS II pigments. The factor is required for the estimation of electron transport rates based on the effective quantum yield of PS II, Y(II). For leaves, the default value for the ETR factor is 0.84/2. The numerator 0.84 corresponds to the average fraction of absorbed light in the visible range (400-700 nm) of

many green leaves, the denominator 2 assumes that absorbed light is equally distributed between PS I and PS II. However, even for leaves deviations from 2 may be observed depending on the light intensity the plant was acclimated to. For suspensions, the leaf ETR factor does not apply, as light absorption depends strongly on cell density and light color. Furthermore, PS I/PS II absorption ratios may vary substantially between different types of algae and cyanobacteria.

In dilute suspensions, electron transport rates per PS II can be determined according to Schreiber *et al.* (2011)*.

*https://www.walz.com/files/downloads/pan/PAN11001.pdf on the walz.com website

by:

(1) Deriving PS II specific absorption, Sigma(II), from the O-I_1 fluorescence rise kinetics of the dark-acclimated sample.

(2) Calculating the rate of quantum absorption by PS II, PAR(II), from incident PAR and Sigma(II).

PAR(II) = Sigma(II)·L·PAR, where L is Avogadro's constant ($6.022*10^{23}$), where Sigma(II) is expressed as nm^2 per PS II-absorbed quantum.

(3) Calculating PS II-specific ETR in the illuminated state by considering the lowering of the effective quantum yield, Y(II), with respect to the dark value, F_V/F_M:

$ETR(II) = PAR(II) \cdot Y(II)/(F_V/F_M)$

Step by step instruction for determination of ETR(II) via fast kinetics measurements of the so-called O-I_1 rise and for transformation of PAR into PAR(II) are given in PAN11001.pdf referred to above, and in the Help texts of PamWin-4 for Calc/O-I_1 rise,

Open/Close Sigma file and PAR(II) as well as Klughammer C and Schreiber U, Photosynth Res 123 (2015) 77-92. Further practical information on the Sigma(II) determination is given in the appendix.

AL Current/PAR Lists

A full set of PAR lists contains data on photosynthetically active radiation, PAR (µmol quanta·m^{-2}·s^{-1}), for all colors and intensity settings of actinic illuminations (AL) and multiple turn-over pulses (MT). Far-red intensities of the MCP-II-E emitter head depend on current values of the same “PS1 far-red” list.

Because most of the emission wavelengths of the far-red LEDs (Fig. 3 page 14) are outside the photosynthetically active spectral range (~400 nm - ~700 nm), the far-red list does not indicate PAR values. In the MULTI-COLOR-PAM-I the lowest intensity of far-red light (FR1) did not measurably excite PS II and was, therefore, ideal for background illumination to keep samples in State 1. The far-red light sources of the MULTI-COLOR-PAM-II seem to be somewhat stronger. Using the FR Duty Cycle option (see below) the effective far-red light intensity can be further reduced to 10% of FR1. Measuring the period-4 oscillations with and without FR allows the user to test if FR-background light affects the redox composition of the PS II donor side and if yes, how strongly (shifting of the oscillations to the left, damping of the amplitudes of the oscillations).

Default PAR lists are provided for setups of suspension and leaf measurements (default_leaf.par [MCP-II-BK] and default_MC.par, respectively). For the STK flashlamp the applied intensity is for suspension and leaf applications the same. In the PAR list window, clicking on the comment icon (🗎) opens information on the measuring protocol used to create a particular set of PAR lists. Sets of PAR lists can be saved as “*.par”

(click: ). A previously stored PAR file can be opened and used as the current set of PAR lists via the <folder icon> ().

Each PAR list consists of three columns. The first column, “Settings”, refers to the 20 “Meas. Light” or “Act. Light” intensities that can be set in the “General Settings” menu. The second column, “current”, provides a relative scale for the current flowing through the LEDs associated with the current PAR list. The LED current scale has an 8-bit resolution, that is, the number of 255 corresponds to 100% of the maximum allowed current. The third column, “PAR”, lists the PAR values measured with the corresponding LED current, except at setting “0” where the actinic current is set to zero, and the PAR values correspond to the measuring light intensity at setting 10 and 100 kHz modulation frequency.

When the PAR sensor is not connected to the MULTI-COLOR-PAM-II, or the PAR sensor is logged via the software (General Settings), the actual PAR is automatically computed as the sum of actinic PAR plus the PAR of the measuring light at the present intensity and frequency settings. The dependence of PAR on the settings is described by color-specific 3rd order polynoms used by the program.

Table 1: Schematic Representation of the PAR List Window.

default_MC.par


Open PAR file


Save PAR file


Show comment

Help
Show help text

AL color 1

[PAR Lists – Drop-Down Menu]

Setting	Current	PAR
0	0	577
1	1	7
20	255	2765

Default PAR-lists represent standard values which may deviate from the PAR values of your particular MULTI-COLOR-PAM-II. The user is responsible for updating the PAR-lists of his/her instrument, which is most conveniently done with a Walz PAR sensor (US-SQS/WB or US-MQS/WB) connected to the MULTI-COLOR-PAM-II using the <Measure PAR Lists> routine available in the “Multi Color” window.

PAR lists can be edited manually using other PAR sensors. Editing of PAR values requires highlighting the field to be edited by mouse click and typing in the new number. Make sure that the PAR value for measuring light (the PAR at setting “0”) is determined under standard conditions, that is, intensity setting 10 and 100 kHz modulation frequency. This means that for the example in given in Table 1 effective light intensity at 100 kHz and

ML Setting 10 is 577 µmol photons m^{-2} s^{-1}. This value is important for the determination of the ML-contribution to the effective light intensity in the case of for example O-I_1-I_2-P measurements. For further information on PAR lists, see the Help text under Options → AL Current/PAR Lists.

The PAR lists can also be used to change to set of available light intensities. For example, the AL list gives 20 current values between 0 and 255. The user may not like the available light intensities. Maybe, the user wants more choice at lower light intensities. It is possible to change the PAR-values manually, but that will not change the effective light intensities. To change the intensity at a particular setting, the "current" has to be changed. For the new set of current values subsequently the PAR values have to be determined. These values can also be estimated by making a plot of PAR versus Current and then determining the newly set PAR values by interpolation.

FR Duty Cycle

The FR Duty Cycle (Fig. 19) gives the percentage of the time the FR-light is on. By reducing the percentage from 100% – always on – to 10% – 10% of the time on – the effective far-red light intensity can be reduced by 90%. The FR-light intensity of the MULTI-COLOR-PAM-II can be quite strong and even FR1 may be already too strong for the use as background FR-light. The FR Duty Cycle allows the user to lower the effective FR-intensity further to a level suitable for background illumination.

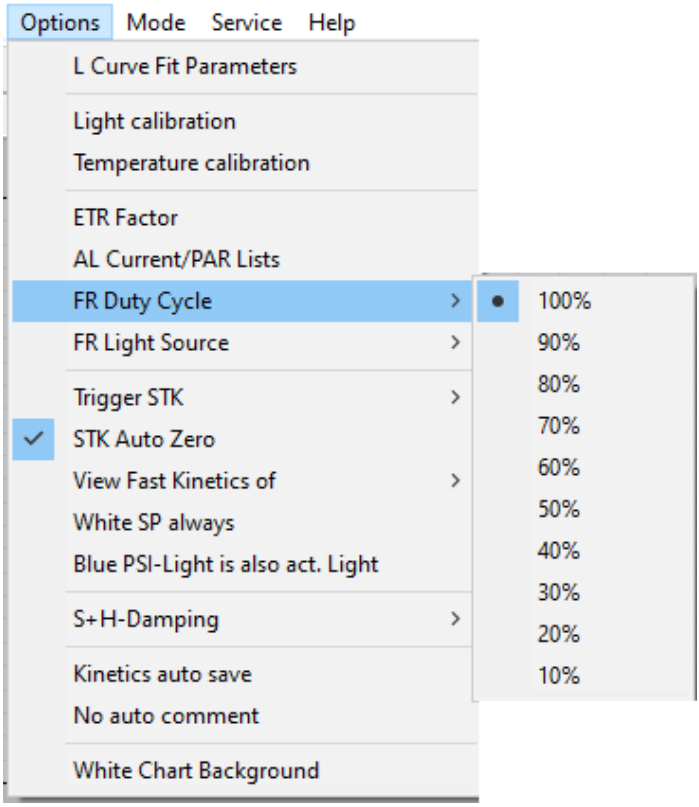


Fig. 19: The FR Duty Cycle-menu to modulate the effective FR-intensity by up to a factor 10

FR Light Source

If the complete version of the MULTI-COLOR-PAM-II is used, two FR-light sources are available (Fig. 20). The option ML emitter refers to the FR-source of the MCP-II-E and the option ST emitter refers to the MCP-II-EDST head. The user can check either option or both FR-sources at the same time.

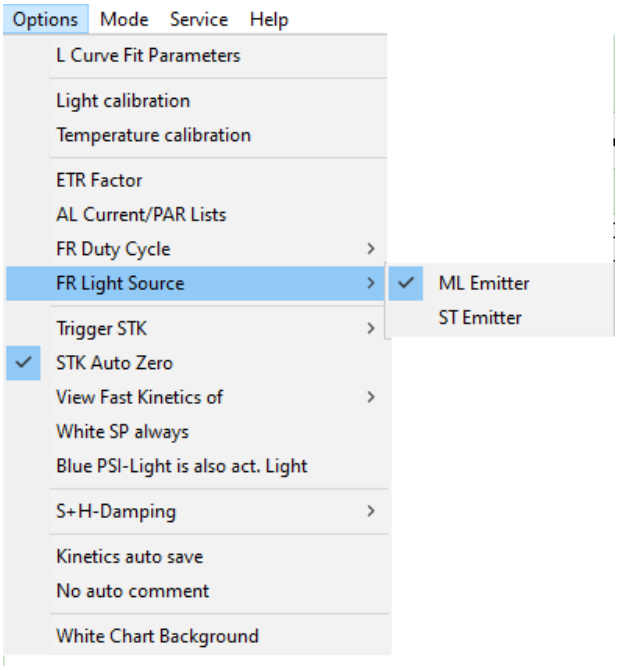


Fig. 20: FR Light Source selection: either the multi-color ML emitter, the ST emitter or both (checking both boxes).

Trigger STK

The Trigger STK menu item gives access to a submenu, where the user can choose between three options: None, before SP and after SP, i.e., a single turnover flash can be triggered either not, before a saturation pulse or after a saturation pulse.

STK autozero

Checking/selecting this option, means that the software will autozero the fluorescence signal before each flash. If this option is deselected, a slight baseline drift may occur (or maybe not).

View fast kinetics of

This menu item gives access to a submenu where the user can choose between F1, F2 and F1+F2 (Fig. 21). In the full configuration both detector heads record the fluorescence signal at the wavelength range determined by the filters placed in front of the detector. F1 refers to the fluorescence signal recorded by MCP-II-D1, F2 to the signal recorded by MCP-II-D2DST and if both heads are used the option F1+F2 can be chosen.

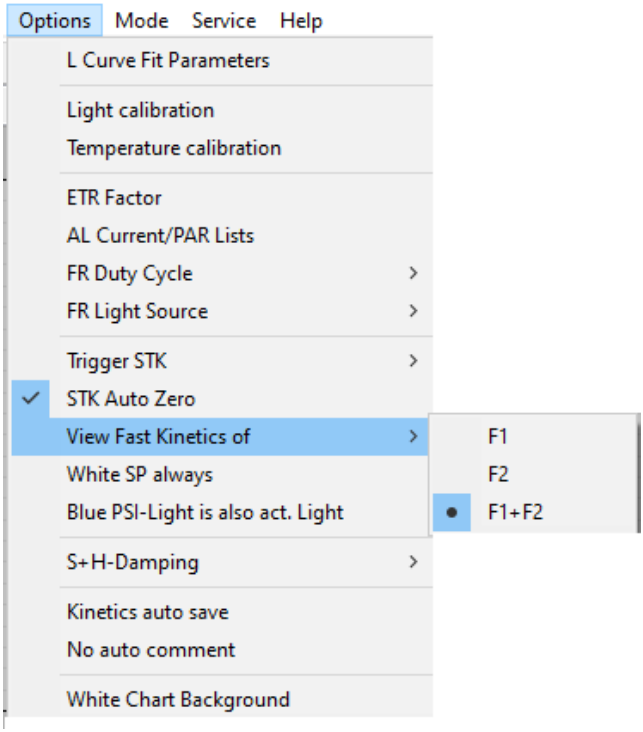


Fig. 21: In the "View Fast Kinetics of" menu a choice between the recording of one fluorescence channel (F1, MCP-II-D1), the other fluorescence channel (F2, MCP-II-D2DST), or both channels is given

White SP always

One reason to work with a MULTI-COLOR-PAM-II may be that it allows the use of different excitation wavelengths. <White SP always> means that the instrument will create a stronger saturation pulse by combining the different excitation wavelengths. The stronger SP means then that the wavelength dependence is lost.

Blue PS I-Light is also act. light

In the “General Settings” window the user can choose between FR and blue light as wavelength to preferentially excite PS I. If blue is chosen, the Options menu item “Blue PS I-Light is also act. light” can be activated to let the software know that this blue light should also be considered as actinic light.

S+H-Damping

<S+H-Damping> button allows the adjustment of the response characteristic of the “sample and hold” circuit of the system. This circuit keeps the original analog signal for a short time to allow digitization. When <Automatic> is chosen, damping setting D1 is used in the <Fast Kinetics> mode, and damping setting D2 in the <SP-Analysis> mode. The response time of the system depends not only on the damping setting (D1-D4) but also on the measuring frequency. Table 2 shows time constants for instrument response after a step input for all S+H damping settings and the three highest modulation frequencies.

Table 2: Time constants (τ) of system response for sample and hold damping settings D1 to D4, and various modulation frequencies of measuring light (50 to 200 kHz). $Damping_{General\ Settings}$ = 1

Damping	D1	D2	D3	D4
Frequency	τ	τ	τ	T
50 kHz	24 µs	252 µs	809 µs	1012 µs
100 kHz	18 µs	131 µs	424 µs	520 µs
200 kHz	15 µs	68 µs	213 µs	261 µs

Kinetics auto save

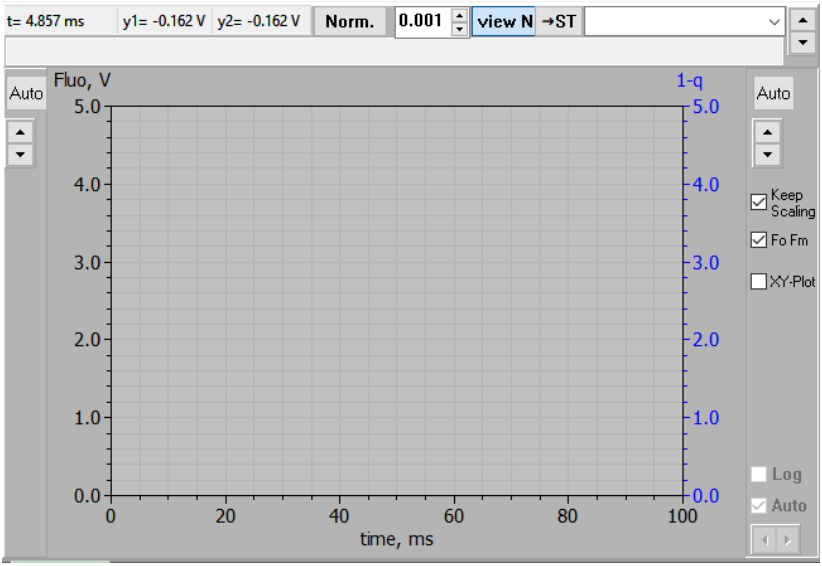
The <kinetics auto save> option is an option the user has to consider carefully. If this option is checked, each fast kinetic measurement is saved immediately following execution. This way the risk that data are lost is minimized, but there can be a high price. Let's assume the user carries out a double pulse experiment, where an O-I_1-I_2-P transient is measured and then a second O-I_1-I_2-P transient 1 s later. When the <Kinetics auto save> option is checked, the software will after the first measurement first save these data and how long that takes depends on the number of measurements already made earlier. Even if it is the first measurement, the effective delta t may be 2 s instead of 1 s and if already some measurements have been accumulated delta t may become, for example, 3 s instead of 1 s. In other words, in the case of a double pulse experiment it is critical that the <Kinetics auto save> option is not active. For fast flash measurements this is even more obvious.

No auto comment

the user can provide information on an experiment in the comment line. The software may provide automatically a comment. The user can decide that he or she does not want this and turn this off by checking this option.

Whitechart background

The PamWin-4 software offers the user two different graphical background options (Fig. 22). The default option is a grey background, but by checking the <White Chart Background> option, the grey background color is changed to white:



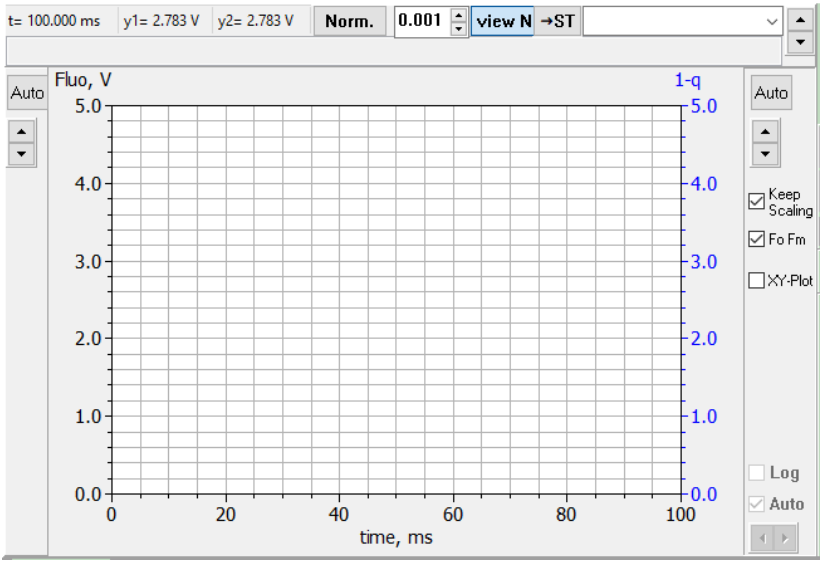


Fig. 22: Whitechart background option allows toggling between a grey background (default) shown at the top and a white background shown at the bottom.

Mode Menu

The two items of the menu, MEASURE and VIEW, are equivalent to the buttons **MEASURE-mode** / **VIEW-mode** in the "General Settings" window. The <VIEW-mode> button is inactive during (slow) fluorescence measurements.

Service Menu

Controller Service

See Section 3.5, page 30.

Read Firmware Version

Retrieves firmware version. The same information is also found in the title bar (see Section 0, page 32).

Trigger out with SP

When checked, a 5 Volt trigger pulse is applied to the AUX socket (see Section 3.1.1, page 8). The pulse length can be set in the “Pulse widths” window. To access this window, right-click on the <MT>, <ST> or <TR> button.

Check devices

When clicking on this menu item, a popup window will indicate the connected devices, for example, the ST detector. The same information is found in the title bar.

Help Menu

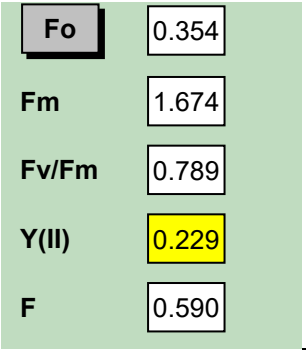
Tooltips

“Tooltips” (default state is: checked) enables help tags. Place the mouse on a function in the software, wait for the tag to show up type F1 or @ to read the help text associated with the tag.

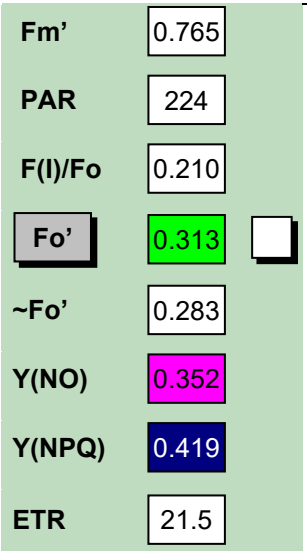
Info

Displays version number of current PamWin-4 software and the names of the authors of the software.

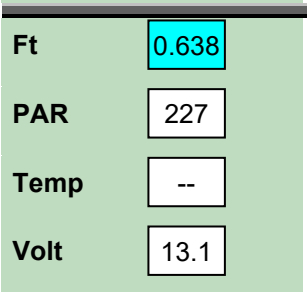
4.2.2 Side Bar



The upper 13 parameters (F_0 to ETR) are related to saturation pulse analysis. Only the F_0 or F_0' buttons can trigger measurements of these two parameters. The F_0 and F_M fluorescence levels should be determined on quasi-dark-acclimated samples (i.e., in the case of algae and cyanobacteria preferentially in presence of weak far-red background light: FR1); the F, F_M' and F_0' after light exposure of the same



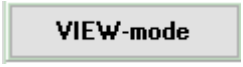
sample. The F_0' checkbox must be checked to activate the F_0' routine consisting of 5 s of far-red illumination and determination of F_0' as the minimum fluorescence during this period. The $\sim F_0'$ data are estimates for F_0' derived from F_0, F_M and F_M' according to Oxborough and Baker (Photosynth Res 54 (1997) 135-142). The $F(I)/F_0$ (before C/F_0) quantifies the contribution of a constant fluorescence component (e.g., phycocyanin and/or PS I fluorescence) to the total F_0 fluorescence. $F_V/F_M=(F_M-F_0)/F_M$ is the maximum and $Y(II)=(F_M'-F)/F_M'$ is the effective photochemical quantum yield of PS II. Detailed information on fluorescence ratio parameters is compiled in Section 9.2, page 168.



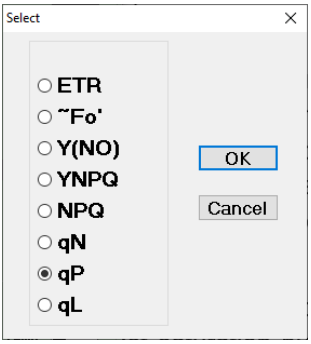
The lower section of the side bar (Ft to Volt) reports continuously on the Ft (current fluorescence level), PAR and the battery voltage values. In the standard setup of the MULTI-COLOR-PAM-II, a temperature sensor is not connected.

Note that values of the three yields: Y(II), Y(NO) and Y(NPQ) by default add up to 1 (Kramer et al. Photosynth Res 79 (2004) 209-218).

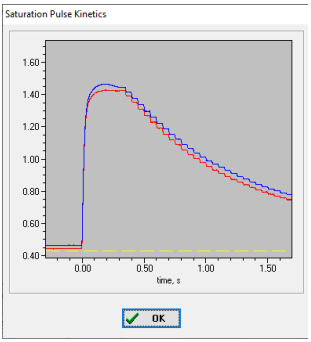
Clicking the <VIEW-mode> button interrupts communication with the



MULTI-COLOR-PAM-II and activates the “VIEW-mode” for off-line data analysis.



Below $F_0´$ in the side bar four parameters are displayed, but there are eight available. To open the menu shown on the right-hand side, double-click on one of the numerical fields of the four parameters below $F_0´$. Select a parameter and click OK.

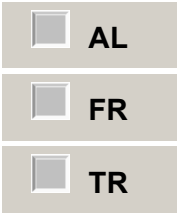


The fluorescence kinetics triggered by the last saturation pulse are displayed in a miniature panel at the bottom of the side bar. In the panel, the F_0 fluorescence level is indicated as a dashed yellow line. The panel shows a dual channel measurement. Double-click on the miniature panel to enlarge the graph. The y-axis of the enlarged graph is the original voltage scale of the fluorescence signal.

4.2.3 Bottom Bar

On/off button for modulated measuring light, ML. PAM fluorometers measure selectively the modulated fluorescence signal induced by ML. The red square indicates that ML is switched on. Likewise, a red square signifies the on-state for functions <MF-H> to <TR> (see below).

On/off button for high modulation frequency of measuring light <MF-H>. When <Auto MF-High> is active (General Settings window), 'switching on' of the actinic illumination, switches MF-H on automatically.



On/off button for actinic light <AL>, far-red light <FR> and 5 V trigger signal applied to AUX socket <TR> (see 3.1.1, page 8, for pin assignments). Intensity and duration of illumination is defined in the "General Settings" window. When the <Width> is set to zero, illumination must be switched off manually. More importantly, when <Width> is not set to zero the light source will be on for a limited time defined by <Width>. Far-red light predominantly excites PS I in higher plants and algae. In cyanobacteria, blue is frequently used for PS I-selective excitation, but also in that case the user can decide to use FR.

MT

ST

TR Pulse

Trigger for a multiple turnover pulse <MT> and single turnover flashes <ST>, as well as the trigger for 5V pulses applied to the AUX socket <TR Pulse>. Pointing the mouse cursor at one of the buttons displays the current pulse length. The color of MT pulses and ST flashes (unless <Multi Color ST> is checked in the "Multi Color" window) is identical to the color of the actinic light. It is wise to make "<Multi Color ST> checked" the default setting. MT intensity equates the saturation pulse (SP) intensity set in the "General Settings" window and ST intensity is always the maximum possible intensity but can be modulated by changing the ST width (but also, in practice, by choosing between the different AL colors, with <Multi Color ST> unchecked).

TR, MT, ST Pulse Widths

To edit pulse lengths, the "Pulse Widths" window has to be opened: click with the right-hand button of the mouse on one of the pulse buttons (<MT>, <ST> or <TR pulse>). The <length of trigger>, <multiple turnover> and <single turnover> pulses can be adjusted via drop-down lists. For <TR Pulse Width>, two drop-down lists are provided: one offers pulse lengths > 1 ms, the other covers shorter intervals and is available when the <Sub ms> box is checked. The settings for "pulse width" made here are active in the <SP-Analysis> mode. They do not affect the trigger pattern in the <Fast Acquisition> mode.

Additional options are available for single turnover flashes. To suppress false signals and detector overload caused by

fluorescence induced by the extremely strong single turnover flashes, the sample and hold circuit S+H is switched off during a flash. Thereafter, the sample and hold circuit will stay switched off for the time interval selected from the <Extended S+H off time> list when <S+H off> is checked. A prolonged off phase may be useful for removing artefacts in some applications, but at the same time leads to loss of kinetic resolution. [The MCP-II-D2DST and the detector in the MCP-II-EDST can cope with the extremely strong flash intensities and record the flash kinetics.]

Sequences of up to 20 single turnover flashes with interflash times <Widths, ms> 10-500 ms can be specified in the field <ST Pulse Sequence>. However, for the MCP-II-E flash lamp a time interval of more than 100 ms is needed to completely regenerate the flash intensity. Flash sequences are triggered in the <Fast Acquisition> mode by a mouse click on the <ST> button. When <Trigger Fast Kinetics after Sequence> is selected, the currently active trigger pattern (FTM-file) is executed after the pulse sequence with a delay corresponding to the <Width, ms> setting. Flash trains of much higher quality can be obtained with MCP-II-EDST- MCP-II-D2DST emitter-detector combination.

AL + Y

AL + F.K.

The <AL + Y> command first actinically illuminates the sample and then performs a saturation pulse analysis. Intensity and time interval of actinic illumination are defined in the “General Settings” window. Given that the standard AL width is in most cases 0 [light source remains on until e.g. the user turns it of] a width has to be set. For intervals < 3 s, the <AL + Y> command is unavailable. In the <Fast Acquisition> mode, <AL + Y> is

replaced by <AL + F.K.>, which executes the active trigger pattern (FTM-file) after actinic illumination.

FR + Y

FR + F.K.

The <FR + Y> command is in all respects similar to the <AL + Y> command except actinic is replaced by far-red pre-illumination (FR) with the same 3 s width limitation. The <SP-Analysis> or <Fast Acquisition> measuring modes determine if saturation pulse analysis or a fast kinetics measurement is performed after pre-illumination.

Fo, Fm

SAT-Pulse

The <F_0, F_M> button is used to determine the minimum fluorescence yield (F_0) of a quasi-dark-acclimated sample at low measuring light frequency prior to the application of a saturation pulse, which then induces the maximum fluorescence intensity (F_M). From these two parameters, the F_V/F_M (maximum photochemical quantum yield of PS II) is calculated. In algae and cyanobacteria “dark”-acclimation to weak far-red background light (FR1) is recommended to establish and maintain state 1, characterized by maximal values of F_V/F_M. These F_0 and F_M values are subsequently used as reference values for the determination of qN and NPQ.

The <Sat-Pulse> button also triggers a saturation pulse analysis. However, the software does not treat these F_0 and F_M values as reference values. In the case of light exposed

samples, the analysis starts with the determination of F, which is the fluorescence intensity at the actinic light intensity used; subsequently, F_M' is induced by a saturation pulse. If the <F_0' mode> box in the side bar is checked, the saturation pulse is followed by 5 s of illumination with PS I light (often far-red light) to determine F_0'. The F_M' and F fluorescence intensities suffice to calculate Y(II), the effective photochemical quantum yield of PS II.

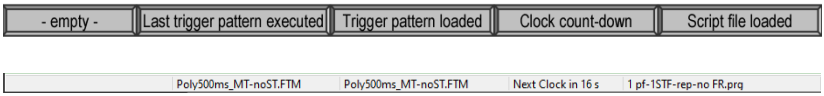
Script

Load

Run

The script file button <Load> opens C:\Program Files (86x)\PamWin Folder\Script Files, the default directory for PamWin-4 script files. If script files are stored elsewhere, it is possible to navigate from there to another directory. Pressing the <Cancel> button generates a blank script file window in which a new script file can be created. To execute a script file, close the Script file window and then click <Run>. Details on script files are outlined later (Chapter 6, page 129).

Status Bar



The status bar found completely at the bottom of the software window displays information on the loaded Fast Kinetics trigger patterns (FTM-files), Clock count-down (next clock trigger in .. s) if the clock-function is active, and Script files (the name of the active script file). The response measured with the last

executed FTM-file is displayed in <Fast Acquisition> mode in the Fast Kinetics window. The time indicated by the Clock count-down shows the time left until the next Clock-triggered event.

4.3 General Settings

In the General Settings window, changes in the settings of the different light sources and measuring conditions can be made:

- The modulation frequency of the measuring light is selected from a drop-down menu.
- All other hardware parameters can be set by mouse click via the pair of upward/downward arrows. Alternatively (and in most cases preferentially), a double-click on the number to be adjusted opens a small window in which the new setting (= number) can be typed.

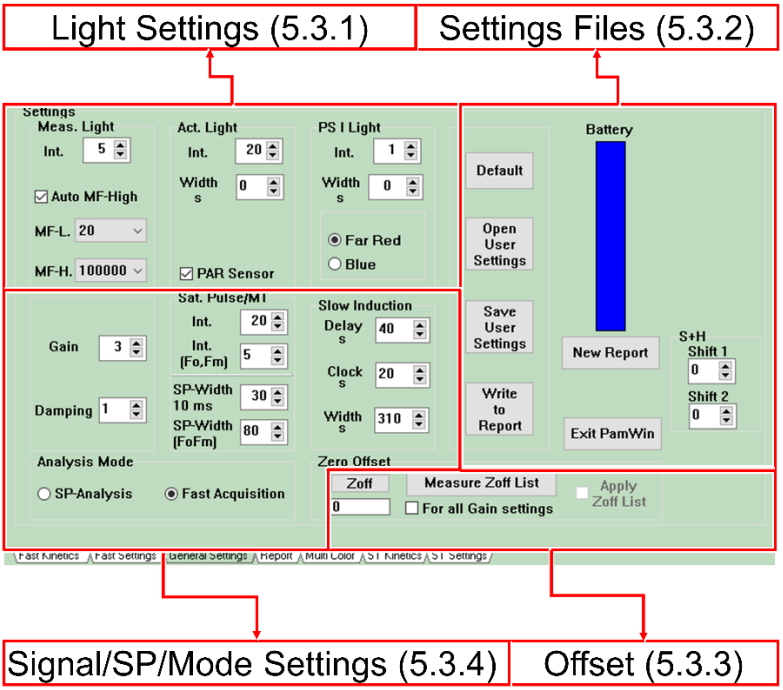


Fig. 23: General Settings.

4.3.1 Light Settings

Measuring light (Meas. Light)

This section discusses changes in intensity (Int., 20 levels), low pulse modulation frequency (MF-L., 10 to 5000 Hz) and high pulse modulation frequency (MF-H., 1 kHz to 100 kHz) of measuring light. Additionally, measuring light can be modulated at a frequency of 200 kHz (<MF-max>) in the <Fast Acquisition> mode. Factory values for the intensity of measuring light at intensity level 10 and frequency 100 kHz are given in the first line

of the respective PAR lists (see 4.2.1). When <Auto MF-high> is selected, the high pulse modulation frequency is always active when actinic light is on. Basically, increasing the modulation frequency improves time resolution and signal/noise ratio. At the same time, however, the actinic effect of the ML is increased. Therefore, low frequency measuring light must be used to correctly determine F_0 and F_0' fluorescence unless trigger files are used in which the high frequency measuring light is turned on less than 100 µs before the actinic light is turned on.

Actinic light (Act. Light)

Two parameters define actinic illumination: intensity (Int., 20 levels: 1 to 20) and duration (Width); allowed widths range from 1 to 900 s. If Width=0 is chosen, actinic light has to be switched on/off manually or via a script.

Actinic light for PS I excitation (PS I Light)

The PS I light is set as described above for actinic light. For F_0' detection, samples are illuminated for 5 seconds with PS I light. This 5 s interval is not affected by the “Width” set here.

Read PAR from Sensor (PAR Sensor)

When a PAR sensor is connected to the socket “EXT. SENSOR” of The MULTI-COLOR-PAM-II, the function <PAR Sensor> is automatically activated (corresponding box is checked = ☑). With the active PAR sensor, PAR lists are conveniently measured using the corresponding command in the “Multi Color” window of PamWin-4. Once PAR lists have been measured/established and saved, the <PAR Sensor> button can be unchecked; PAR values are then derived from the established PAR lists.

4.3.2 Settings File

Default

Open User Settings

Save User Settings

Write to Report

New Report

The term “settings” refers to all current settings of hardware parameters in PamWin-4 together. Most settings can be saved by the command <Save User Settings>. By default, setting files are stored in directory C:\Program Files\PamWin Folder\User Settings; their file name format is Filename.DEF. The Zero offset value is not stored. ETR factor, offset values for light and temperature sensors, and calibration factor for the light sensor are stored in a configuration file which is read at each start of PamWin-4. The command <Open User Settings> recalls stored settings including the factory default settings for work with suspensions “Walz_MC.DEF”. Factory default settings are also loaded by the <Default> command. The <Write to Report> command creates a line describing current settings in the Report file (see Table 3). <New Report> saves the current Report and associated kinetics and starts a new Report.

Battery

The blue bar indicator displays the charge status of the MULTI-COLOR-PAM-II battery. However, in the standard version of the MULTI-COLOR-PAM-II the battery has been replaced by condensators. In the absence of a battery the blue bar has lost its meaning.

Table 3: Documentation of Settings in the Report window

Date	Time	No.	ML	Temp.	PAR	F	Fm'	Y(II)	
06.12.11	15:41:48	FL 10	MI 3	G 2	AI 6	BI 4	FR/BL FR		
		FH 10000	Z 0	D 4	FI 1	SI 10	AL-Src 440 nm	ML-Src 440 nm	SI(Fm) 5
06.12.11	15:41:48	ML On	MFH Off	MFLog Off	AL Off	FR Off	BL Off		

Abbreviations used in Report

FL *Low frequency of measuring light, Hz*	**MI** *Measuring light intensity*	**G** *Signal amplification (Gain)*	**AI** *Actinic light intensity*	**BI** *Intensity of optional PS I lamp*	**FR/BL** *PS I light selected*		
FH *High frequency measuring light, Hz*	**Z** *Fluorescence offset (Zoff)*	**D** *Signal damping*	**FI** Far-red light intensity	**SI** *Saturation pulse intensity*	**AL-Src** *Actinic light source, nm*	**ML-Src** *Measuring light source, nm*	**SI(Fm)** *Saturation pulse intensity* for Fo,Fm determination

ML	**MFH**	**MFLog**	**AL**	**FR**	**BL**
On/Off Information on status of:					
Measuring light	*High frequency of measuring light*	*Logarithmic frequency decrease of measuring light (Fast settings)*	*Actinic light*	*Far-red light*	*Blue light from optional PS I lamp*

4.3.3 Offset

The <Zoff> (Zero offset) and <Measure Zoff List> commands determine background signals for subtraction from the total signal. Background signals must possess the modulation characteristics of measuring light to be recognized by the differential amplifier. These signals can arise from:

- Fluorescence from suspension media or detector filter excited by measuring light.
- Traces of modulated excitation light transmitted by the detector filter.
- Non-optical modulated "electronic noise".

In general, the background signals are different for the various colors of measuring light and increase almost linearly with measuring light intensity and gain factor.

Note: All measuring conditions for Zoff determination must be identical to those for sample investigation.

To determine the zero offset for experiments with suspensions, place a cuvette containing pure medium in the optical unit ED-101US/MD, and select one of the three options for zero offset determination (see Table 4, page 66).

Table 4: Three Levels of Zero Offset Determination for Fluorescence Measurements.

Command:	Zoff	Measure Zoff List	Measure Zoff List ☑ For all Gain Settings
Measuring Light Intensity	Current	All	All
Measuring Light Color	Current	All	All
Gain	Current	Current	All

The time required for the zero offset determinations increases with the number of measurements and is highest when all

possible settings are considered (<Measure Zoff List> + <For all Gain Settings>). For experiments with constant measuring light and gain, the simple <Zoff> command can be used. The <Zoff> command also allows entering manually a value for zero offset; in this way, the zero offset value can also be reset to zero.

The Zoff Lists are saved as Zero_Offset_Col.ini in the folder C:\Program Files (x86)\PamWin Folder\Data_MC.

Offset lists can be activated by checking <Apply Zoff List>. Then, the appropriate offset is subtracted from the measured signal. Offset subtraction is inactive/inactivated when the measuring light is switched off.

4.3.4 Signal/SP/Mode Settings

Gain

The setting <Gain> determines the amplification factor of the fluorescence signal but also of electronic noise. Therefore, improving the fluorescence signal by increasing measuring light intensity should be considered in case of low signal/noise ratios. Even very high measuring light intensities do not have much actinic effect at modulation frequencies of 100 Hz or lower. Increasing <Gain> also amplifies the background signals, which requires redetermination of the zero offset <Zoff> unless a complete background determination was performed.

Damping

“Damping” is a factor determining the system’s speed of response to fluorescence changes. The overall system response also depends on the setting of the sample and hold circuit damping and the modulation frequency of the measuring light. Table 5 (page 68) lists response times measured with sample

and hold damping set to D2 (default for slow kinetics when *automatic* sample and hold damping is selected), and 10 and 100 kHz pulse modulation frequency.

Table 5 shows that at 10 Hz modulation frequency, the response is independent of the damping. The sluggish response is determined by the low modulation frequency. At 100 kHz, the system response is affected at damping setting 5 and higher.

Table 5: Gain Factors and Time Constants at Two Modulation Frequencies and S+H-Damping = D2.

Gain setting	Gain factor	Damping setting	Time constant (τ) at 10 Hz modulation frequency	Time constant (τ) at 100 kHz modulation frequency
1	1.0	1	1210 ms	0.13 ms
2	2.0	2	1220 ms	0.13 ms
3	2.7	3	1180 ms	0.14 ms
4	3.4	4	1200 ms	0.18 ms
5	4.2	5	1040 ms	0.29 ms
6	5.1	6	1230 ms	0.60 ms
7	5.8	7	1210 ms	1.66 ms
8	7.0	8	1270 ms	4.76 ms
9	7.7			
10	8.6			

<Sat. Pulse>/<MT>

Saturation pulses <Sat. Pulse> are applied for quenching analyses, and multiple turnover pulses <MT> are typically used to induce fast fluorescence changes in the <Fast Acquisition> mode. The purpose of an SP is to induce F_M, whereas the

purpose of an MT can be to induce the O-I_1 rise or to reduce the PQ pool; reaching F_M is not necessarily goal. The intensity of both is adjusted in the <Int.> field. A special case is the intensity of the saturation pulse used for F_0, F_M determination which is adjusted in the <Int. (F_0,F_M)> field. The determination of F_M requires a much lower light intensity than the determination of F_M´. The software allows a differential treatment of the two cases. However, with the discovery of HIQ (Schreiber et al. Photosynth Res 142 (2019) 35-50) the user should take into account that a large difference in SP-intensity between the two cases may lead to a differential induction of HIQ and a small systematic underestimation of F_M´ compared to F_M.

The duration of saturation pulses is set by <SP-Width>. Pulse widths between 100 and 800 ms are available. The length of multiple turnover pulses is set in the Pulse Width window which opens by a right mouse click on the <MT>, <ST> or <TR pulse> buttons of the bottom bar.

Slow Induction

PamWin-4 defines the time course of long (slow) fluorescence induction curves by three parameters: (1) <Delay>, (2) <Clock>, and (3) <Width>. A slow induction curve is started by selection of <Ind.Curve> in the drop-down menu of the slow kinetics window, followed by pressing the <Start> button. The definitions of the three parameters are:

Delay, s determines the dark interval between the initial F_V/F_M determination and onset of actinic illumination. The default interval is 40 s which normally assures that the pre-illumination effect of the saturation pulse is relaxed. However, if a full re-oxidation of the photosynthetic electron transport chain is required, a 100 s dark time interval may be a better choice.

Table 6: Intervals between Saturation Pulses during Dark Recovery.

Pulse number	Time interval
1	15 s
2	18 s
3	22 s
4	25 s
5	31 s
6	37 s
7	44 s
8	53 s
9	64 s
10	76 s
11	92 s
12	110 s
13	132 s
14	158 s
	Σ 877 s (15 min)

Clock, s defines the interval between two consecutive saturation pulses during actinic illumination. The default interval is 20 s, which normally allows evaluating the kinetic changes of saturation pulse data in the light with negligible effects of the preceding saturation pulses.

Width, s is the total duration of the slow induction curve (time interval the actinic light is on). Width assumes only distinct values which are defined by

$$\mathbf{Width = (n \cdot Clock) + 10}$$

where n corresponds to the number of intervals between saturation pulses during actinic illumination. The extra 10 seconds result from 5 s prior to the first saturation pulse after start of

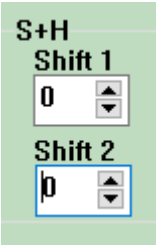
illumination plus 5 s at the end of illumination. The total experimental time is 15 minutes longer when monitoring of fluorescence recovery has been activated by selecting <Ind.+Rec.> in the drop-down menu of the slow kinetics window.

Table 6 (page 70) lists the roughly exponentially increasing intervals between pulses during the fluorescence recovery phase.

Exit PamWin

Saves current Report and kinetic data, writes current settings in a configuration file (INI file), and shuts down the PamWin-4 software.

S+H



The S+H shift 1 and shift 2 functions allow the user to fine tune the ML-signal detection. The two S+H-windows can be shifted slightly forward (positive number) or backward (negative number). In practice, the ML can be switched on and the two values can be adjusted in such a way that the signal becomes maximal.

Analysis Mode

Field for selection of <SP-Analysis> or <Fast Acquisition> modes. The two modes are shortly characterized in Table 7, page 72.

Table 7: Analysis Modes.

	Analysis Mode ⦿ SP-Analysis	⦿ Fast Acquisition
Saturation pulse analysis	Photochemical and non-photo-chemical fluorescence quenching	F_V/F_M determination
Type of kinetic measurements	Slow (seconds to hours)	Fast (µs to seconds)
Automatic rou-tines	Dark-light fluorescence induction and light response curves	Fast trigger files,
Script files	Yes	Yes
Time resolution	Low	High
Goal	Measurement extremes	Measurement kinetics

4.4 Slow Kinetics Window

This window displays slow fluorescence kinetics recorded in the <SP-Analysis> mode. The scaling of the graph can be modified in four ways as outlined in Fig. 24 (page 73).

- Zoom in by clicking with the left mouse key on one corner of the rectangular area to be magnified, keeping the key depressed move to the opposite corner of the rectangle, and release key.
- Zoom out by right mouse click anywhere on the chart.
- Shift y-axis by moving the mouse cursor vertically with the shift key depressed. Shift x-axis by moving mouse cursor horizontally with the control key depressed.

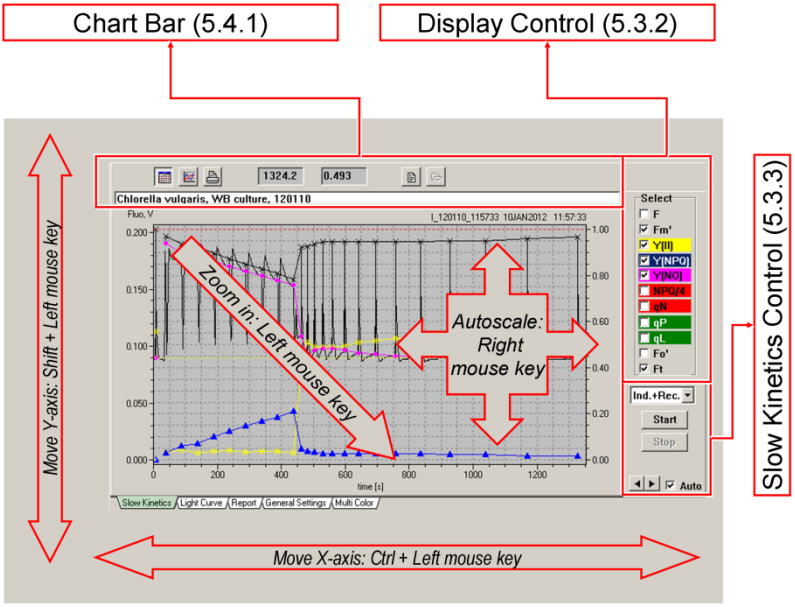


Fig. 24: Slow Kinetics Window.

4.4.1 Chart Bar

The chart bar displays the XY coordinates of the current pointer position and contains various buttons with the following functions:

On/off switch for grid display.

Zoom out: display of complete graph after zoom in. Equivalent to right mouse click on chart.

Prints a black-and-white copy of the graph for documentation.

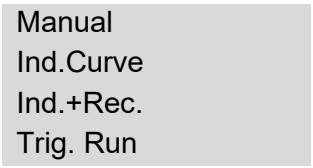
Displays notes and comments associated with current slow kinetics.

Opens a Slow Kinetics file for display. The icon is active only in <VIEW> mode.

4.4.2 Display Control

Eleven different fluorescence parameters can be selected for display. The display is controlled by checkboxes. Data points and background colors of the corresponding checkbox labels have identical colors.

4.4.3 Slow Kinetics Control



The “Slow Kinetics Control” (cf. Fig. 24, page 73) provides a drop-down menu consisting of 4 items: <Manual> permits complete manual control of the experiment. <Ind.Curve> corresponds to slow dark-light fluorescence induction curve performed according to the settings in “General Settings”. When <Ind.+Rec.> is selected, the induction curve is followed by a dark recovery phase. During recovery, saturation pulse analysis is conducted at increasing time intervals (see

Table 6, page 70).

The **Start** button triggers the selected type of slow kinetics, the **Stop** button terminates the experiment, which will, however end by itself when the protocol comes to an end.

Recordings of dark-to-light induction curves automatically start with determination of the F_0 and F_M levels. Then, the left voltage scale is scaled to fully display the fluorescence signal. All fluorescence ratio parameters are plotted using the right y-axis which ranges from 0 to 1. In case of the non-photochemical quenching, values of NPQ/4 are displayed because the original data can assume values above 1 (but normally do not exceed 4), which would exceed the latter y-axis range. Manual recordings can be carried out without initial F_0, F_M measurements. In this case, automatic scaling according to the F_M level does not occur.

The arrow keys in “Slow Kinetics Control” (Fig. 24, page 73) increase or decrease the x-axis range. Employing the arrow keys deactivates the <Auto> function which otherwise automatically adjusts the x-axis for full display of the fluorescence trace.

4.5 Light Curve Window

The Light Curve window (Fig. 25) provides the functional elements for control, display and analysis of light response curves. A light curve consists of a pre-programmed series of illuminations, normally with increasing intensities. At the end of each illumination step, a saturation pulse is applied for quenching analysis. The light curve chart plots fluorescence yields and saturation pulse data against light intensity. In parallel, data are plotted on a time axis in the Slow Kinetics window.

The PAR values used as x-values correspond to those of the active internal PAR list. It is not recommended to measure PAR online, as the sensor shades part of the sample. If a PAR sensor is connected, it should be removed from the sample and its reading disabled in the General Settings window by removing the checkmark from the PAR Sensor checkbox (below Act. Light).

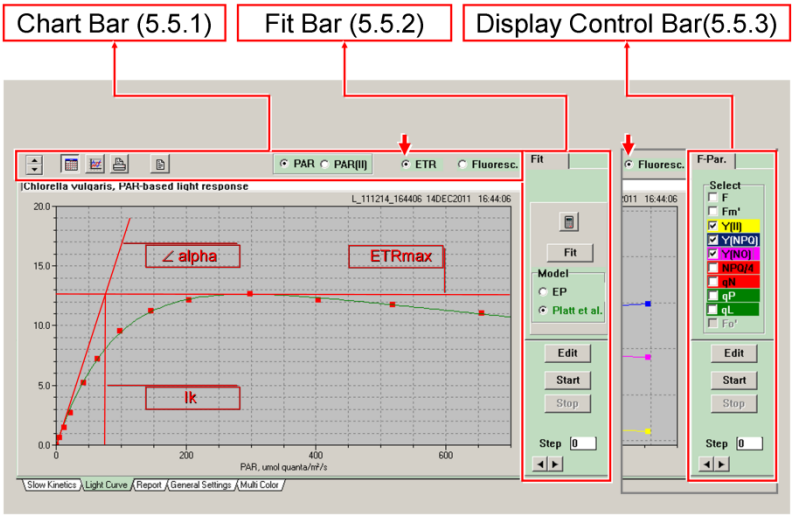


Fig. 25: Light Curve Window, when ETR is checked ("Fit Bar") or when Fluorescence is checked ("Display Control Bar").

4.5.1 Chart Bar

The left part of the chart bar provides 4 buttons already known from the Slow Kinetics window. In addition, vertical arrows permit changes in x-axis scaling. These arrows, together with the horizontal pair of arrows (bottom, right),

permit the scaling up or down of the initial part of the light curve. Auto scaling of both axes by the right mouse button functions as described for the Slow Kinetics window. Zooming in by moving the mouse cursor within the chart is not possible.

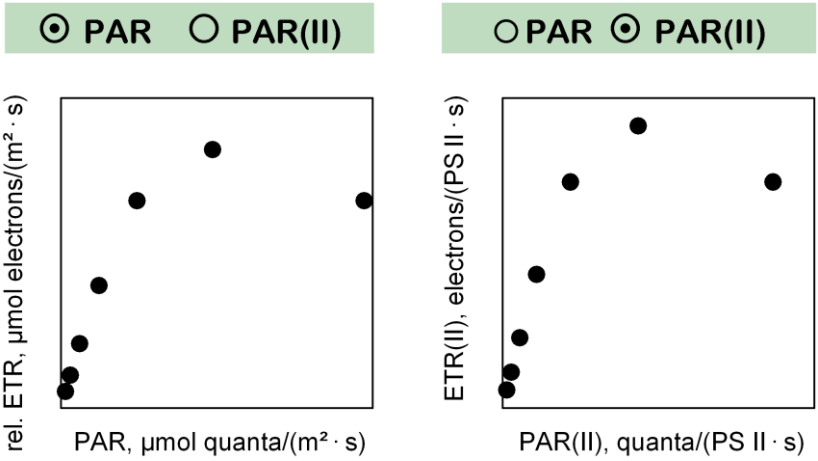


Fig. 26: Two Different Types of Light Curves.

Different types of y-axes can be selected. To view the relationship between relative photosynthetic rates and light intensities, select ”ETR” (electron transport rate). To plot various fluorescence levels ($F_0´$, F, and $F_M´$) or fluorescence ratio parameters as a function of the light intensity, choose “Fluoresc.”. If ETR is selected, the side bar provides tools to fit a theoretical function to data of a light response curve (“Fit Bar”). In the other case, the side bar controls display of fluorescence data (“Display Control Bar”).

Also, different x-axes are available (Fig. 26, page 77). If “PAR” is checked the x-axis units are quantum flux densities of incident radiation; when “PAR(II)” is selected, the x-axis is the PS II-specific quantum flux rate. In the latter case, ETR(II) in units of electrons per PS II per s is the dependent variable. ETR(II) is

calculated according to: ETR(II) = PAR(II)·Y(II)/(F_V/F_M), where Y(II) and F_V/F_M are the photochemical yields of PS II in the light-exposed and dark-acclimated state, respectively (see Chapter 9.1, page 166).

Values of PAR(II) correspond to PAR multiplied by the functional absorption cross-section of PS II, $Sigma(II)_\lambda$, and Avogadro's constant. For determination of $Sigma(II)_\lambda$, PamWin-4 provides a fitting routine which analyzes the fluorescence rise from the O to the I_1 level. This fitting routine will be reviewed in section "Fast Acquisition" (Chapter 5, page 92).

4.5.2 Fit Bar/Light Curve Edit

Buttons for the Edit, Start and Stop commands are available on both side bars of the Light Curve window.

The <Edit> command opens the window for configuration of the light curve measurements (Light Curve Edit, Table 8, page 79). The other commands start and stop a light curve measurement.

Standard lcp-files (light curve program files) optimized for various types of samples and colors of illumination are provided with the PamWin-4 Setup program (e.g., Walz_cyano_625.lcp and Walz_leaf_440.lcp).

These light curve programs can be further optimized by the user for particular applications and saved under new names. After modification of light curve parameters, the original name is maintained (unless changed by the user) and the name is marked by an asterisk. An asterisk also is added to the name when the color, <ML-intensity>, <MF-Low> or <MF-High> settings are changed.

Table 8: Light Curve Program Files (LCP)

Light Curve Edit

Open and save commands for LCP (light curve program) files.

LCP files contain information on setting of actinic light intensity ("Intens.") and duration ("Time/10s") for each of the 20 light steps available. Also, information on all settings affecting the light intensity is stored and automatically installed upon loading an LCP file (actinic light color as well as color, intensity and frequency of measuring light). PAR values result from both the setting programmed for each light step and the various measuring light parameters. Light curve information is linked to the current color of actinic light. Thus, changing actinic light color will change the light curve response.

Step	PAR	Intens.	Time/10s
1	0	MF1K	3
2	11	MF100K	3
3		1	0
20	2006	20	

✓OK
× Cancel
✓ Uniform time
Default

Editable columns

To edit, click in target field, type in the new data, and click elsewhere in editable columns. Measuring or actinic light LEDs can be used to drive photosynthesis. The effective intensity of measuring light depends on intensity setting and pulse frequency. In light curves, high pulse frequencies are used to cover the lowest PAR range. For example, entering MF100K in the "Intensity" column sets modulation frequency to 100 kHz. All 7 MF-H frequencies can be addressed this way. The actinic light source is controlled by entering a number between 0 and 20. The time interval of a step is entered as multiples of 10 seconds in column "Time/10s". To change the length of all 20 steps at once, check "uniform Time". A step with time=0 is not executed and terminates the light curve. Before entering 0, disable <Uniform time>.

Light curve data can be analyzed using the EP model (Eilers PHC, Peeters JCH, Ecol Model 42 (1988) 199-215) or the Platt

et al. model (Platt T, Gallegos CL, Harrison WG, J Mar Res 38 (1980) 687-701).

The <Fit> button starts an iterative process during which the free parameters of the selected model equations (EP or Platt et al.) are varied until the best fit between theory and experiment is obtained. Fitting results are represented graphically by a curved line; the three cardinal points of this calculated line are indicated in Fig. 25 (page 76). The values of cardinal points, and an F_V/F_M-derived parameter, are displayed after clicking the calculator button. Table 9 (page 80) summarizes the units of these parameters.

Table 9: LC Fit Parameters. 

		PAR	PAR(II)
F_V/F_M x ETR factor	Estimated alpha, based on F_V/F_M.		
alpha	Initial slope of light curve.	$\frac{\text{electrons}}{\text{quanta}}$	$\frac{\text{electrons}}{\text{quanta}}$
ETRmax	Maximum electron transport rate	$\frac{\mu\text{mol electrons}}{m^2 \cdot s}$	$\frac{\text{electrons}}{PSII \cdot s}$
I_k	Photon flux at which light-saturation of photosynthesis sets in.	$\frac{\mu\text{mol quanta}}{m^2 \cdot s}$	$\frac{\text{quanta}}{PSII \cdot s}$

4.5.3 Display Control Bar

To plot fluorescence levels (F_0', F, and F_M') or fluorescence ratio parameters against light intensities, choose “Fluoresc.”. Display of data is controlled by the check boxes on the side bar for display control.

4.5.4 Some Comments about Light Curves

Only if illumination steps are long enough to reach steady state photosynthesis, light curves will contain the same information as classical light response curves (P-I curves). Naturally, any limitation due to an insufficient CO_2 supply must be avoided during such long light curve experiments.

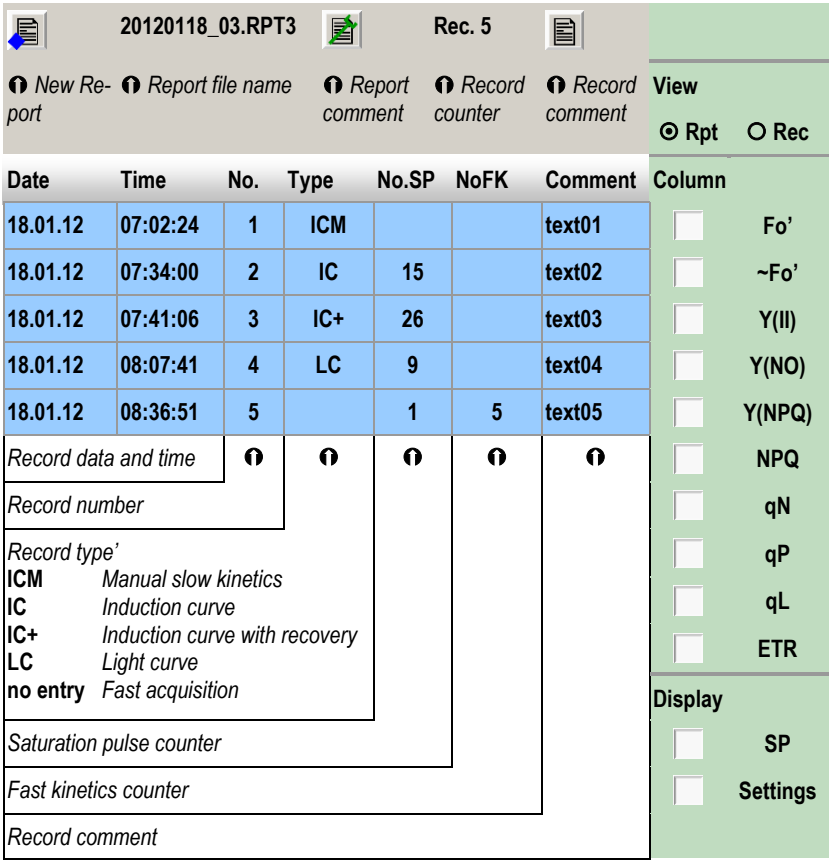
Rapid light curves (RLC) use short illumination steps (down to 10 s) to assess the acclimation state of the photosynthetic apparatus. In this case, it is essential that the sample is quickly transferred from its natural light environment to the sample holder of the PAM fluorometer. Alternatively, a pre-illumination is needed to activate the Calvin-Benson cycle. In that case, F_0 and F_M can be determined before the pre-illumination. Typically, the RLC starts at a PAR value somewhat below that of the natural environment. Without a dark-acclimation period, F_0 and F_M cannot be determined. For the calculation of Y(II), on which calculation of ETR is based, this is not necessary. Without F_0, F_0' and F_M, parameters like NPQ and qP cannot be calculated.

Light curves measured with dilute suspensions of algae and cyanobacteria carry particularly valuable information because PAR gradients are virtually absent.

4.6 Report Window

In the “Report” file all key experimental data are documented and saved automatically. The Report view (View Rpt) displays all experiments (“Records”) contained in the “Report” with information on type of experiment, number of saturation pulse analyses and fast kinetics as well as text written into the diagram headline (see Table 10, page 82).

Table 10: Report Window: Report View



Report comments are entered in a text window, which can be opened by clicking on the Report comment icon (). Record titles are written in the headline above the current graph (slow kinetics, light curve, and fast kinetics). The Record comments window can be edited after a slow kinetics experiment.

VIEW-mode (Chapter 8, page 158) is required to access older Report comments via the Report comment button on top of the Report window (). Also in VIEW-mode, comment lines can be inserted in a Record by the <Insert Comment> command, which is part of a menu opened by a right mouse click on the Report data field (clicking inside the Report data area).

To view all available information of an individual Record, double click on the relevant data line in Report view, which then switches to the Record view mode of the window.

The Record view <View Rec> provides detailed information on an individual experiment (“Record”, see Table 11, page 84). Minimum number of column headlines in the data field is 8 (Date, Time, No., ML, Temp. PAR, F, F_M'). Columns for F_0' fluorescence and fluorescence ratio parameters can be added by selecting the item on the side bar by ticking checkmarks below “Column”. The values of F_0 and F_M are listed in the F and F_M' columns, respectively (Table 11). They are distinguished from other data by column subtitles F_0 and F_M.

The panel “Display” offers options to extend the Record information. Checking <SP> displays date, time and file name of all saturation pulse kinetics of the Record (Table 11). Checking <Settings> displays all information on instrument settings, the currently active PAR list, and all changes of settings during the experiment. Abbreviations of settings (bordered cells in Table 11) are explained in Table 3 (page 65).

Table 11: Report Window: Record View

20120118_03.RPT3 Rec. 5

New Report | *Report file name* | *Report comment* | *Record counter* | *Record comment*

View: ○ Rpt ⊙ Rec

Text line: Enter Record title here

Date	Time	No.	ML	Temp.	PAR	F	Fm'	Y(II)	Y(NPQ)
18.01.12	14:48:27	Type: ICM (or IC, IC+, LC, no entry)							
18.01.12	14:48:28	FL	MI	G	AI	BI	FR/BL		
		10	3	2		4	FR		
		FH	Z	D	FI	SI	AL-Src	ML-Src	SI(Fm)
		10000	0	4	1	10	440 nm	440 nm	5
18.01.12	14:48:28	ML	MFH	MFLog	AL	FR	BL		
		On	Off	Off	Off	Off	Off		
18.01.12	14:48:28	PAR List: default_MC.par							
18.01.12	14:48:27	1	3	0.0	0	Fo 0.855	Fm 1.971		
18.01.12	14:48:27	1	3	0.0	0	0.855	1.971	0.566	0.000
18.01.12	14:48:26	File: S_120118_144826							
18.01.12	14:49:06	MF-H: on							
18.01.12	14:49:06	Act. Light: on							
18.01.12	14:49:08	2	3	0.0	128	1.405	2.007	0.313	0.000
18.01.12	14:49:07	File: S_120118_144907							

Column: ☐ Fo' ☐ ~Fo' ☑ Y(II) ☑ Y(NPQ) ☐ Y(NO) ☐ NPQ ☐ qN ☐ qP ☐ qL ☐ ETR

Display: ☑ SP ☑ Set-tings

4.6.1 Report Context Menu

Clicking in the Report window (in Record View) with the right mouse key opens a menu with Report-specific commands:

Menu item	Available	Action
Delete Del	Always	Deletes selected row.
Undo Delete File Name Esc	After Delete	Restores last deleted line.
Choose Sigma File Name	Always	Associates functional absorption cross-sections of PS II stored in “Sigma File” with current Report.
Insert Comment Ins	Always	Inserts a comment line (Cmt.:) below marked line.
Set Settings to Instrument	Measure mode + Settings line[1] marked	Installs settings of selected line in control unit.
Set Status to Instrument	Measure mode + Status line[2] marked	Sets control unit to status defined in selected line.
Set Marker in Chart	Protocol line[3] marked	Writes message of marked line into Slow Kinetics graph.

[1] **Settings line**

18.01.12	14:48:28	FL	MI	G	AI	BI	FR/BL	
		10	3	2		4	FR	
		FH	Z	D	FI	SI	AL-Src	ML-Src
		10000	0	4	1	10	440 nm	440 nm

[2] **Status line**

18.01.12	14:48:28	ML	MFH	MFLog	AL	FR
		On	Off	Off	Off	Off

[3] **Protocol lines**	18.01.12 14:53:12 Act. Light: on 18.01.12 14:54:23 Act. Light: off

4.7 “Multi Color” Window

The major functions of the “Multi Color” window (Fig. 27) are the selection of light colors, recording of intensities of internal light sources, and handling of Sigma(II)$_\lambda$, which is the (wavelength-dependent) functional absorption cross-section of PS II.

4.7.1 Light

The same 5 colors are available for measuring and actinic light (Table 12, page 87). These colors are numbered consecutively from 1 to 5. In addition, measuring light peaking at 400 nm and white actinic light is available.

By checking the <Auto AL-Color> box AL and ML light are linked. Selecting AL 590 nm instead of AL 625 will automatically lead to the selection of ML 590 nm. Unchecking this box breaks the link between AL and ML wavelength selection. Then, a choice of a different ML color will not affect the set associated AL color and the same is true for the choice of a different AL color.

When <Multi Color ST> is checked, all actinic LEDs contribute to a single turnover flash which is then particularly strong. The <Stirrer on> checkbox controls the status of an optional magnetic stirrer (PHYTO-MS). An equivalent command is part of the script file language (Section 6.3, page 132).

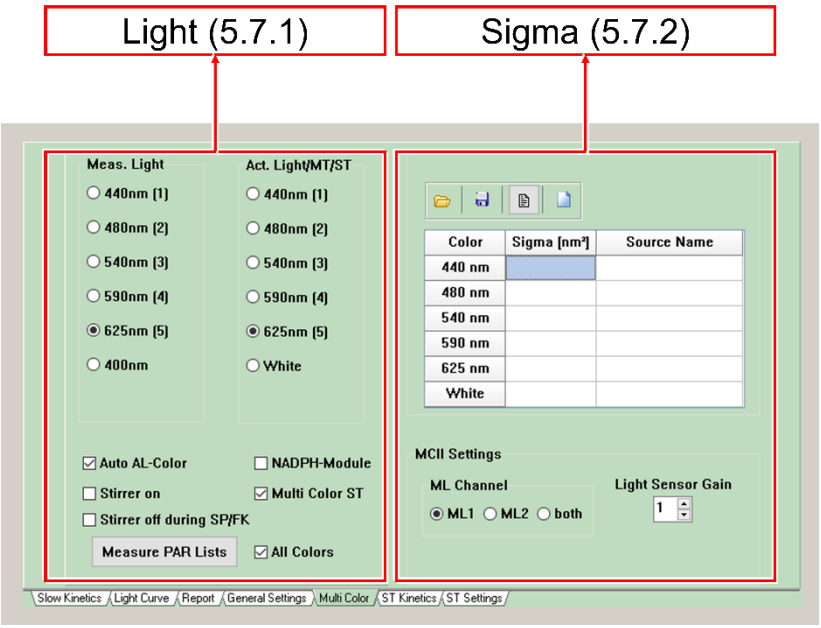


Fig. 27: "Multi Color" Window

A break in stirring is required when the moving stir bar disturbs the signal. In that case, the <Stirrer off during SP/FK> box can be checked. In most (fluorescence) applications, signal disturbance caused by the stirring is negligibly small and continuous stirring is possible.

Table 12: Light sources

Measuring light	Actinic light
Peak wavelength, nm	
440 (1)	440 (1)
480 (2)	480 (2)
540 (3)	540 (3)
590 (4)	590 (4)
625 (5)	625 (5)
400	420-645

Note that effective stirring requires close proximity between the inductive drive system of the PHYTO-MS stirrer and the magnetic stir bar. Therefore, the system must be moved to the

highest position in the bottom port of the optical unit, and the cuvette must be placed in the lowest possible position.

Measure PAR Lists The command <Measure PAR Lists> is executed only when a PAR sensor is connected. The command starts a routine, which measures the PAR values for the currently selected actinic light color. Checking the <All Colors> box next to the command will lead to the measurement of PAR for all actinic light colors. For each color, PAR is measured for all 20 intensity settings of actinic light, for all 20 intensity settings of multiple turnover pulses, and for measuring light at 100 kHz frequency and intensity setting 10. These PAR values are entered in PAR lists that can be accessed via the “Options menu”. For AL color 1 to AL color 5, the PAR value in the first line of the list corresponds to the PAR value established for measuring light. Based on this value, the program calculates the PAR for all combinations of measuring light intensity and frequency.

A PAR list is determined for the actinic light color selected, if one of the colors numbered 1 to 5 is selected (cf. Table 12, page 87), the measuring light color automatically assumes the actinic light color. If white is selected, each of the 6 measuring light colors can be chosen. The PAR value of the selected measuring light source, set to intensity level 10 and 100 kHz frequency, is the value representing setting 0 in the PAR list for white actinic light.

Setting 0 of the PAR list for white actinic light corresponds to the intensity of 400 nm measuring light under standard conditions (100 kHz, intensity setting 10). When PAR lists for white actinic light are measured, 400 nm measuring light should be selected. The spectral sensitivity of Walz PAR sensors is mostly flat in the range from 400 to 700 nm but steeply sloped around 400 nm.

Therefore, sensor calibration is invalid when 400 nm light is measured. In principle, this error can be corrected for according to:

$$\text{Setting } 0_{\text{Corrected}} = \frac{\text{Calibration Constant AIR}}{\text{Spectral Sensitivity at 400 nm}} \cdot \text{Setting } 0$$

The “Calibration Constant AIR” and “Spectral Sensitivity at 400 nm” are documented by the calibration certificate of the sensor. Setting 0 is the non-corrected PAR value at position 0 of the PAR list for white actinic light which is then replaced by Setting $0_{\text{Corrected}}$.

Newly measured PAR lists overwrite the current lists unless they are saved under a separate name. If PAR lists are not recorded for all colors, the current set of PAR lists consists of newly measured and old data. The current set of PAR lists can be saved together with annotations (menu Options → AL Current/PAR Lists).

The PAR acting on a sample is derived from measured PAR lists provided that the PAR sensor is unplugged or inactivated. Then the PAR value is calculated by considering light color and intensity as well as measuring light frequency. Calculation of PAR also works for different colors, e.g., green measuring light and red actinic light.

Correct PAR lists are not only important for PAR measurements as such, but also for assessment of the wavelength- and sample dependent optical cross-section Sigma(II) and determination of effective PS II turnover rate ETR(II). Therefore, users are encouraged to update the PAR lists of their individual instruments before measuring a new Sigma file. This should be done under close to identical optical conditions as the Fast Kinetics measurements of the O-I_1 rise (see below).

For measurements using the Optical Unit ED-101US/MD (suspensions), the spherical sensor has to be mounted with the diffuser sphere centered in the 10x10x10mm sample volume. For measurements using the Optical Unit MCP-II-BK (with leaf clip), the planar sensor has to be fixed in the cylindrical opening of the clip holder, with the diffuser plate in the sample plane (pushed against the emitter Perspex rod at an angle of 10°).

4.7.1.1 NADPH-module

It is now possible to use the MULTI-COLOR-PAM(-II) in combination with the NADPH-module. If the NADPH-Module box is checked the NADPH emitter and detector heads can be connected to the control unit of the MULTI-COLOR-PAM-II and in that case only the red 625 nm actinic light is available to prevent the 440 and 480 nm light from damaging the photomultiplier.

4.7.2 Sigma(II)

This window section is used for managing of Sigma files: existing files can be opened and assigned to the current Report. Also, files can be annotated, saved, or removed from the window. Sigma files contain information on $Sigma(II)_{\lambda}$ which is the wavelength-dependent functional absorption cross-section of PS II of a particular sample for up to 6 different colors of light. Values of $Sigma(II)_{\lambda}$ are obtained by fitting a kinetic model describing the reactions occurring during the first 2.3 ms following a dark-to-light transition to the fluorescence rise from the O to the I_1 level. This procedure will be explained in <Fast Acquisition> mode (Chapter 5, page 92). Sigma files are stored in C:\Program Files\PamWin Folder\Data_MC\Report\Sigma.

4.7.2.1 MCII Settings

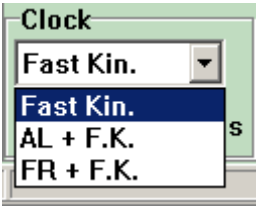
A new feature is the ML channel selection in the MCII Settings section. If two Emitter heads are connected to the Control Unit of the MULTI-COLOR-PAM-II, the user can decide if he/she wants to use the ML of the one unit by selecting <ML1>, the other unit by selecting <ML2> or decide to use both sources of measuring light by selecting <both>.

4.7.2.2 Light Sensor Gain

To the right of the ML Channel selection, there is a Light Sensor Gain selector. Levels 1-4 can be chosen, and this selector can only be used in combination with a light sensor specifically developed for the MULTI-COLOR-PAM-II.

5 Fast Acquisition

The <Fast Acquisition> mode permits the recording of fluorescence kinetics in the sub-msec to sec range at a maximum time resolution of 10 µs. This time resolution can be determined on the basis of the signal response time (time to reach 100% signal) of a fluorescence standard. Illumination protocols can be programmed at increments of 2.5 µs and repeated measurements can be averaged to obtain high signal quality even with weakly fluorescent samples.



Saturation pulse analysis, that is the quantification of measured data, is limited to F_V/F_M in <Fast Acquisition> mode. Events that can be repetitively triggered (Clock) are (1) fast kinetics, (2) fast kinetics with actinic (pre-)illumination, and (3) fast kinetics with far-red (pre-)illumination. Manual triggering of these three events is possible via the buttons (1) **Start**, (2) **AL + F.K.**, and (3) **FR + F.K.**.

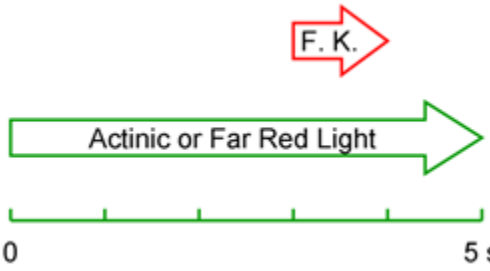


Fig. 28: Example of a time pattern for "Fast kinetics with actinic illumination" or "Fast Kinetics with far-red illumination". Illumination interval (actinic or far-red): 5 s. Fast kinetics start 2 s before end of illumination.

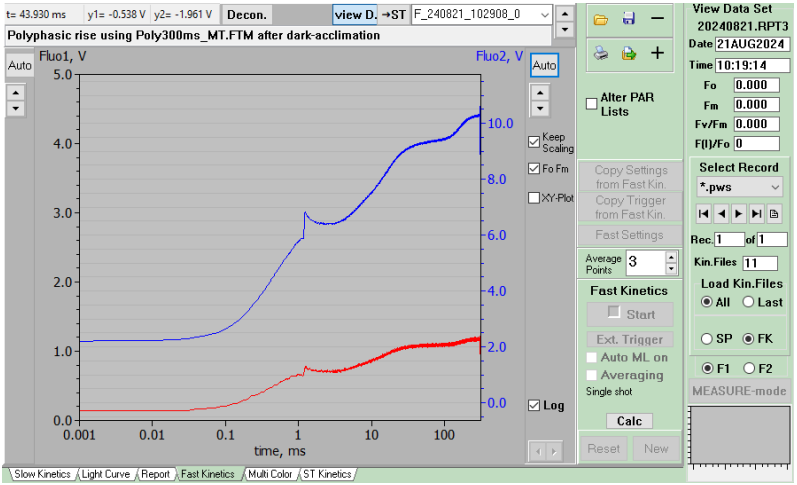
Note that actinic or far-red illumination stays on during <fast kinetics with actinic illumination> or <fast kinetics with far-red illumination>. Thus, these protocols differ from pre-

trigger illumination in Fast Settings, which terminates before a trigger pattern is executed.

Fast kinetics with illumination is only available when the respective time interval (actinic or far-red light) is 3 s or more. Then, the fast kinetics will be performed 2 seconds before illumination ends (see Fig. 28).

5.1 Fast Kinetics Window

The display of the fast kinetics graph (Fig. 29) can be changed as described for the Slow Kinetics window. The new features of the Fast Kinetics window are listed in s (y2) the position of the



cursor.

Next, the <Decon.> button, with which FI(v) and FII(v) contributions to O-I_1-I_2-P transients can be analyzed. A prerequisite for the use of the Decon. button is the simultaneous measurement of the polyphasic rise kinetics at wavelengths longer than 700 nm and shorter than 710 nm. Both for the measurement and when the data are analyzed in View-mode the option <Detectors 1+2> has to be

selected when opening the PamWin-4 software. Before clicking the Decon. button the intervals for the O-I_1 equalization and I_2-P equalization have to be defined. The dialogue window for <Intervals for O-I_1 Equalization> opens via right mouse click. Good results were obtained using F_0-time= -0.02 and width = 0.0.2. The I_1-time is defined by the time where the ST is applied and a suitable width = 0..1 ms. The dialogue window for <Intervals for I_2-P Equalization> opens via Shift + right mouse click. The optimal times for I_2 and P have to be estimated from the original curves. Good results were obtained with an I_2-width = 5 ms and a P-width = 30 ms.

Clicking the Decon. button, the O-I_1 amplitudes of the two curves are equalized and the equalization factor is displayed. It is the factor by which the <710 nm curve has to be multiplied to get the same O-I_1 amplitude as that of the >700 nm curve. The O-I_1 amplitude is also called the photosynthetic phase and is thought to be a pure PS II signal directly related to the reduction of Q_A. By double normalizing the data to the O-I_1-amplitude a scaling for PS II fluorescence is created. The software subtracts the F <710 nm curve (less PS I fluorescence) from the F >700 nm curve (enriched in PS I fluorescence) and creates thereby an F_V(I) induction curve. The amplitude of the F_V(I) changes is larger than the amplitude of the kinetic changes observed for difference curve because an appreciable amount of F_V(I) is contained in the F <710 nm curve. In vivo, LHCII has been shown to be bound to PS I, and the PS I fluorescence emission spectrum of PS I was shown to have side band around 675-680 nm and the 700-710 nm range contains contributions from the PS I fluorescence main band peaking around 730 nm. The analysis goes one step further, not showing the induction kinetics of F_V(I), but instead a F_V(I), F_V(II) deconvolution of F >700 nm based on the assumption that all fluorescence of the I_2-P phase is F_V(I). For this purpose, it equalizes the I_2-P-phase of both curves, i.e., that of the F_V(I) induction spectrum just obtained and the F >700 nm curve. The normalized F_V(I) induction transient is then

shown in red. This curve is subtracted from the F >700 nm kinetics to obtain the F_V(II) induction transient shown in blue.

In a leaf, the F <710 nm and the F >700 nm due to strong self-absorption of the F <710 nm fluorescence signal, are not emitted by the same chloroplasts. This would be problematic for the analysis. This self-absorption effect may also play a role in denser suspensions of cells. It is, therefore, important that the optical density of the sample is sufficiently low (Chl content 200-300 µg/l or less) to avoid density effects affecting the analysis. At higher Chl concentrations the O-I_1 rise curves are slower when measuring the F >700 nm compared with the F <710 nm signal. To a lesser extent this is also true for the I_1-I_2 rise. This will lead to oscillations in the F_V(I) kinetics and potential confusion.

In order to obtain low noise fluorescence responses at low Chl content the maximal 440 nm ML intensity (ML20) is recommended. In addition, it is a good idea to use the Clock function to trigger multiple Fast Kinetics recordings spaced 3-5 min apart.

The <view D.> button swaps the deconvoluted data created by the <Decon.> button and the original measurements. A third button, <→ST> representing the command “Show ST kinetics”.

Table 13, page 93.

Fig. 29: Fast Kinetics Window.

The band above the graphical window gives for the x-axis, the detector 1 y-axis (y1) and the detector 2 y-axis (y2) the position of the cursor.

Next, the <Decon.> button, with which FI(v) and FII(v) contributions to O-I_1-I_2-P transients can be analyzed. A prerequisite for the use of the Decon. button is the simultaneous measurement of the polyphasic rise kinetics at wavelengths longer than 700 nm and shorter than 710 nm. Both for the measurement and when the data are analyzed in View-mode the option <Detectors 1+2> has to be

selected when opening the PamWin-4 software. Before clicking the Decon. button the intervals for the O-I_1 equalization and I_2-P equalization have to be defined. The dialogue window for <Intervals for O-I_1 Equalization> opens via right mouse click. Good results were obtained using F_0-time= -0.02 and width = 0.0.2. The I_1-time is defined by the time where the ST is applied and a suitable width = 0..1 ms. The dialogue window for <Intervals for I_2-P Equalization> opens via Shift + right mouse click. The optimal times for I_2 and P have to be estimated from the original curves. Good results were obtained with an I_2-width = 5 ms and a P-width = 30 ms.

Clicking the Decon. button, the O-I_1 amplitudes of the two curves are equalized and the equalization factor is displayed. It is the factor by which the <710 nm curve has to be multiplied to get the same O-I_1 amplitude as that of the >700 nm curve. The O-I_1 amplitude is also called the photosynthetic phase and is thought to be a pure PS II signal directly related to the reduction of Q_A. By double normalizing the data to the O-I_1-amplitude a scaling for PS II fluorescence is created. The software subtracts the F <710 nm curve (less PS I fluorescence) from the F >700 nm curve (enriched in PS I fluorescence) and creates thereby an F_V(I) induction curve. The amplitude of the F_V(I) changes is larger than the amplitude of the kinetic changes observed for difference curve because an appreciable amount of F_V(I) is contained in the F <710 nm curve. In vivo, LHCII has been shown to be bound to PS I, and the PS I fluorescence emission spectrum of PS I was shown to have side band around 675-680 nm and the 700-710 nm range contains contributions from the PS I fluorescence main band peaking around 730 nm. The analysis goes one step further, not showing the induction kinetics of F_V(I), but instead a F_V(I), F_V(II) deconvolution of F >700 nm based on the assumption that all fluorescence of the I_2-P phase is F_V(I). For this purpose, it equalizes the I_2-P-phase of both curves, i.e., that of the F_V(I) induction spectrum just obtained and the F >700 nm curve. The normalized F_V(I) induction transient is then

shown in red. This curve is subtracted from the F >700 nm kinetics to obtain the F_V(II) induction transient shown in blue.

In a leaf, the F <710 nm and the F >700 nm due to strong self-absorption of the F <710 nm fluorescence signal, are not emitted by the same chloroplasts. This would be problematic for the analysis. This self-absorption effect may also play a role in denser suspensions of cells. It is, therefore, important that the optical density of the sample is sufficiently low (Chl content 200-300 µg/l or less) to avoid density effects affecting the analysis. At higher Chl concentrations the O-I_1 rise curves are slower when measuring the F >700 nm compared with the F <710 nm signal. To a lesser extent this is also true for the I_1-I_2 rise. This will lead to oscillations in the F_V(I) kinetics and potential confusion.

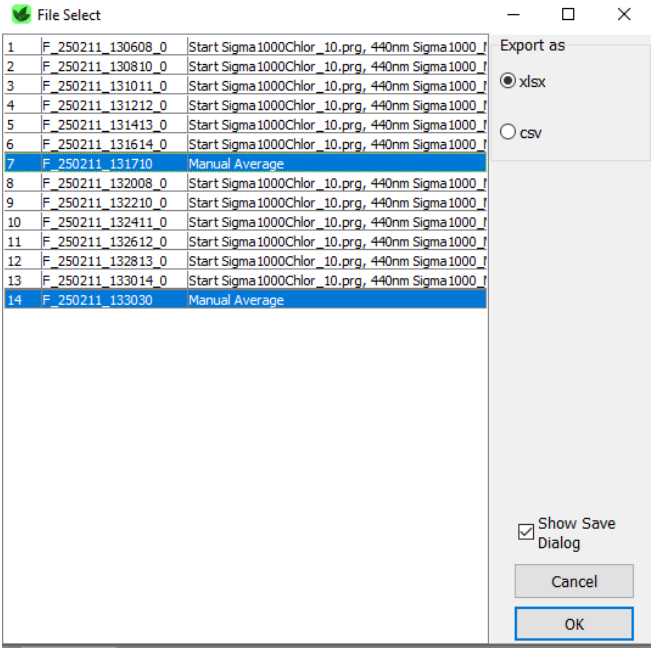
In order to obtain low noise fluorescence responses at low Chl content the maximal 440 nm ML intensity (ML20) is recommended. In addition, it is a good idea to use the Clock function to trigger multiple Fast Kinetics recordings spaced 3-5 min apart.

The <view D.> button swaps the deconvoluted data created by the <Decon.> button and the original measurements. A third button, <→ST> representing the command “Show ST kinetics”.

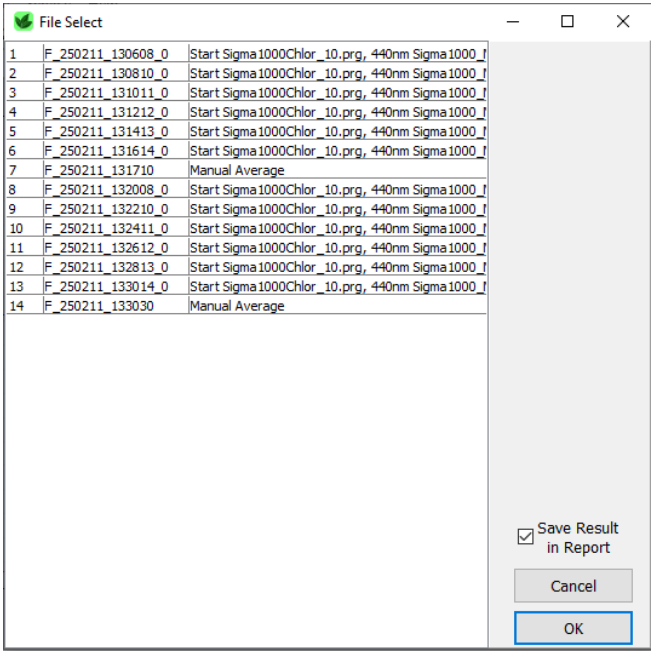
Table 13: Fast Kinetics Commands

Control	Command
Keep Scale	Keep scaling of fluorescence axis constant, prevents auto scaling of x-axis.
Log	Plot fluorescence on a logarithmic time scale.

Control	Command
	Open a PamWin-4 fast kinetic file. Fast kinetics data files contain information on the fluorescence kinetics but also on the fast trigger pattern and settings present during recording of the fast kinetics. To load the fast trigger pattern, click <Copy Trigger from Fast Kin.> after loading the fast kinetics file; to load only the settings, click <Copy Settings from Fast Kin.> Settings include a) status (on or off) of measuring light, actinic light, far-red light, high measuring light frequency, logarithmically decreasing measuring pulse frequency (MF log), b) low and high frequency of measuring light pulses <MF-L> and <MF-H>. Default directory is: C:\Program Files (x86)\PamWin Folder\Data_MCII\Report\YYYYMMDD_## (## counter for Reports created on the same date). File format of saturation pulse kinetics is S_YYMMTT_HHMMSS.PFK File format of fast kinetics is F_YYMMTT_HHMMSS_# .PFK (# counter for fast kinetics if more files have the same time).
	Save fast kinetics file. This command can be used to save files which are created by calculations (see below). Original files are saved automatically.
	Print current graph.

Control	Command
	Export selected fast kinetics data as *.CSV data (comma-separated values).  By default, the file name of fast kinetics is used and files are saved to the directory: C:\Program Files (x86)\PamWin Folder\Data_MC\Export. If <Show Save Dialog> is checked, the target directory is made visible and different directories can be selected. When only one kinetic dataset is exported, the file name can be edited.
	Subtract one of the fast kinetics datasets from the Record of the currently displayed fast kinetics. To conduct subtraction, click on minus (-) icon to view the list of fast kinetics, select by mouse click the fast kinetics to be subtracted, and close window by clicking <OK> to display the difference kinetics. To automatically save difference kinetics in the actually used Report, check <Save Result in Report> in the window for file selection.

Control	Command
+	Calculate the average of selected files.



To select files to average, click the plus (+) icon to view the list of fast kinetics. To pick a set of files to average, click with left mouse key on the first file of the/a series, keep mouse button depressed and move to the last file of that series, and release. Alternatively, click on the first file of the/a series, keep the shift key depressed and with the downward arrow key of the keyboard select files. Several non-neighboring files can be selected or deselected – by left mouse click on files – with the Ctrl key depressed. After picking files for averaging, click <OK> to display the result of averaging. The averaged dataset is added at the end of the list of fast kinetics and denoted “Manual Average”. Manual Average files are automatically saved provided that “Save Result in Report” has been checked.

Copy Settings from Fast Kin.

Each fast kinetics is saved with its trigger pattern and instrument settings. This particular trigger pattern and settings can be extracted and subsequently pasted by the <Copy Settings from Fast Kin.> command. The settings parameters concerned are summarized in s (y2) the position of the cursor.

Next, the <Decon.> button, with which FI(v) and FII(v) contributions to O-I_1-I_2-P transients can be analyzed. A prerequisite for the use of the Decon. button is the simultaneous measurement of the polyphasic rise kinetics at wavelengths longer than 700 nm and shorter than 710 nm. Both for the measurement and when the data are analyzed in View-mode the option <Detectors 1+2> has to be selected when opening the PamWin-4 software. Before clicking the Decon. button the intervals for the O-I_1 equalization and I_2-P equalization have to be defined. The dialogue window for <Intervals for O-I_1 Equalization> opens via right mouse click. Good results were obtained using F_0-time= -0.02 and width = 0.0.2. The I_1-time is defined by the time where the ST is applied and a suitable width = 0..1 ms. The dialogue window for <Intervals for I_2-P Equalization> opens via Shift + right mouse click. The optimal times for I_2 and P have to be estimated from the original curves. Good results were obtained with an I_2-width = 5 ms and a P-width = 30 ms.

Clicking the Decon. button, the O-I_1 amplitudes of the two curves are equalized and the equalization factor is displayed. It is the factor by which the <710 nm curve has to be multiplied to get the same O-I_1 amplitude as that of the >700 nm curve. The O-I_1 amplitude is also called the photosynthetic phase and is thought to be a pure PS II signal directly related to the reduction of Q_A. By double normalizing the data to the O-I_1-amplitude a scaling for PS II fluorescence is created. The software subtracts the F <710 nm curve (less PS I fluorescence) from the F >700 nm curve (enriched in PS I fluorescence) and creates thereby an F_V(I) induction curve. The amplitude of the F_V(I) changes is larger than the amplitude of the kinetic changes observed for difference curve because an

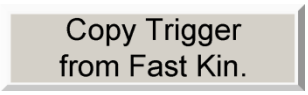
appreciable amount of $F_V(I)$ is contained in the F <710 nm curve. In vivo, LHCII has been shown to be bound to PS I, and the PS I fluorescence emission spectrum of PS I was shown to have side band around 675-680 nm and the 700-710 nm range contains contributions from the PS I fluorescence main band peaking around 730 nm. The analysis goes one step further, not showing the induction kinetics of $F_V(I)$, but instead a $F_V(I)$, $F_V(II)$ deconvolution of F >700 nm based on the assumption that all fluorescence of the I_2-P phase is $F_V(I)$. For this purpose, it equalizes the I_2-P-phase of both curves, i.e., that of the $F_V(I)$ induction spectrum just obtained and the F >700 nm curve. The normalized $F_V(I)$ induction transient is then shown in red. This curve is subtracted from the F >700 nm kinetics to obtain the $F_V(II)$ induction transient shown in blue.

In a leaf, the F <710 nm and the F >700 nm due to strong self-absorption of the F <710 nm fluorescence signal, are not emitted by the same chloroplasts. This would be problematic for the analysis. This self-absorption effect may also play a role in denser suspensions of cells. It is, therefore, important that the optical density of the sample is sufficiently low (Chl content 200-300 µg/l or less) to avoid density effects affecting the analysis. At higher Chl concentrations the O-I_1 rise curves are slower when measuring the F >700 nm compared with the F <710 nm signal. To a lesser extent this is also true for the I_1-I_2 rise. This will lead to oscillations in the $F_V(I)$ kinetics and potential confusion.

In order to obtain low noise fluorescence responses at low Chl content the maximal 440 nm ML intensity (ML20) is recommended. In addition, it is a good idea to use the Clock function to trigger multiple Fast Kinetics recordings spaced 3-5 min apart.

The <view D.> button swaps the deconvoluted data created by the <Decon.> button and the original measurements. A third button, <→ST> representing the command “Show ST kinetics”.

Table 13, page 93. To reproduce the trigger pattern of a previously saved PFK file, switch to <VIEW> mode, open the Report and Record containing the Fast Kinetics of interest, display Fast Kinetics, and click <Copy Settings from Fast Kin.>. Then return to <MEASURE> mode.



This command works similarly as the previous one, except that only the trigger pattern is copied.

Opens the Fast Settings window.

Point averaging leads to noise reduction at the cost of time resolution. Hence, point averaging is equivalent to signal damping. In contrast to the analog “Damping” (General Settings) and the “S&H” damping (Options), point averaging can be applied after actual measurements, so that an optimal compromise between time resolution and signal-to-noise ratio may be obtained. Loss of time resolution may be acceptable when the original data were recorded at higher time resolution than required for data analysis. The size of exported CSV files decreases with increasing number of averaged points. Clicking the command <Averaging> on this side bar starts calculation of the mean of repeated fast kinetic curves which results in noise reduction without loss of time resolution.

The <Start> button on the side panel of the Fast Kinetics window executes the currently loaded trigger pattern (Fast Settings window) and records the resulting fluorescence kinetics. The button is equivalent to the <Start> button of the bottom panel.

The command <Ext. Trigger> opens the window “External Trigger Control” which contains the button <Start External Triggering>. After clicking on this button, a 5 V DC signal applied to the trigger input socket of the control unit sets off the currently active trigger pattern and the recording of the fluorescence signal. To return to the normal state, click <Stop External Triggering> and <Close>.

Auto ML on

<Auto ML on> turns the measuring light on just before the start of a fast kinetics measurement and switches measuring light off immediately thereafter. This function is useful to avoid actinic effects of strong measuring light on the (redox) state of the electron transport chain of the sample. Alternatively, switching on the measuring light can also be included in trigger patterns imported in fast kinetics protocols (scripts).

Averaging

<Averaging> calculates the arithmetic mean of up to “n” consecutively measured fluorescence kinetics. Averaging of kinetics files reduces noise without decreasing time resolution. However, the operation assumes that the sample returns each time to the same starting conditions. This is only true if sufficient time between repetitions is given. Only the averaged kinetic data are saved. Individual kinetics can be triggered by the <Start> buttons (side or bottom bar) or by the <Clock> (bottom bar). The

number “n” of individual kinetic measurements is set as <Target Average> in the “Fast Settings” window. The clock stops automatically once “n” kinetics are averaged. In the average mode, two additional buttons are available:

which stops averaging and deletes averaged kinetic data.

which stops averaging and saves current averaged kinetic data. This command is useful when the number of measurements that were needed to reach a good signal/noise quality was smaller than the set <Target Average> number.

The <Calc> (calculations) button gives access to various methods to quantitatively analyze fluorescence kinetics and, not unimportantly, to the <Draw> option that allows the viewing of several fast kinetics overlaid on top of each other. These analyses will be reviewed in Section 5.3, page 112.

5.2 Fast Settings Window

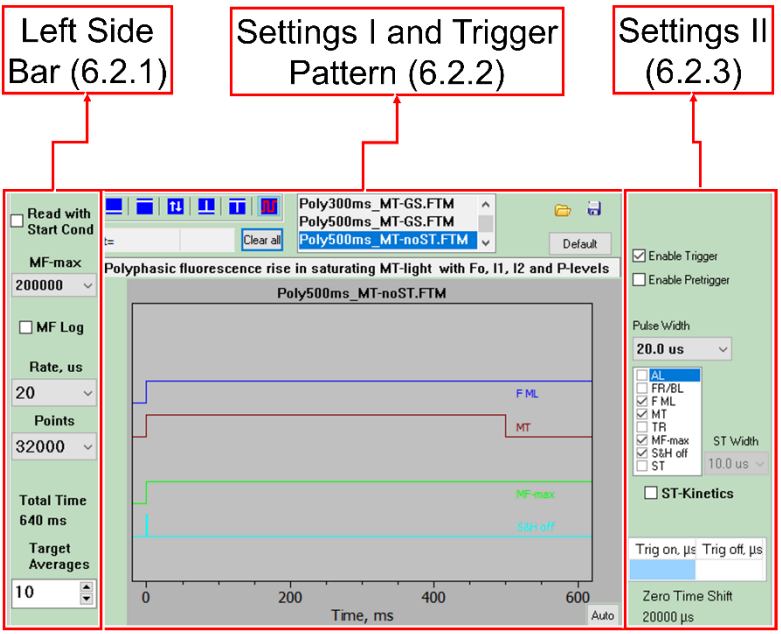


Fig. 30: Fast Settings Window.

Timing and light conditions of Fast Kinetics recordings can be adjusted in the “Fast Settings” window (Fig. 30). The window provides various tools and parameters to design a particular Fast Kinetics trigger pattern, which is graphically illustrated in the “Fast Settings” Chart.

5.2.1 Left Side Bar

Read with Start Cond

Similarly, as described for fast kinetic files, trigger files contain information on instrument settings (On/Off status of measuring light, actinic light, far-red light, high measuring light frequency, logarithmically decreasing measuring pulse frequency: MF log). Further settings saved are low and high frequency of measuring

light pulses (MF-L and MF-H).) When <Read with Start Conditions> is enabled, these settings become active/are applied.

MF-max

The available pulse frequencies for <MF-max> are the same as for <MF-H> (General Settings). The exception is the highest frequency of 200 000 Hz (providing the highest time resolution of 5 µs), which can only be set by the MF-max trigger.

MF-log

The <MF-log> setting decreases the measuring pulse frequency logarithmically with time. <MF-log> is triggered by termination of <MF-max>. If the trigger pattern does not involve <MF-max> or <MF-max> is not terminated within the fast kinetics time window, <MF-log> is not available. The <MF-log> function minimizes the actinic effect of measuring light as a function of time, which is particularly important during fluorescence relaxation kinetics following a single turnover flash (ST).

Rate, µs

The “Rate” in µs corresponds to the time interval between two consecutive measuring light pulses. The total time interval of fluorescence kinetics is the product of rate and the number of “Points”. The maximum number of points for a trigger program is 32 000 (but see below on “Ext. time”). The overall fluorescence kinetics may consist of up to 128 000 points.

For example, at a rate of 10 µs and 64 000 points, the duration of the trigger pattern is 320 ms followed by another 320 ms for, e.g., recording of the fluorescence decay.

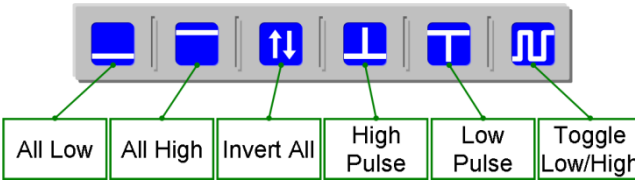
Ext. time

The extended time function can be used when the wished for number of data points is > 32 000. The function increases total experimental time by increasing the acquisition rate at point 32 001, and, for 128 000 point kinetics, again at point 64 001 (Table 14, page 104).

Table 14: Extended Time (Fast Kinetics).

Points total	128000	128000	128000
Rate, µs (selected)	2.5	2.5	5
Ext. time OFF ( ) or ON (✓)	☐	☑	☑
Rate, µs (true)			
(1 - 31999 points)	2.5	2.5	5
(32000 - 63999 points)	2.5	5	10
(64000 - 128000 points)	2.5	10	10
Total time, ms	320	880	1120

6 Trigger Icons



8 Events

- ☑ AL
- ☑ FR/BL
- ☑ F ML
- ☐ MT
- ☐ TR
- ☑ MF-max
- ☑ SH off
- ☑ ST

Target Averages

<Target Averages> determines the number of fluorescence kinetics to be averaged. Curve averaging is activated in the Fast Kinetics window by checking the <Averaging> box. The number of kinetics to be averaged can be changed even during curve averaging. Setting a higher number for target averages than

used in the previous average process requires pressing the <New> button on the Fast Kinetics window; as explained above, <New> can also be used to stop the averaging process; if <New> is not pressed, new measurements will be added to the existing average until the target average is reached.

5.2.2 Settings I and Trigger Pattern

A trigger pattern consists of time courses of trigger signals. A trigger signal can be either low or high which corresponds to “off” or “on”, respectively. The switching between on and off is extremely fast and can be/is programmed with 2.5 µs resolution (see section 13.1, page 200 for practical trigger pattern instructions).

- Six different tool icons are provided to design trigger patterns, and 8 events can be triggered.
- To edit a trigger pattern, check all events which should be triggered. This leads to the display of the corresponding trigger lines in the graph. <Clear all> sets all trigger events to off (low, flat lines).
- To increase the time scale of a trigger graph, move mouse pointer with left mouse button depressed parallel to the x-axis, along the time interval to be zoomed in on. Release of the mouse button will display the selected time interval. The <Auto> button (lower right corner) zooms out to full scale view.

Table 15: Trigger - Program Tools

Action	Icon	Key & mouse combinations	Events
Main Trigger Chart			
High pulse*		<Shift> + <right mouse key>**	All
Low pulse*		<Shift> + <right mouse key>**	All
Switch to high		<Shift> + <left mouse key>**	All but ST
Switch to low		<Shift> + <right mouse key>**	All but ST
All low		First, select event to be triggered. (Checkbox label must be high-lighted: white letters on blue back-ground).	All
All high			All but ST
Invert levels			All
Pretrigger Chart			
Toggle between always high and always low		<right mouse key>	All but ST

* For all graphically added events, the length of pulses depends on the time selected from the drop-down list “Pulse Width” in the bar on the right-hand side of the graph (10 to 2000 µs) except ST flash length, which is determined by the time selected for “ST Width”.

** Trigger setting *via* these shortcuts does not require selection of an event. Simply click on a trace (while pressing shift) to select the associated event.

- Editing trigger signals using icons <All Low>, <All High> and <Invert All> requires selection of the event by clicking on its checkbox label. Selection is indicated by the

highlighted checkbox label (white letters on blue background; see “AL” above).

- To trigger pulses and change trigger levels, select the appropriate trigger icon (red line in the icons indicates selection). Then, events can be selected by mouse click on the trigger line using the instructions of Table 15, page 106. Alternatively, select the event and enter time points in the <Trig on> and <Trig off> fields on the left side bar. Graphical editing of trigger lines is facilitated by the numerical displays of the mouse cursor position (numerical displays below trigger icons), however, in practice entering the time points is more precise.
- The ST (single turnover flash) is a unique event, as it must be particularly short to induce a charge separation in all PS II reaction centers only once. Therefore, only the <High Pulse> and <All Low> buttons are available for configuring the ST.
- The length of high and low pulses is determined by <Pulse Width> for all events except <ST> for which <ST Width> (5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 µs) applies. Dropdown menus for both pulse widths are adapted to the acquisition rate selected.
- Trigger patterns control only the “on/off” status of an event. Most events concern the internal light sources of the fluorometer. Light source properties can be set in the settings windows introduced earlier (Fig. 23, page 62) and summarized in Table 16, page 108.

 ,

Trigger files can be saved and recalled. The file extension is “FTM”, and the default directory is “C:\Program Files (x86)\Pam-Win Folder\Fast Kin Trigger”.

Table 16: Triggered Events - Settings

Label	Event	Settings	Window
☑ AL	Actinic light	Color: Intensity	Multi Color. General Settings.
☑ FR/BL	Far-red or blue light	Color: Intensity:	General Settings or FR/Blue button. General Settings.
☑ F ML	Measuring light	Color: Intensity: Frequencies: ([MF-L] and [MF-H] on/off buttons define start conditions of kinetics).	Multi Color. General Settings. General Settings.
☑ MT	Multiple turn-over pulse	Color: Intensity:	Multi Color. General Settings.
☑ TR	Trigger out	-	
☑ MF-max	Maximum frequency of measuring light	Color: Frequency: Intensity:	Multi Color. MFmax in Fast Settings. General Settings.
☑ S&H off	Sample and hold circuit off	-	
☑ ST	Single turn-over flash	Color: Intensity	General settings. (Always maximal).

Default

<Default> opens FastKin.FTM which triggers a 300 ms polyphasic fluorescence rise typically showing 4 fluorescence levels and which is called O-I_1-I_2-P or O-J-I-P. <Default> also activates experimental settings for the polyphasic rise kinetics, that is,

measuring light (ML) switched off and high frequency measuring light (MF-H) enabled.

5.2.3 Settings II

Enable Trigger

Should be checked during normal operation. The command is disabled for service purposes (associated with the service menu) only.

Enable Pretrigger

The Pretrigger feature provides the option to expose samples to pre-illumination. The length of exposure (1-10 s) can be selected from a drop-down menu, which is activated after <Enable Pretrigger> has been checked. Also, a narrow chart window, entitled <Pre>, to the left of the major trigger graph window is activated in which a trigger signal can be toggled on/off by left-clicking on a particular trigger line. The pre-trigger interval is immediately followed by the fast kinetics. Note that the <Clear All> button does not affect the pre-trigger settings.

Zero Time Shift

To change the zero-time shift, double-click on the number displayed below the <Zero Time Shift> (e.g., 300 µs). Time in µs can be entered manually or by using the pair of vertical arrows beside the number field. Time shift moves the zero point of the time axis so that recording of a fast kinetics starts at negative time values. By keeping the onset of actinic light at point zero, fluorescence kinetics data can be plotted on a logarithmic time scale.

5.2.4 Example of a Trigger Pattern

The trigger pattern displayed in Fig. 31 induces a fluorescence increase from the O level to close to the I_1 level, which is then attained by a saturating single turnover flash, ST. Specific settings were:

- Rate = 2.5 µs and points = 1000. The two settings yield a total experimental time of 2.5 ms.
- Zero Time shift = 300 µs. The x-axis starts at -300 µs.

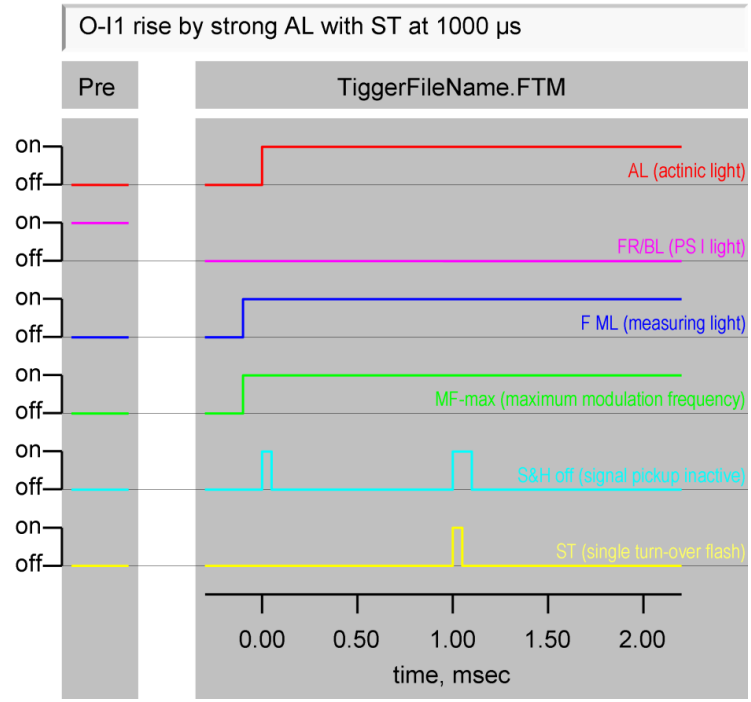


Fig. 31: Example Trigger Pattern.

- Pretrigger enabled. The “Pre” window is activated.

- Six events are triggered; multiple turnover pulse (MT) and trigger out (TR) are hidden/inactive.
- Other settings which provide suitable conditions for the measurement of fluorescence induction are:
    - "Act. Light Int." (actinic light intensity) = 20, to drive fluorescence inducting with strong light.
    - ML button off (measuring light switched off) to start fast kinetics without measuring light.
    - Far-red selected.

The example trigger pattern has the following characteristics:

FR (PS I Light)

Pre-illumination with PS I light is terminated at the start of the trigger pattern.

AL (actinic light)

The AL to drive fluorescence induction sets in at time zero. (In reality, the "Trig on" time is set to -5 µs because of a small delay between the trigger time point and onset of actinic light. Similarly, a 5 µs shift is needed in the case of a multiple turnover pulse.)

F ML (measuring light) and MF-max (maximum modulation frequency)

To measure F_0 level fluorescence, F ML and MF-max are switched on at time -100 µs, that is before onset of actinic light.

ST (single turnover flash)

To induce the I_1-level, a 50 µs ST flash (1000 to 1050 µs) was applied.

S&H off (signal processing inactivated)

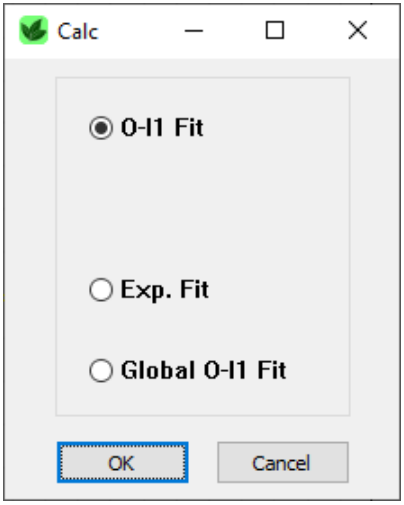
To suppress signal disturbance by sudden increases in absolute fluorescence intensity, the sample-and-hold (S&H) circuit is inactivated from -20 to 5 µs which corresponds to the onset of strong actinic light. Additionally, the S&H was switched off in the interval from 990 to 1085 µs, thus suppressing the disturbing effect of the single turnover flash.

5.3 Fast Kinetics Analyses

Data analysis options of PamWin-4 as well as the <Draw> option (under O-I_1 Fit) are available via the <Calc> button in the Fast Kinetics window.

5.3.1 Calc Window

The first step of data analysis is the selection of a method: O-I_1 Fit, Exp. Fit, or, after applying Ctrl A, Global O-I_1 Fit. Both methods vary the free parameters of theoretical functions until a best fit between theoretical function and experiment is obtained. Exp. Fit can be used for the analysis of the fluorescence decay kinetics following an ST.



Note that the letter “O” of the O-I_1 Fit symbolizes the minimum fluorescence level of fast fluorescence kinetics which is determined briefly after onset of measuring light during the 20 µs time period preceding onset of

actinic illumination. This fluorescence level corresponds to the F_0 measured with low frequency measuring light. Therefore, O and F_0 are used as synonyms in this section.

5.3.2 O-I_1 Fit Window

The O-I_1 Fit window allows an analysis of the O-I_1 rise kinetics. A kinetic model describing the reactions that play a role during the first 2.3 ms following a dark-to-light transition is fit to the O-I_1 rise. One of the fitting parameters is the functional optical cross section of PS II: Sigma(II), which is wavelength- and sample-specific. The original routine was based on a six-state model, which did not consider the role of the redox states of the water splitting complex on the PS II donor side (Klughammer C, Schreiber U, Photosynth Res 123 (2015) 77-92). This is still the standard fit model. More recently, a new routine based on a 48-state model, which allows the inclusion of the PS II donor side reactions, was developed. In both cases, the routine is based on the reversible radical pair model of PS II (Schatz et al., 1988, Biophys J 54: 397-405; Lavergne and Trissl, 1995, Biophys J 68: 2474-2492). In the 6-state model reoxidation of the primary PS II acceptor Q_A as well as energy transfer between PS II units (connectivity) were taken into account. Important prerequisites for analysis of the rise kinetics are:

a) Low chlorophyll content (< 300 µg/L for 440 nm excitation) to avoid light gradients.

b) Oxidized PS II acceptor-side (Q_A and PQ-pool) achieved by low intensity far-red background light or far-red pre-illumination (which induces a state with approx. 50% Q_B^-).

c) Accurate determination of the O and I_1-levels (turning on ML just before onset of AL). This means that the O-level is

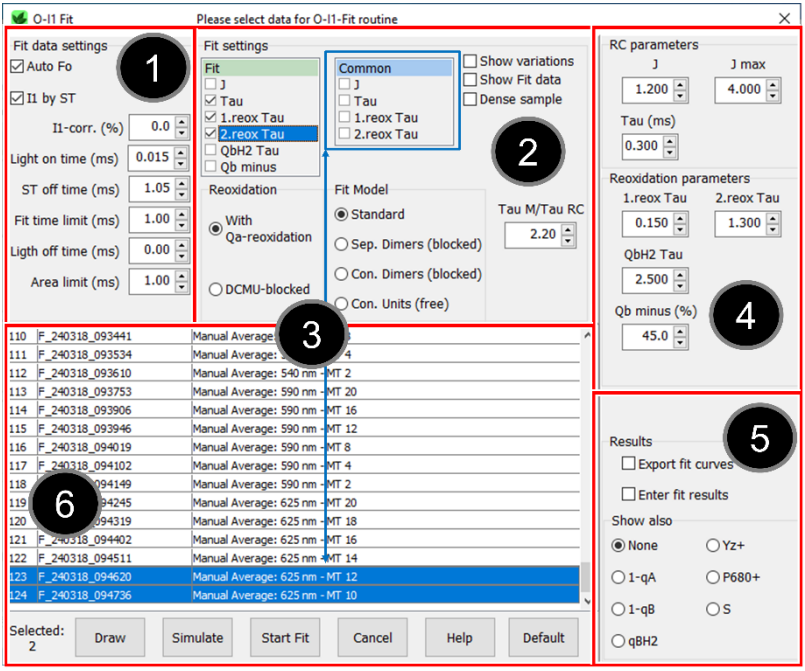


Fig. 32: Extended O-I$_1$ Fit Window (Ctrl A).

determined just before onset of actinic illumination and the I_1-level is the fluorescence level reached by a single turnover saturating flash <Multi Color ST> at the end of the illumination period. With a 50 µs ST, the maximum I_1 level is induced. A yellow sloped line is fit against the downward slope observed following the ST. The I_1-level is then determined by back extrapolation to 1.05 ms. The latter procedure is required because primary data acquisitioning was switched off during the single turnover flash to avoid signal artefacts.

The O-I_1 Fit window illustrated in Fig. 32 can be split in several sections.

1 In section 1 several technical parameters defining the fit are found.

If <I1 by ST> is checked, the fluorescence level induced by the ST given after 1 ms of illumination (e.g. as in the standard trigger files Sigma1000_AL.FTM and Sigma1000_MT.FTM) is taken as I_1. If <I1 by ST> is not checked the fluorescence level at the end of the recording is assigned to I_1. This is reasonable for DCMU-inhibited samples).

If a 50 µs ST is given after 1 ms, the <ST off time (ms)> should be 1.05 (as is the case in the above-mentioned standard trigger files).

Sometimes there is a mismatch between the starting point of the fit and the starting point of the measurement kinetics. In that case, the starting point for the fit can be shifted by the <Light on time (ms)>. By checking <Log> to the right-hand side of the graph window the user can more easily see if there is a mismatch between fit and measurement.

The fit should not consider the kinetics induced by the ST and, therefore, <Fit time limit (ms)> should be set to a value below 1 ms.

One of the parameters the fit determines is the area above the O-I_1 rise. This area is also delimited by the position of the ST, i.e., <Area limit (ms)> should be set to 1 ms (or less).

If the user positions the ST at another time point all the above-mentioned times should be modified accordingly.

Section 2 defines the type of experiment: an uninhibited sample in which forward electron transport to Q_B occurs (check <with Qa reoxidation>); or forward electron transport is blocked (check <DCMU-blocked>).

Further, here the user can define which parameters the fit routine should vary. Four or six parameters can be chosen for this purpose: J, Tau, 1.reox Tau, 2.reox Tau, (QbH2 Tau, Qb

minus). With the key combination Ctrl A, the user can toggle between a basic and an extended fit window.

J is the (energetic) connectivity parameter defining the sigmoidicity of the O-I_1 rise; Tau defines the fluorescence rise time; 1.reox Tau is the rate of electron transfer from Q_A^- to Q_B; 2.reox Tau is the rate of electron transfer from Q_A^- to Q_B^-; QbH2 Tau is the rate of exchange of double reduced Q_B for an oxidized PQ molecule; Qb minus is the percentage of PS II reaction centers containing Q_B^-.

In section 2, three additional functions can be 'activated': 1. 'show variations', 2. 'show fit data' and 3. 'Dense sample'. These are discussed below.

At the moment only the standard fit option is functionally available. The other three options (Separate dimers (blocked), Connected Dimers (blocked), Connected Units (free) and the parameter TauM/TauRC) are related to a 48 state model including the different S-states in the fit model, which is still under development.

When two or more measurements are fit at the same time, a table of common parameters shows up (nr. 3 in blue). For one batch of cells J, 1.reox Tau and 2.reox Tau are in principle the same for all measurements. Theoretically, these 3 parameters can be set as common parameters, and this could improve the global fit of the measurements that are fit at the same time.

In section 4 starting values for fit parameters and expected values for the parameters that are not fit are defined.

There is no consensus in the literature what the value of J is. Joliot and Joliot (1964) found the equivalent of J = 1.2 in their

study. Given that Sigma(II) and J interact, higher J-values give higher Sigma(II) values, it may be a good idea to fix J (maybe try a few values) to avoid variability due to the J-Sigma(II) interaction. J is an intrinsic parameter for a given state of a sample. It should, e.g., be independent of the light intensity with which the O-I_1 transient is induced.

The parameter 1.reox Tau has a value in the range 150-300 µs, 2.reox Tau in the range 800-1300 µs and the parameter QbH2 has a value in the ms range. This parameter is sensitive to PQ pool size and PQ pool redox state.

The parameter Qb minus in the mixed state (25% of each donor side S-state, see next section) is in principle 50%. For dark-acclimated samples it can be sample and species dependent with 25% as the default value set for, e.g., *Chlorella*. When FR1 background light is used a value of ~50% would be expected for this parameter.

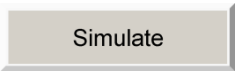
In section 5, it is possible to visualize the kinetics of the parameters 1-Q_A, 1-Q_B and Q_BH_2 on the basis of the fit by selecting one of these three parameters. The parameters Y_Z^+, $P680^+$ and S are only functional in relationship to the 48-state model mentioned above. Two further functions that can be activated in this section are <Export fit curves> and <Enter fit results>. By checking <Export fit curves> the fit curve will be saved as a *.csv file. Checking <Enter fit results> means that when clicking <Simulate> the results of the last fit will be used for the simulation.

In section 6, the measurements found in the active record are shown and measurements to be fit/simulated/drawn, etc., can be selected here. To analyze several curves concomitantly, select the additional curves by

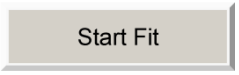
mouse click with the Ctrl key depressed. Using the Shift key, a sequence of curves can be selected by mouse click.

At the bottom of the section six different functions can be triggered: <Draw>, <Simulate>, <Start Fit>, <Cancel>, <Help>, <Default>.

<Draw> has nothing to do with fits or simulations but is an important option because it allows the plotting of different measurements on top of each other and thereby allows the comparison of the kinetics of different measurements.

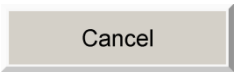


<Simulate> calculates an O-I_1 transient on the basis of the parameters chosen in the Fit Window. If <Enter fit results> is checked and the results of a previous fit are available, the <Simulate> function will use these data for the simulation. With this option it is possible to visualize the effects of changes in one or more of the parameters on the kinetics of the O-I_1 transient.

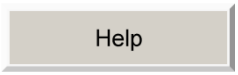


<Start Fit> will start the fit routine. The routine uses an iterative process in which the values of the fit parameters are each time a little bit changed, an O-I_1 transient is calculated and then the deviation from the measured transient is determined. If the goodness of the fit matches certain criteria the fit process is stopped. The results are compiled in the O-I_1 Fit data table. Simultaneously, original traces and curve fits are shown in the Fast Kinetics window. The error is a measure for the fit quality, however it is always important also to look at the 1.reox Tau and

2.reox Tau values. A perfect fit and totally unrealistic parameter values still points at an incorrect fit.



<Cancel> closes the O-I_1 fit window and returns the user to the Fast Kinetics window.

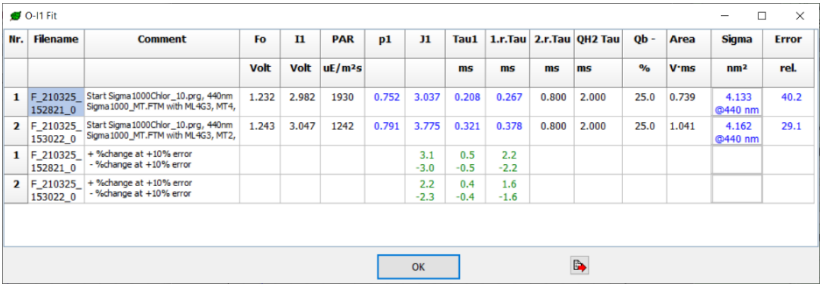


<Help> opens a window with a text similar to the text in this manual.

<Default> returns the different fit parameter values to their default values.

<Show variations> and <Show fit data>

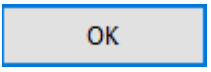
In section two, three other options can be activated by checking: <Show variations>, <Show fit data> and <Dense sample>. The options <Show variations> and <Show fit data> provide quantitative (Fig. 33) and graphical information (Fig. 34), respectively, on the fit error/quality. With respect to Fig. 33, note that small signal variations are indicative of a reliable fit. These are printed in green. Values printed in red, suggest a parameter limit reached during the fitting process.


O-I1 Fit

Nr.	Filename	Comment	Fo	I1	PAR	p1	J1	Tau1	1.r.Tau	2.r.Tau	QH2 Tau	Qb -	Area	Sigma	Error
			Volt	Volt	uE/m²s			ms	ms	ms	ms	%	V·ms	nm²	rel.
1	F_210325_152821_0	Start Sigma1000Chlor_10.prg, 440nm Sigma1000_MT.FTM with ML4G3, MT4,	1.232	2.982	1930	0.752	3.037	0.208	0.267	0.800	2.000	25.0	0.739	4.133 @440 nm	40.2
2	F_210325_153022_0	Start Sigma1000Chlor_10.prg, 440nm Sigma1000_MT.FTM with ML4G3, MT2,	1.243	3.047	1242	0.791	3.775	0.321	0.378	0.800	2.000	25.0	1.041	4.162 @440 nm	29.1
1	F_210325_152821_0	+ %change at +10% error - %change at +10% error					3.1 -3.0	0.5 -0.5	2.2 -2.2						
2	F_210325_153022_0	+ %change at +10% error - %change at +10% error					2.2 -2.3	0.4 -0.4	1.6 -1.6						

OK

Fig. 33: <Show variations> checked. In the example given two measurements were fit and, in that case, <Show variations> adds two lines to the table in which for each fit parameter is indicated how much (in %) the fit parameter would be smaller or larger when the error would be 10%. This gives information on the fit quality of the individual fitted parameters.

 and 

of Fig. 33

The export button (left) saves fitting results as *.csv file. The export directory is C:\PamWin_4\Data_MC\Export. The <OK> button induces the return to the Fast Kinetics window.

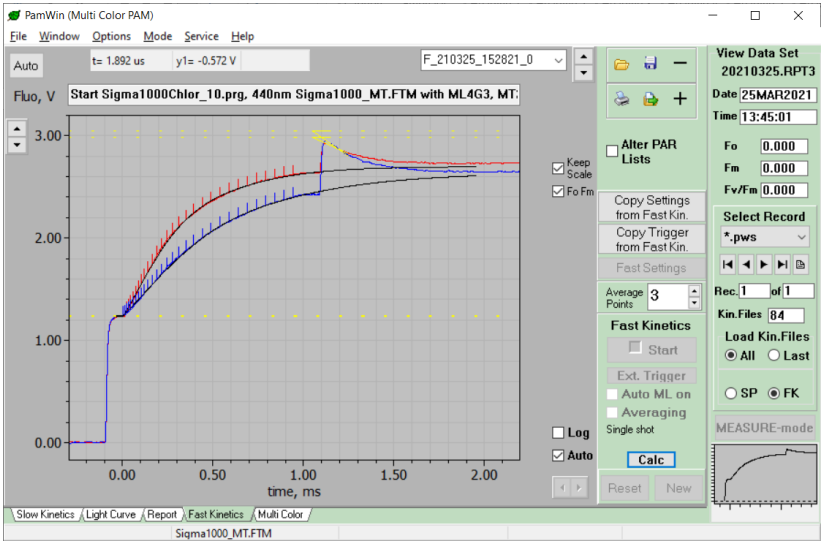


Fig. 34: <Show fit data> checked. In the example, two measurements made at two light intensities are shown with their fit lines and the vertical line segments as a measure for the fit quality made visible by checking <show fit data>.

If possible, Sigma(II) determinations should be made on dilute suspensions in which the light gradient plays no role. If dense samples (for example leaves) are measured, the light gradient does play a role and the Sigma(II) determination has to be corrected for this. Checking <Dense sample> will activate such a

correction. The user is referred to Klughammer and Schreiber (Photosynth Res 123 (2015) 77-92) for a treatment of the dense sample case.

From the O-I_1 rise the functional absorption cross section of PS II (Sigma(II)) can be derived. Sigma(II) is one of the parameters determining the O-I_1 rise and a fit of those kinetics also allows the determination of Sigma(II). One of the required inputs for the fit is the incident PAR driving this rise (saved in the PAR Lists). If the values in the PAR Lists do not match the actual values of the incident PAR it will lead to an over- or underestimation of Sigma(II).

Knowledge of Sigma(II) permits transformation of incident PAR (in µmol quanta · m^{-2} · s^{-1}) into the rate of quantum absorption per PS II complex, PAR(II) (in quanta · PS II^{-1} · s^{-1}). Then, the rate of linear electron transport per PS II reaction center, ETR(II) (electrons · PS II^{-1} · s^{-1}) can be estimated according to

ETR(II)=PAR(II) · Y(II)/(F_V/F_M) (see Section 4.5.1, page 77),

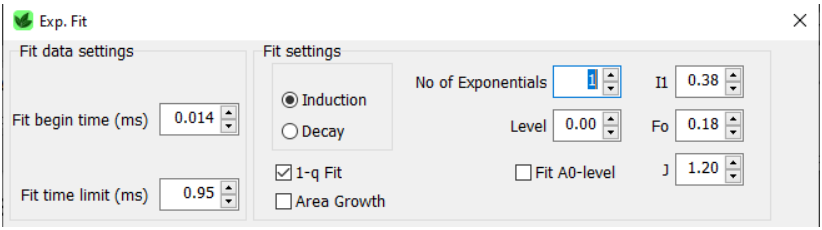
where Y(II) and F_V/F_M are the photochemical yields of PS II in the light-exposed and quasi-dark-acclimated state (i.e., with weak far-red background light), respectively. The parameters that control the O-I_1 fitting procedure are reviewed below; see for the parameters the Fit window shown in

Fig. 32.

5.3.3 1-q

In the older versions of PamWin-3 the 1-q fit had its own window. In PamWin-4 a 1-q fit can be generated in the Exp. Fit window. However, it is better to apply this function only to decay

curves. For O-I_1 induction curves the 1-Q_A simulation that can be generated together with an O-I_1 fit should be chosen.



Exp. Fit window, with parameters chosen for a 1-q fit of an O-I_1 transient.

When fitting the fluorescence decay following a single turnover flash, the A_0-level is the semi-stable fluorescence level reached after a few ms. Slow recombination reaction will lead to a further fluorescence decay on a hundreds of ms timescale. These/this slow decay phase(s) are treated as an offset (A_0). Fit A_0-level means that the fast exponential decay phases preceding A_0 are fit.

The parameter q corresponds to the fraction of open reaction centers characterized by the oxidized primary acceptor Q_A. Setting the sum of open and closed reaction centers to one, 1-q is the fraction of closed PS II reaction centers with reduced primary acceptor Q_A^-. 1-q is non-linearly related to fluorescence yield, F, due to transfer of excitation energy between PS II units (connectivity). Connectivity explains why F increases more slowly than 1-q during the initial part of the O-I_1 rise (sigmoidicity).

In the PS II excitation model by Lavergne and Trissl (1995, Biophys J 68: 2474-2492), on which the present O-I_1 analysis is based, the extent of connectivity is described by the parameter J. This J is related to the empirical sigmoidicity parameter p (Joliot and Joliot, 1964, C R Acad Sci Paris 258: 4622-4625) by the equation J = p/(1-p).

Calculations of 1-q require parameters from the fitting analysis of the O-I_1 rise, specifically, the parameters I_1, F_0 and J. Therefore, analysis of the O-I_1 rise should precede 1-q calculations. Three different 1-q calculations can be performed, based on different models and suited to different applications. The resulting 1-q plots are drawn in different colors.

O-I_1 Fit Model (displayed in blue)

Calculation is based on the model underlying the O-I_1 Fit. This model takes account of Q_A reoxidation and, hence, is also valid for analysis of rise kinetics in the absence of PS II inhibitors (like DCMU).

Based on F and J (displayed in green)

Values of 1-q are calculated according to the following equation (Lavergne and Trissl, 1995, Biophys J 68: 2474-2492. Joliot and Joliot, 1964, C R Acad Sci Paris 258: 4622-4625):

$$\frac{F(t)-F_0}{I_1-F_0}=\frac{1-q}{1+J\cdot q}\rightarrow q=\frac{I_1-F(t)}{(F(t)-F_0)\cdot J+(I_1-F_0)}$$

where F(t), F_0, and I_1 are the fluorescence curve and its O and I_1 levels, respectively. This model does not take into account Q_A reoxidation and, therefore, it is valid only for analysis of rise kinetics in the presence of PS II inhibitors (like DCMU) or at extremely high actinic intensities at which the rate of PS II charge separation is much higher than the rate of Q_A reoxidation. In the latter case, however, the quantum efficiency of PS II may be affected (Rappaport et al, Biochim Biophys Acta 1767 (2007) 56-67) and HIQ (high intensity fluorescence quenching) will occur (Schreiber et al, Photosynth Res 142 (2019) 35-50).

Area Growth (displayed in yellow)

The 1-q curves are derived by the area growth method (Malkin and Kok, Biochim Biophys Acta 126 (1966) 413-432; Murata, Nishimura, Takamiya, Biochim Biophys Acta 120 (1966) 23-33). The method is valid only when reoxidation of reduced primary acceptor Q_A is prevented, e.g., by presence of DCMU.

5.3.4 Exp. Fit

The <Exp. Fit> routine permits analysis of decay kinetics following termination of actinic illumination (actinic light, multiple turnover or single turnover flashes). Settings and conditions for this fitting procedure are entered into the “Exp. Fit” window (Fig. 35) as explained below (numbers 1, 2 and 3 refer to sections in Fig. 35).

Fit Data Settings 1

Light off time (ms)

Switch-off time of illumination.

Fit begin time (ms)

Lower time limit of data considered for curve-fitting.

Fit time limit (ms)

Upper time limit of data considered for curve-fitting.

Fit Settings 2

1-q Fit

This function transforms the fluorescence kinetics into changes in 1-q (cf. Section 5.3.3, page 121) and the kinetic analysis refers to the 1-q relaxation kinetics.

No of Exponentials

The relaxation kinetics can be described by the sum of up to three exponentials. The corresponding free parameters are the amplitudes A1, A2 and A3, and the time constants Tau 1, Tau 2 and Tau 3.

Tau 1 of the relaxation kinetics corresponds to 1. reox Tau of the O-I_1 fit, i.e., the time constant of Q_A^- reoxidation by Q_B.

Tau 2 of the relaxation kinetics corresponds to 2. reox Tau of the O-I_1 fit, i.e., the time constant of Q_A^- reoxidation by Q_B^-.

Tau 3 of the relaxation kinetics corresponds to the time constant with which double-reduced Q_B^{2-} is replaced by fully oxidized Q_B (involving protonation, release and rebinding of a PQ-molecule) or the binding of a PQ molecule to an empty Q_B site, which is more likely for a dark-adapted sample.

Level

"Level" stands for the amplitude A_0. A_0 corresponds to the fraction of closed reaction centers (1-q) that is not re-oxidized during the fit time. A_0 is considered to reflect the so-called "inactive PS II's" in which Q_A to Q_B electron transport does not occur. However, e.g., a reduced PQ pool will lead to vacant Q_B binding sites, which will prevent Q_A to Q_B electron transport. The slow recombination between Q_B^- and the PS II donor side will also lead to some fluorescence decay. As A_0 often displays values of 40-50% in perfectly active samples, it is likely that A_0 represents a mix of true "inactive PS IIs" and physiological conditions that lead to vacant Q_B binding sites.

I_1, F_0

Exponential fitting requires the determination of the parameters F_0 and I_1. These are conveniently estimated by recording an O-I_1 rise curve with the same sample under identical measuring light conditions. After O-I_1 fitting analysis, the F_0 and I_1 parameters are automatically written into the Exp. Fit window. Both values can also be manually entered.

J

The sigmoidicity parameter J is required when the “1-q Fit” is active. Similarly as for F_0 and I_1, the J-value is taken from a preceding O-I_1 curve fit but can also be entered manually.

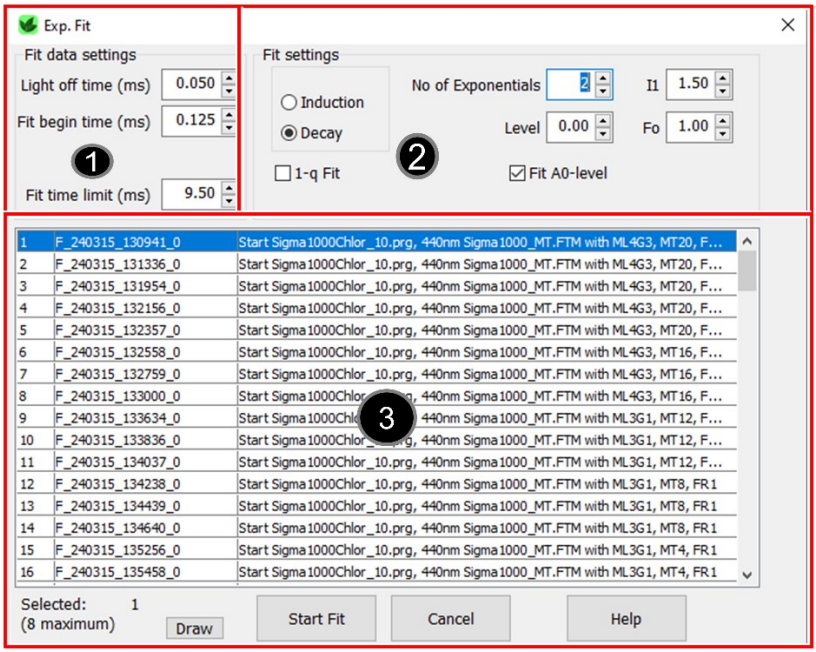


Fig. 35: Exp. Fit Window.

Data List 3

The Exp. Fit window lists all kinetics of the current Record. Pick curve for analysis by clicking on it with the mouse. Several curves can be analyzed simultaneously (maximum is 8): simply select the curves of interest by mouse click while pressing the Ctrl key; a series of curves can be selected using the mouse cursor in combination with the Shift key.

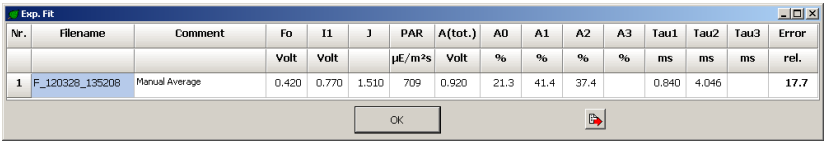
Draw

Draws selected curves in the same Fast Kinetics window. This function can be also used independently of the Exp. Fit routine for display of superimposed Fast Kinetics.

Start Fit

The button starts fitting exponential curves to the data. The results are compiled in the Exp. Fit data table.

Exp. Fit Table



Nr.	Filename	Comment	Fo	I1	J	PAR	A(tot.)	A0	A1	A2	A3	Tau1	Tau2	Tau3	Error
			Volt	Volt		µE/m²s	Volt	%	%	%	%	ms	ms	ms	rel.
1	F_120328_135208	Manual Average	0.420	0.770	1.510	709	0.920	21.3	41.4	37.4		0.840	4.046		17.7

At the same time, original traces and fitted curves are displayed in the Fast Kinetics window (Fig. 36). Clicking the export button ( ) saves fitting results as CSV data into C:\PamWin_4\Data_MC\Export. To return to the Fast Kinetics window, click <OK>.

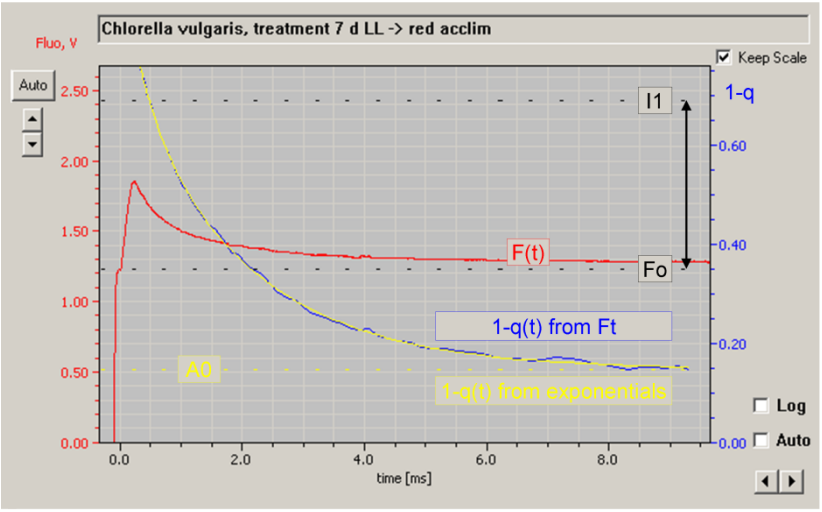


Fig. 36: Fast Kinetics Window: Experimental and Fit Data.

6 Script File Window

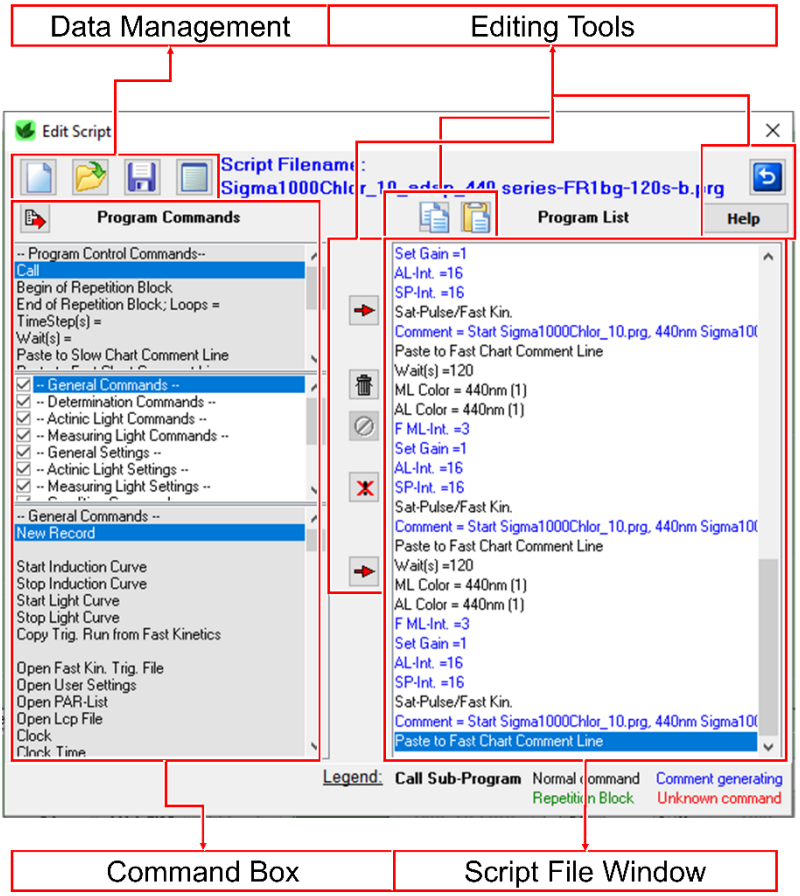


Fig. 37: Script File Window.

Script files are used for automated execution of experimental procedures of various complexities. Particularly, script files are advantageous when the same type of analysis needs to be repeated frequently and when complicated protocols must be exactly reproduced.

Generally, all manual operations in the PamWin-4 software can also be carried out under the control of a Script file. In addition, Script files offer commands for time management, combinations of sub-programs and conditional commands.

To access the script file window, click on the Load button in the bottom bar of the PamWin-4 windows. To execute script files, click on the Run button below the Load button.

6.1 Data Management

Four commands are provided for script file management:

	New Script File. Clears the script file window and prompts for a new script file name.
	Open Script File. Opens a script file with name format “file-name.PRG”. The default directory for script files is C:\Pam-Win_4\Script Files. Other directories can be selected.
	Save Script File. Saves to default or user-defined directory.
	Script File Comment. Displays a text-window for notes on the current script file. The content of the window is saved as text file with name “filename01.TXT” which is associated with the script file “filename01.PRG”.

6.2 Editing Tools

	Copy The command stores one or several lines of the current script file in the clipboard. To execute the copy command, select one or several lines using the mouse cursor (Left-click once to pick one line. Hold down the Shift key and select the first and last line of a series of script file commands. Hold down Ctrl key for the selection of several scattered lines.) Click Copy icon. The selected commands are now available for pasting in the current or into another script file using the Paste command.
	Paste To paste previously copied commands, select a line in the target script file and click the Paste icon. The pasted lines will be added below the selected line.

Insert Generally, the two “Insert” icons transfer commands from the command boxes to the script file window. The upper insert icon transfers commands from the box “Program Control Commands”, the lower icon transfers commands from the “General Commands” box. Double click on the command to be transferred is equivalent to the insert icons.

To insert a new command in the program list:

- Select the command to be inserted with the mouse cursor in the command box (left text window of

Fig. 37, page 129)

- Select with the mouse cursor in the script file window (right text window of

Fig. 37 (page 129) the line below which the command has to be inserted

- Click Insert button.

Delete Select one or several commands to be deleted (see above) and click icon. The command is equivalent to pressing “delete” on the keyboard.

Undo Delete Reverses the last delete action.

Disable/Enable Disables command lines of the current script file or enables previously disabled command lines. Disabled lines are printed in grey. To execute the disable/enable command, select line(s) in script file window and click icon.

BACK Closes script file window. When a sub-program is displayed, the Back button returns to the script file which calls the sub-program.

Export Command list Exports the list of commands to the file Command List.txt

6.3 Command Box (List of Script File Commands)

The command box of the script file window (

Fig. 37, page 129) consists of 3 sections. The upper one contains commands controlling the progress of script files. The middle panel contains the titles of the 8 groups of commands listed in the bottom panel. Unchecking titles in the middle panels hides the corresponding group of commands in the bottom panel.

NB. Any parameter not defined in the script will take its value from the settings in the Settings window(s).

See section 13.2, page 201 for some comments on writing script files.

PARAMETER	COMMAND, COMMENT	INPUT
Section 1: -- Program Control Commands --		
Call	Executes another PamWin-4 script file as a sub-program. A sub-program can be displayed by double-clicking an existing calling line. Use the Back button (⮌) to return to main script file. Sub-programs may call further sub-programs.	Script file name (file-name.prg)
Begin of Repetition Block	Marks the beginning of a series of commands (repetition block) which have to be repeated.	Name of repetition block
End of Repetition Block; Loops =	Marks the end of a repetition block. A repetition block may contain other repetition blocks. Loops = defines the number of repetitions. Repetitions can also be terminated depending on the levels of Ft or temperature (see section “Condition Commands” at the end of this list).	Number of repetitions
TimeStep(s) =	Defines the time interval between the beginnings of two consecutive events; if the TimeStep command is not preceded by an event, the time interval starts with script file execution. A series of time steps forms a time scale along which actions can be defined. [Precise]	Time interval in seconds

PARAMETER	COMMAND, COMMENT	INPUT
Wait(s)	Defines the time interval between completion of the last command and the execution of the next command. Wait steps can be placed inside of a TimeStep interval provided that the total time required for command execution plus wait steps is shorter than the time step interval. [Less precise]	Time interval in seconds
Paste to Slow Chart Comment Line	Writes the content of the last comment-generating line (printed in blue) into the title line of the Slow Kinetics window. The title line is saved in the “Record Comment File” (see Report window). When a script program contains several “Paste to Slow Chart” commands, only the last pasted line will be recorded in the “Record Comment File”.	None
Paste to Fast Chart Comment Line	Paste the content of the last comment generating line (typed in blue) into the title line of the Fast Kinetics window, which can be examined in “VIEW-mode”. Execution of the command requires that a Fast Kinetics measurement has been started.	None
Paste to ST-Kinetics Comment Line	Paste the content of the last comment generating line (typed in blue) into the title line of the ST-Kinetics window, which can be examined in “VIEW-mode”. Execution of the command requires that a ST-kinetics measurement has been started	

PARAMETER	COMMAND, COMMENT	INPUT
Message =	Halts script execution and displays a message. Click OK on the message window or hit the Enter key to continue execution of the script file.	Message title and message text.
Comment =	Inserts a comment line in the script file. A comment line can be written to the slow chart title using the “Paste to Slow Chart” command or to the fast chart title using the “Paste to Fast Chart” command.	Comment text
Spacer	Inserts an empty line after the currently selected line.	None
Exit	Terminates and exits a script file.	None
Section: -- General Commands --		
New Record	Starts a new Record.	None
Start Induction Curve	Starts recording of a slow kinetics measurement: equivalent to the <Start> button in the slow kinetics window.	None
Stop Induction Curve	Stops recording of a slow kinetics measurement: equivalent to the <Stop> button in the slow kinetics window.	None
Start Light Curve	Starts recording of a light curve: equivalent to the <Start> button in the light curve window.	None
Stop Light Curve	Stops recording of a light curve: equivalent to the <Stop> button in the light curve window.	None

PARAMETER	COMMAND, COMMENT	INPUT
Copy Trig. Run from Fast Kinetics	Copies the trigger pattern of the Fast Kinetics Curve currently displayed. Note that fast kinetics files contain fluorescence data as well as relevant trigger pattern and instrument settings.	None
Open Fast Kin. Trig. File	Activates trigger pattern of trigger file “filename.FTM” in directory “C:\PamWin_4\Fast Kin Trigger”	File name
Open User Settings	Activates stored instrument settings. File name format: filename.DEF. Default settings: Walz_MC.DEF.	File name
Open PAR-List	Activates a particular PAR-List, allows toggling between different PAR-Lists established for particular measurement conditions (e.g., high and low light).	File name
Open Lcp File	Opens standard light curve program file optimized for various types of samples.	File name
Clock	Starts or stops the repetitive trigger.	Check/uncheck
Clock Time	Sets or modifies clock interval.	Clock interval in s (=s). Increment in s to decrease (-s) or increase (+s) the current clock interval
Clock Mode=	Selects the action to be triggered by the clock. Note that the drop-down list for triggered events differ between the SP-Analysis and Fast Acquisition modes.	Select from drop-down list
eSP Mode	In the eSP mode the $F_M´$ value is calculated on the basis of the O-I_1 amplitude	Checked is on/unchecked is off

PARAMETER	COMMAND, COMMENT	INPUT
Stirrer	Switches stirrer on/off. Stirring is brought in standby mode by the stirrer switch on the front panel of the control unit.	Check on/off
Measure PAR Lists	Measures for all 20 intensity settings and the active measuring light color the PAR of measuring light, actinic light and multiple turnover pulses.	None
Measure Zoff List	Measures zero offset for all measuring light colors at current gain setting.	None
Zoff List for all gain settings	As indicated, this command determines the Zoff lists for all gain settings. It is an extension of the previous command.	Checked is on/unchecked is off
Kinetics auto save	Saves kinetics to hard disk immediately after acquisition.	Check on/off
PAR sensor	Reads the PAR value of the connected PAR sensor.	Checked is on/unchecked is off
Section 2: -- Determination Commands --		
Fo,Fm	Determines F_0 and F_M fluorescence level and calculates F_V/F_M.	None
Sat-Pulse/Fast Kin.	Performs saturation pulse analysis (in SP Analysis Mode). Performs fast kinetics (in Fast Kinetics Mode) as defined by the loaded trigger file.	None
Fo	Determines F_0 fluorescence level.	None
Fo´	Determines F_0' fluorescence level.	None
F_0´-mode	Saturation Pulse is followed by 5 s of far-red light to determine F_0´.	None

PARAMETER	COMMAND, COMMENT	INPUT
FR+Yield/Fast Kin.	Performs far-red pre-illumination followed by saturation pulse analysis (in SP Analysis Mode) or followed by fast kinetics (in Fast Kinetics Mode) as defined by the loaded trigger file.	None
AL+Yield/Fast Kin.	Performs pre-illumination by actinic light followed by saturation pulse analysis (in SP Analysis Mode) or followed by fast kinetics (in Fast Kinetics Mode) as defined by loaded trigger file.	None
Start ST-flash procedure	Executes the flash protocol defined in the ST settings window.	None
Section 3: -- Actinic Light Commands --		
AL	Switches actinic light on/off.	Check on/off
FR	Switches far-red light on/off.	Check on/off
BL	Switches blue light on/off. This command can, at the moment, not be used.	Check on/off
TR	Switches trigger on/off	Check on/off
PS I Light =	Switches between far-red and blue PS I light. This command can, at the moment, not be used.	Check Far red/Blue
Act. Light =	Switches between red and blue light	Check Red/Blue
ST	Triggers single turnover flash but does not record it!	None
MT	Triggers multiple turnover pulse but does not record it!	None
TR Pulse	Applies 5 V trigger pulse at AUX socket.	None

PARAMETER	COMMAND, COMMENT	INPUT
Section 4: -- Measuring Light Commands --		
F ML	Switches measuring light on/off.	Check on/off
MF-H	Switches measuring light frequency between low and high as defined in the settings window.	Check on/off
Section 5: -- General Settings --		
Analysis Mode	Selects between SP-Analysis and Fast Acquisition mode.	Check selection
Recording Mode	Selects type of slow kinetics measurement (manual, induction, or induction and recovery).	Drop-down list
Set Gain	Sets or modifies gain (1 to 10).	Gain setting (= #). Increment for decrease (- #) or increase (+ #)
Set Damping	Sets or modifies damping (1 to 8).	Damping level (= #). Increment for decrease (- #) or increase (+ #)
Fast Kin. Averaging	Averages a series of kinetic measurements.	Checkbox
Target Averages	Sets or modifies the number of fast kinetics measurements to be averaged.	Number of kinetics (= #). Increment number for decrease (- #) or increase (+ #)
New Fast Kin. Average	Stops averaging of fast kinetics measurements and saves averaged kinetics.	None

PARAMETER	COMMAND, COMMENT	INPUT
Auto ML on	Turns on measuring light just before start fast kinetics measurements, and switches measuring light off immediately thereafter.	Checkbox
Keep F_0,F_M	Off: Determines F_0 and F_M values at start of each induction or light curve. On: Uses the initial F_0 and F_M values for all subsequent saturation pulse analyses.	Check on/off
Read with Start Cond	Activates instrument settings of a trigger file when a trigger file is loaded. See also: Copy Settings from Fast Kin.	Check on/off
Apply Zoff List	Activates offset subtraction from fluorescence signal	Check on/off
Set ST-flash Target Nbr	This number defines the number of flashes in a flash train	1 to 19
ST-flash repetition time =	Time interval between the flashes in a flash train	50, 100, 200 … 1000 ms
Section 6: -- Actinic Light Settings --		
AL Color	Select actinic light color.	Check one of 6 colors
Multi Color ST	Triggers a single-turnover flash that makes use of all actinic LEDs.	Check on/off
	Sets intensity levels of:	
AL-Int.	Actinic light,	1 to 20
BL-Int.	Blue light, [cannot be used]	1 to 20
FR-Int.	Far-red light,	1 to 20
SP-Int.	Saturation pulse,	1 to 20
Fm-SP-int.	Saturation pulse for dark-acclimated sample	1 to 20

PARAMETER	COMMAND, COMMENT	INPUT
	Sets illumination time interval for	
AL Width	Actinic light	0 - 900 s
BL Width	Blue light [cannot be used]	0 - 900 s
FR Width	Far-red light	0 - 10 s
SP Width	Saturation pulse	0.1 - 0.8 s
SP Width (FoFm)	Saturation pulse for dark-acclimated sample	0.1 - 0.8 s
	Sets time interval of	Drop-down list
ST Pulse Width =	Single turnover flash.	5 - 50 µs
MT Pulse Width =	Multiple turnover pulse.	1 - 300 ms
TR us-Pulse Width =	Short (sub ms) 5 V trigger pulse.	10 - 250 µs
TR ms-Pulse Width =	5 V trigger pulse (sub s).	1 - 1000 ms
	Sets increase/decrease time interval of:	
ST Pulse Width Step =	Single turnover flash (increment = 5 µs),	Increment number to decrease (- #) or to increase (+ #)
MT Pulse Width Step =	Multiple turnover pulse (increment = 1 ms for width < 10 ms else 10 ms),	
TR us-Pulse Width Step =	Effective for short (sub ms) 5 V trigger pulse (increment = 10 µs),	
TR ms-Pulse Width Step =	Effective for longer (sub s range) 5 V trigger pulse (increment = 1 ms for width < 10 ms else 10 or 20 ms).	
ST sequence number	Defines the number of single turnover flashes in a flash sequence or increases/decreases the flash number of a flash sequence.	Number of flashes; decrease (- #) or increase (+ #) number of flashes. Valid entries: 1 to 20

PARAMETER	COMMAND, COMMENT	INPUT
ST sequence width	Defines time interval between consecutive single turnover flashes or increases/decreases time interval.	10 to 500 ms, time interval of decrease (- # ms) or increase (+ #ms)
Fast Kin. after ST sequence	Starts fast fluorescence kinetics measurement after sequence of single turnover flashes.	Check on/off
Section 7: -- Measuring Light Settings --		
Auto AL Color Selection	Links or unlinks the wavelengths of ML and AL	Check Auto AL Color
ML Color	Sets color or measuring light	Check color
MF-H =	High measuring light frequency (Hz).	1000-100000 (Drop-down list)
MF-L =	Low measuring light frequency (Hz).	10-5000 (Drop-down list)
MF-H Step	Stepwise increases/decreases the high measuring light frequency (variable increments depending on present frequency).	Increment number to decrease (- #) or to increase (+ #)
MF-L Step	Stepwise increases/decreases the low measuring light frequency (variable increments depending on present frequency).	Increment number to decrease (- #) or to increase (+ #)
F ML-Int =	Sets Intensity of fluorescence measuring light.	1 - 20
AutoMF_H	Switches to high measuring light frequency when actinic light is on.	Check on/off
MF Log	Decreases measuring light frequency in logarithmic fashion after termination of MF-max in fast kinetics measurements.	Check on/off

PARAMETER	COMMAND, COMMENT	INPUT
Section 8: -- Condition Commands --		
If Ft	Executes next command if Ft is greater (>) / smaller (<) than the threshold value entered	Ft in Volt
If Temp	Executes next command if temperature is greater (>) / smaller (<) than the threshold value entered	Temperature in °C
Else	2nd part of an If … Else argument; provides an alternative choice, defined in the subsequent line, which is executed when the If argument is not met.	None
Wait until Ft	Interrupts script file execution until Ft is greater (>) / smaller (<) than the threshold value entered	Ft in Volt
Wait until Temp	Interrupts script file execution until temperature is greater (>) / smaller (<) than the threshold value entered	Temperature in °C
End of Rep. Block; Repeat until Ft	Terminates a repetition block if Ft is greater (>)/ smaller (<) than the threshold value entered	Ft in Volt
End of Rep. Block; Repeat until Temp	Terminates a repetition block if temperature is greater (>) / smaller (<) than the threshold value entered	Temperature in °C
Wait until clock counter	If the clock counter reaches the set value, the next command is executed	Number
Section 9: -- ST-settings --		
ST Rep. Time =	Time between flashes in the flash train	10 and 2000 ms

PARAMETER	COMMAND, COMMENT	INPUT
ST Target Nr.	Defines the number of flashes in a flash train	1 to 100 flashes
ADC-resolution =	Conversion of analog to digital signal	12 or 14 bits
Signal Offset =	Sets or modifies the Offset value	Offset setting (= #). Increment for decrease (- #) or increase (+ #)
Signal Gain =	Sets or modifies the gain (1 to 4)	Gain setting (= #). Increment for decrease (- #) or increase (+ #)
Block Points =	Nr of measuring points that will be acquired	100 to 15000 points
ADC-Frequency =	Measuring point acquisition rate	1 to 40 MHz
Pulse Amplitude 1	The flash intensity of the 1st flash	800 to 3300 mV
Pulse Width 1	Length of the 1st flash	1 to 14000 µs
Pulse Delay 1	Time interval between 1st and 2nd flash	1 to 10000 µs
2nd Pulse	Activation of 2nd and more flashes	Check 2nd Pulse
Pulse Amplitude 2	The flash intensity of the 2nd and subsequent flashes	800 to 3300 mV
Pulse Width 2	Length of the second and subsequent flashes	1 to 10000 µs
Pulse Delay 2	Time interval between second and subsequent flashes	1 to 10000 µs

PARAMETER	COMMAND, COMMENT	INPUT
Pre-Flashes	Number of pre-flashes	1 to 100
Pre-Flash Amplitude	The pre-flash intensity in mV	800 to 3300 mV
Pre-Flash Width	Length of the pre-flash in µs	1 to 100 µs
Pre-Flash Rep. time	Time interval between pre-flashes	10 to 2000 ms
Pre-Flash Delay	Time interval between pre-flashes and measurement (flash(es) or flash train)	20 to 10000 ms
Section 10: -- ST Commands --		
Start ST-recording	Executes an ST-Kinetics protocol either defined in the ST Settings Tab or by the script	None

7 ST-kinetics

7.1 ST settings window

A completely new Tab is the ST Settings Tab where the user can define his/her flash measurements.

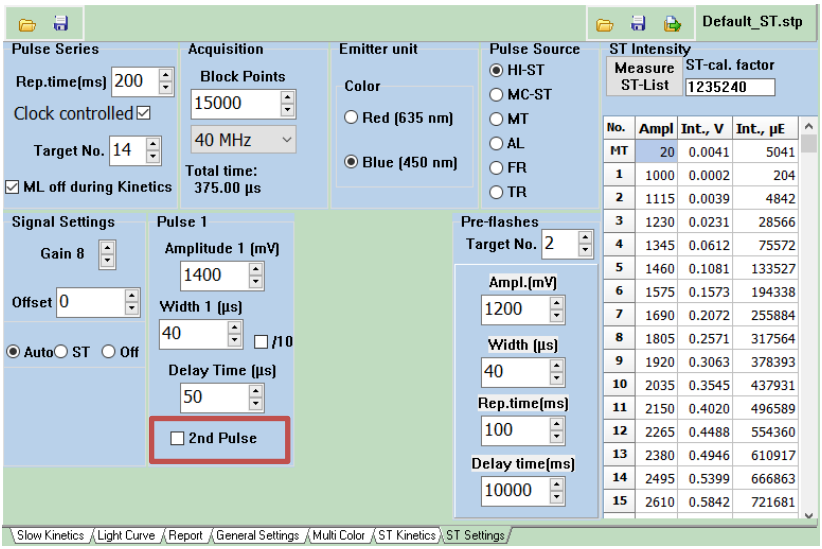


Fig. 38: If the <2nd pulse> box is unchecked, the associated Pulse 2 [ST] section is invisible.

Not all sections are visible at all times. Unchecking the 2nd Pulse box will make the Pulse 2 [ST] section disappear (Fig. 38, pag. 143).

And only a <Rep. No> of 2 or higher will make the Rep. Mode menu at the bottom of the Pulse 2 [ST] section visible (Fig. 39, page 144).

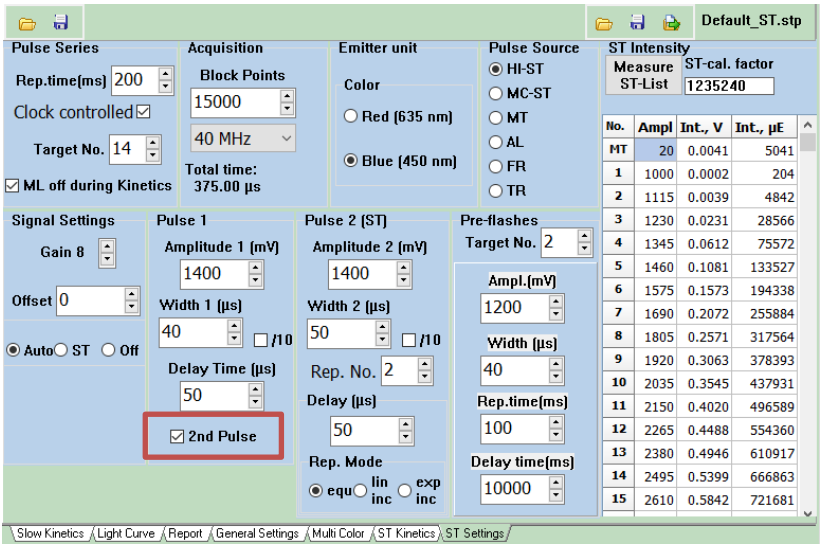


Fig. 39: Checking the <2nd pulse> box makes the Pulse 2 section visible.

Section Pulse 1 – this part of the page defines a first or single flash. <Amplitude 1 [mV]> defines the flash intensity, where values below 800 mV are too low to induce a flash and the maximum flash intensity is set if 3300 mV is chosen. At the highest flash intensities, the fluorescence signal is likely to go off-scale. Using pinholes, reducing the area measured, the signal can be brought back on scale. The only two other tools the user has, to bring the signal back on-scale are a lowering of the <Gain> or of the “Amplitude”. <Width 1 [µs]> gives the duration of the flash, in Fig. 38 and Fig. 39 40 µs. The minimum value is 1 µs, but if the </10> box is checked, values as low as 0.1 µs are possible. However, the flash lamp needs about 0.5 µs to reach its maximum intensity. The maximum value that can be chosen is 14000 µs. The <Delay Time (µs)> defines the time between the first and a possible second flash. To activate the menu for second pulses the <2nd pulse> box should be checked (Fig. 39).

Section Pulse 2 [ST] – In this section a second (or multiple) flash(es) can be defined. Again, <Amplitude 2 [mV]> defines the flash intensity and <Width 2 [µs]> defines the length of the flash and by checking the </10> the minimum flash length can be reduced to 0.1 µs. <Rep. No.> defines the number of 2nd flashes and <Delay [µs]> defines the time interval between those 2nd flashes. However, via <Rep. mode> (see below) this interval can be further modified.

<Rep. Mode> - giving several 'second pulses' the user can chose 'equ', which stands for 'equal' and means that all flashes are applied the set delay time apart; 'lin inc', which stands for 'linear increase', means that the time between the flashes is increased from 50 to 100 to 150 µs, etc. Choosing 'exp inc', which is 'exponential increase' means that the flash interval would go from 50 to 100 to 200 to 400 µs, etc.

Pulse Series section:

<Rep.time(ms)> - this parameter determines the time interval between flashes in a flash series/train. Values between 10 and 3000 ms can be chosen. For the determination of period-4 oscillations a flash frequency of 20 Hz or less should be chosen. That is a flash interval of 50 ms or more. To get the best oscillation amplitudes, time intervals of around 200 ms may give better results, but the user should test this for his or her samples. However, the flash interval determines as well the time resolution of the flash train. If the process to be probed is faster than the time interval chosen, the process will not be detected. At the highest flash intervals, the period-4 oscillations are no longer detected. Flash intervals of ~10 ms may be used for the stepwise reduction of the photosynthetic electron transport chain. That flash rate exceeds the recombination rate (Q_A^- with the PS II donor side) by more than an order of magnitude. The

reduction of the PQ pool will lead to an increase of the flash-induced fluorescence amplitude.

<Clock controlled> - If the user wants flash intervals that are longer than 3 s, it is possible to check the <Clock controlled> box. Such flash intervals are too long for the measurement of period-4 oscillations, but there may be other applications.

<Target No.> - this term is maybe not immediately obvious, but it simply defines the number of flashes in a flash train. To detect period-4 oscillations a value of ~14 (or more) may be recommendable.

ML off during Kinetics: The measurement of the flashes is non-modulated and, therefore, the measuring light can be off during the measurement of the flashes. If the experiment only consists of flashes, then it is in most cases better to leave the measuring light completely off. However, if the user is interested in the fluorescence decay kinetics following a flash, the measuring light should remain on. See a discussion on STKs embedded in a modulated measurement below.

Flash trains and pre-flashes are a logical combination. With one or two pre-flashes the S_2, respectively, S_3 state can be induced and by giving a flash train Δt later, the decay of these S-sates back to S_1 can be monitored.

Pre-flashes can be defined in the Pre-flashes section.

<Target No.> - Here the number of pre-flashes is defined. If the value 0 is chosen, the other settings in this section will disappear. The maximum value for this setting is 100 pre-flashes. The user should be aware of the fact that the period-4 oscillations dampen as a function of the flash number. Defining a large number of pre-flashes may be meaningful if the user strives for a state in which all S-states make an approximately 25%

contribution. Such a state may be practical when the user wants to minimize flash-dependent contributions of the S-states to the measurements.

The other pre-flash parameters - <Ampl. (mV)> defines the flash intensity, <Width [µs]> defines the length of the flash, <Rep. time [ms]> defines the time interval between the pre-flashes, <Delay time [ms]> defines the time between pre-flashes and probe flash or flash train. The maximum delay time is 10 s. If the user does not use the software full screen, it may happen that the last 1-2 parameters are not visible.

Acquisition section:

For the measurement of the flashes the software allows up to 15000 points per flash is <Block points>. These points can be acquired with a frequency of 1, 2, 5, 10, 20 or 40 MHz. At 40 MHz a flash can be recorded for 375 µs, whereas at 1 MHz this is 15 ms. Since the measurements are non-modulated, the fluorescence intensity drops back to 0 almost instantly after the end of the flash.

Emitter unit section:

At the moment there is only a blue [450 nm] flash lamp available, because only the available blue LEDs are powerful enough to yield flashes of 1 million or more µmol photons m^{-2} s^{-1}. It is imaginable that such strong flashes are also saturating in the case of for example cyanobacteria (which have a low Sigma(II) in the blue part of the spectrum) and the same may be true for other photosynthetic organisms that may differ in the structure and composition of their outer antennae but still have Chl *a* containing core antennae. A red [635 nm] STK-flashlamp may be developed once suitable red LEDs become available.

Pulse Source section:

In the ST Settings Tab the choice of HI-ST [High intensity single turnover flash] pulse source seems to be the most likely, however, the software allows the user to choose as well the multicolor flashes <MC-ST>, multiple turnover pulses <MT>, actinic light <AL>, far-red <FR> or trigger <TR> pulses as pulse source.

ST Intensity section:

<Measure ST-List>-button – The SQS and MQS light meters measure light intensities up to 30000 µmol photons m^{-2} s^{-1}. The flash-light intensities of the MULTI-COLOR-PAM-II are much higher. To determine a PAR list for the ST-intensities, first the light intensity of a reference MT is determined, and this is used as reference for the ST intensities. The measurements are non-modulated and that means that the fluorescence emitted by the fluorescence reference is linearly related to the light intensity:

ST(Ampl)/MT(Ampl)*MT(intensity) = ST(intensity)

The maximum ST(intensity) that can be generated with the MULTI-COLOR-PAM-II flash lamp that could be determined this way was in the order of 1 200 000 µmol photons m^{-2} s^{-1} (is 1.2 mol photons m^{-2} s^{-1}).

On this basis an <ST-cal. factor> is determined.

In Fig. 39, the ST-cal. factor is 1235240 and if this factor is for example multiplied with Int., V value of setting 11 (0.4020), the flash intensity of 496589 µmol photons m^{-2} s^{-1} is obtained.

Signal Settings section:

The last section on the ST settings page is Signal Settings.

For the parameter <Gain> the user can choose between the settings 1, 2, 4 and 8

Below <Gain>, <Offset> is found. Here the user can choose between ‘Auto’, ‘ST’ and ‘Off’. The default choice is ‘Auto’.

Offset refers to the fluorescence offset of the light source used/selected in this tab (signal indicated in the absence of light).

When ‘Auto’ is selected the instrument will detect the light source used and determine the offset. If ‘ST’ is chosen the user indicates that the single turnover flashes of the STK flashlamp are the light source and that for this light source the offset should be determined. If ‘Off’ is chosen, no offset will be determined.

7.2 ST kinetics window

Most of the space in this window is taken up by the graph window. Beyond that, there is a band of icons above the graph window and a column with functions to the right side of the graph window.

Above the ST-kinetics window there is a band with the following function icons:

First icon of the band is the ‘Start ST recording’ icon.

What exactly is recorded is determined by the settings in the ST Settings window. If the number of pre-flashes is >0, the measurement will start with the defined number of pre-flashes. Then, it will execute the flash defined in the Pulse 1 section. If the 2nd Pulse is activated the measurement will continue with the flash or flashes defined in the Pulse 2 section. If in the Pulse Series section the Target No. is >1, then software will repeat the flash

combination defined by Pulse 1 and 2 as many times as indicated by the <Target No.>.

If the user does not want all of this, he/she can for example set the <Target No.> to 1 and/or inactivate the 2nd pulse and or reduce the <Target No.> of the pre-flashes to 0.

The second icon of the band, here with a green point in the middle, is the 'Signal Overflow' icon. If the point in the middle turns red, it indicates that a signal overflow has been detected and that either the flash amplitude and/or the Gain has to be reduced and/or a pinhole has to be inserted.

The third icon, which looks like an empty page, is the 'Clear All' icon. It clears all measurements from the ST Kinetics window. It is comparable to the <New Record> button of the PAM side of the software.

The fourth icon, which suggests the opening of a folder, is the 'Open Files' icon. Clicking this icon opens the STKineticsData folder.

The fifth icon, the diskette icon, saves files selected by the user. These files are found on the right-hand side of the graph window.

The sixth icon, a folder with an arrow pointing to the right, is the 'Export Selected' icon and saves selected files in the form of *.csv or excel files, which can then be imported in a spreadsheet program.

The seventh icon, an empty square (when active a folder with a green arrow pointing up), is the 'Export Interval Data'. It is only active when the interval data of a flash train are being analyzed (the flash dependence of F_0, F_V and F_M, for example). With this button such data can be exported as either *.csv files or Excel files.

The eighth icon, a letter A, is the ‘autoscaling’ button.

The ninth icon, the IA button (see also Fig. 40), is the ‘interval analysis’ button. Clicking it extracts the amplitude for a selected time interval from a flash series and plots the flash dependence in the ST Kinetics window.

The tenth icon is the N button, clicking it allows the normalization of a selected measurement.

The eleventh icon is the + button, clicking it will average measurements selected by the user.

The twelfth icon is the - button, clicking it will subtract or invert a measurement.

The thirteenth icon is the Z button, clicking it, zeroes a selected measurement.

The fourteenth button is the S button, when clicked, the scale is kept.

Finally, the t and F windows monitor the position of the cursor inside the ST kinetics window.

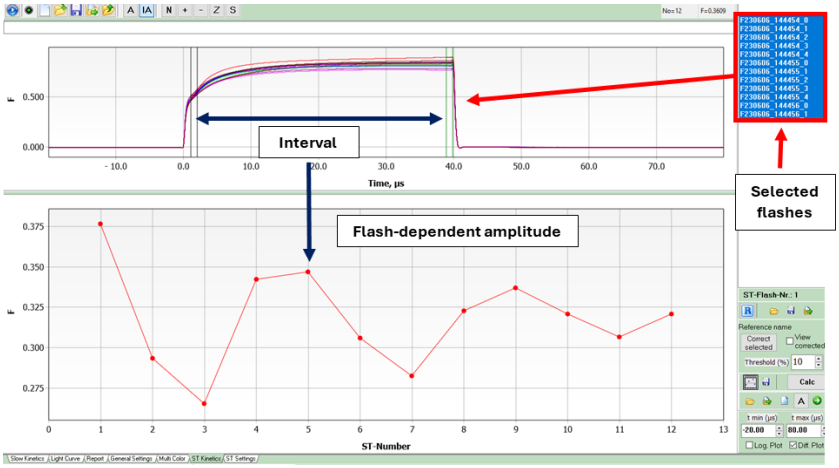
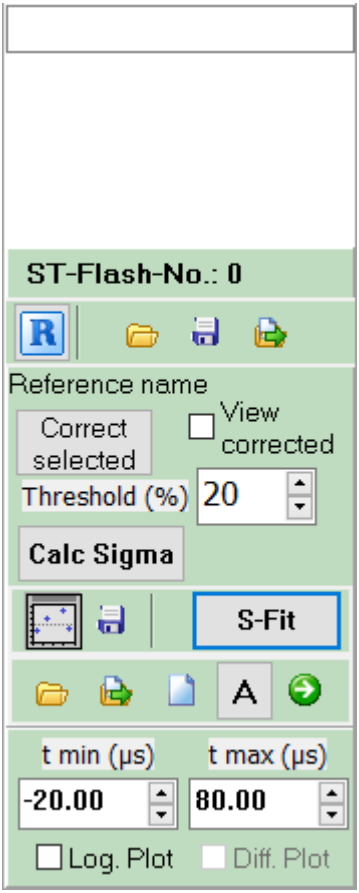


Fig. 40: The Interval Analysis consists of an upper window showing the transients of the selected set of flashes and a bottom window which extracts the average values of the selected windows and in this case determines the difference between maximum and minimum value and plots the difference as a function of the flash number.



Side Bar

ST-Flash-No.

The ST-Flash-No. gives the number of files available for selection. Here this number is 0, because all files were cleared away by clicking the white page icon (see above).

R

If the user selects a measurement and then clicks on <R>, it will set this measurement as reference.

Folder icon

By clicking on the folder icon next to R it is also possible to open a directory (the STKineticsData directory); the user can import a reference measurement from there.

Diskette icon

The Diskette icon on the righthand side can be used to save a reference measurement for later use.

Icon with green arrow

By clicking the folder with green arrow pointing right it is also possible to export a reference measurement (to be imported in a spreadsheet program like Excel)

Correct selected

Clicking this button corrects the selected measurements for the reference measurement and checking the <View corrected> box sees to it that the corrected measurements are plotted in the ST Kinetics window.

Threshold (%)

As mentioned above the flashes need about 0.5 µs to reach their maximum intensity. For this reason, at the shortest times (less than approx. 100 ns) the increasing flashlight intensity is likely to dominate the fluorescence rise so much that interpretation becomes problematic. By choosing a threshold value the measurement data at times shorter than the threshold will be ignored.

Calc Sigma

Clicking the <Calc Sigma> button opens the fit window for O-I1 fits.

The first icon, the ‘get model kinetics’ icon can extract information on the fluorescence rise kinetics of a single flash. The diskette icon can then save this kinetic information.

S-Fit

S-state fit, with this button a fit of the period-4 oscillations can be initiated on the basis of which, for example, the initial S-state distribution can be determined.

The five icons below the ‘get model kinetics’ are related to this topic:

The first icon, the folder icon, is the ‘open model kinetics’ icon with which model kinetics that were saved before can be opened.

Second icon, the icon with the green arrow, is the ‘export model kinetics’ icon which can export the model kinetics data in *.csv format.

The third icon, the empty page icon, clears the model kinetics away.

The fourth icon, the letter A, is the ‘autoscaling’ icon, which allows the optimal use of the graphical area for the presentation of the model kinetics.

The fifth icon, a white right pointing arrow on a green dot, is the fit kinetics icon, which exports the fit data of the period-4 oscillations in *.csv format.

Scale time axis

Below these five icons the user can define the width of the time-axis of the graphical window.

Log. Plot

Checking the <Log. Plot> box changes the linear timescale of the graphical area into a logarithmical timescale

Diff. Plot

If the user makes an interval analysis two time-dependences are initially shown. The ‘F_0’ (or the minimum selected value) and ‘F_M’ (or maximum selected value). Checking the Diff. Plot box

takes the difference between these minimum and maximum values and plots this difference (the “variable fluorescence”).

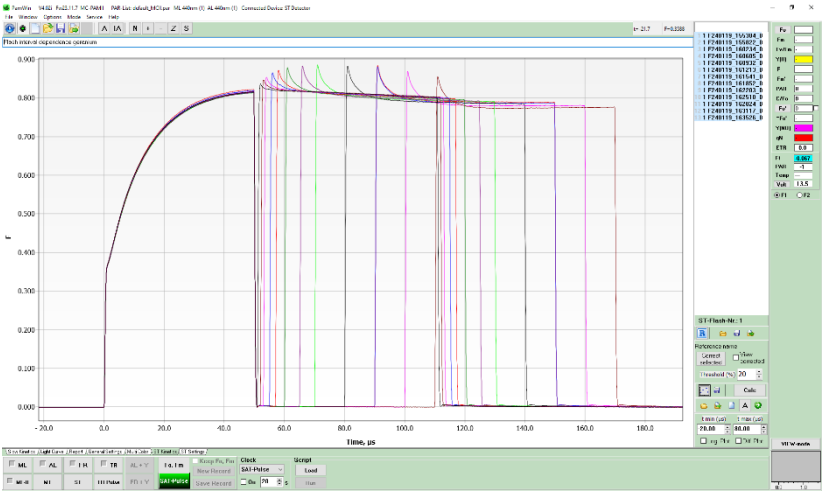


Fig. 41: Example of a double flash measurement. The position of the first flashes shows that the timing of the flashes is very precise. By selecting multiple files, it is possible to overlay these measurements in the graphical window. In this case, the time interval between first and second flash was varied between 1 and 60 µs.

8 VIEW-Mode

VIEW-Mode Windows

Slow Kinetics
Light Curve
Report
Fast Kinetics
Multi Color
ST Kinetics

In “VIEW-mode” six different windows, which have been introduced in previous chapters, are available. In the present chapter, additional features provided by “VIEW-mode” will be explained.

Generally, the “VIEW-mode” of the PamWin-4 software allows offline analyses of fluorescence measurements. This includes editing of titles of records and measurements, as well as file names. If you want to modify the file name of a Report, however, make sure that Report file name (filename.RPT3) and directory name are identical.

Offline analyses can (also) be performed on computers which are not connected to the MULTI-COLOR-PAM-II. To analyze Report data on an offline computer, install PamWin-4 on this computer and copy the Report data folder from the measuring computer (C:\Program Files (x86)\PamWin Folder\Data_MCII\Report\YYYYMMDD) to the corresponding folder on the offline computer.

8.1 Structure of Data

The highest organizational data level in PamWin-4 is called “Report”. A Report consists of one or several Records. A Record file contains experimental parameters like instrument settings and changes of these settings. Further, fluorescence levels and fluorescence ratio parameters of saturation pulse analyses are

part of a Record as well. The Report file refers to different types of data files, which can be analyzed in VIEW-mode. Depending on file type, the name of these data files starts with a different letter:

First Letter of File Name	Type of measurement
F	Fast fluorescence kinetics
I	Dark-light fluorescence induction curve
L	Slow fluorescence kinetics of light curves
M	Manual slow kinetics
S	Saturation pulse-induced fluorescence kinetics

8.2 Menu Bar

The file menu of the menu bar (cf. Section 0, page 32) of the MULTI-COLOR-PAM-II contains two new items: <Open pws-file> and <Export as CSV file>.

File Menu

VIEW-mode	MEASURE-mode
Open Report	New Report
Import Old Report	Import Old Report
Load Script	Load Script
Open pws-file	Run Script
Export as csv-file	
Printer Setup	Printer Setup
Print Graph	Print Graph
Exit	Exit

<Open pws-file> is available for the 'Slow Kinetics' and the 'Light Curve' windows. The corresponding icon is available only in the 'Slow Kinetics' window (see Table below). The command opens a list of all slow kinetics in the current Report. From this list, a slow kinetics file can be selected for display.

The second new feature is <Export as csv-file>. The command stores slow kinetics and corresponding Record data as comma-separated values files (*.CSV) in the export folder of the Pam-Win-4 software (C:\Program Files (x86)\PamWin Folder\ Data_MC\Export). The export dialogue (but not the export icon) offers the option to export all slow kinetics and Record data of the Report. In this case, data will be stored in a new directory that will be created in the export folder. The directory name is that of the current Report.

VIEW-Mode Window	Icon	Command
Slow Kinetics		Open slow kinetics (pws-file)
Slow Kinetics Light Curve Report		Export saturation pulse data of currently selected Record and slow kinetics as CSV file into directory C:\Program Files (x86)\PamWin Folder\Data_MC\Export.
Show Slow Kin. Settings		On clicking this icon, a popup window opens
Show Comment file		On clicking this icon, a popup window opens in which the user can make notes.
Slow kin. Average		This function allows the user to average several slow kinetics measurements.

8.3 Side Bars

8.3.1 Record-Associated Side Bar

General View Control

The main switch with respect to navigation through the data is the general view control (Fig. 42, page 161; see also Table 10, page 82). Selecting “Rpt” provides an overview of the Records of the current Report. Each Record contains key information such as date and time of the measurement and notes; in case of slow kinetic measurements, the type of kinetics and the number of saturation pulse analyses are given.

To view a particular Record, double-click on the corresponding line of the Record or select Record from the “Select Record” drop-down list on the rightmost side bar (Fig. 42, page 161, “General Side Bar”).

$F_M´$ Factor

The $F_M´$ factor is a multiplier for $F_M´$ values only; the F_M is not changed by the $F_M´$ factor. This factor can be set to values > 1 if $F_M´$ is underestimated due to measuring artefacts. Indeed, an increase in the LED-chip temperature during a saturation pulse can decrease the intensity of pulse-modulated measuring light and, thus, result in $F_M´$ underestimation. In the MULTI-COLOR-PAM-II, this underestimation is exceptionally small as ML and SP (=AL) LEDs are separated: even with an SP-intensity setting of 20 and SP-width of 800 ms, underestimation is smaller than 0.5% of $F_M´$ for all colors except 590 nm where the decrease amounts to about 1%. Consequently, the default $F_M´$ factor (1.000) is appropriate in most cases.

The “SP artefact” can be detected using the plastic “fluorescence standard” delivered with the instrument. For a correct determination, however, one needs to consider that the standard foil displays a rather small heat capacity and heating of the fluorescence standard during exposure to the saturation pulse may also cause a small decrease in chlorophyll fluorescence yield.

The latter artefact can be minimized by placing a narrow strip of the fluorescence standard in a water-filled cuvette.

The standard foil is a relatively strong fluorescence emitter, which means that signal saturation can occur. Therefore, if the signal exceeds 6 V, adjust the distance between detector and the fluorescence-guiding Perspex rod appropriately.

Determination of the $F_M´$ factor should be carried out using the same settings of color, SP intensity and SP-width as used for the physiological measurements. It is important that <MF-High> is already switched on before the SP is applied, as an increase of the ML pulse frequency may also cause a temperature increase and, thus, a decrease in measuring light intensity. The latter artefact does not play a role during actinic illumination when <Auto MF high> is selected because the high measuring light frequency <MF-High>, which is automatically triggered, remains on during the SP.

The SP-induced signal decrease can be determined via a manual slow kinetics recording. The $F_M´$ values listed in the Report do not provide the required information, as these values are measured with the help of a peak-detector.

Finally, the F_M' factor can be calculated as the fluorescence level prior to a saturation pulse divided by the fluorescence level immediately following a saturation pulse measured on the standard foil. An F_M' factor continues to be assigned to a Record, unless changed.

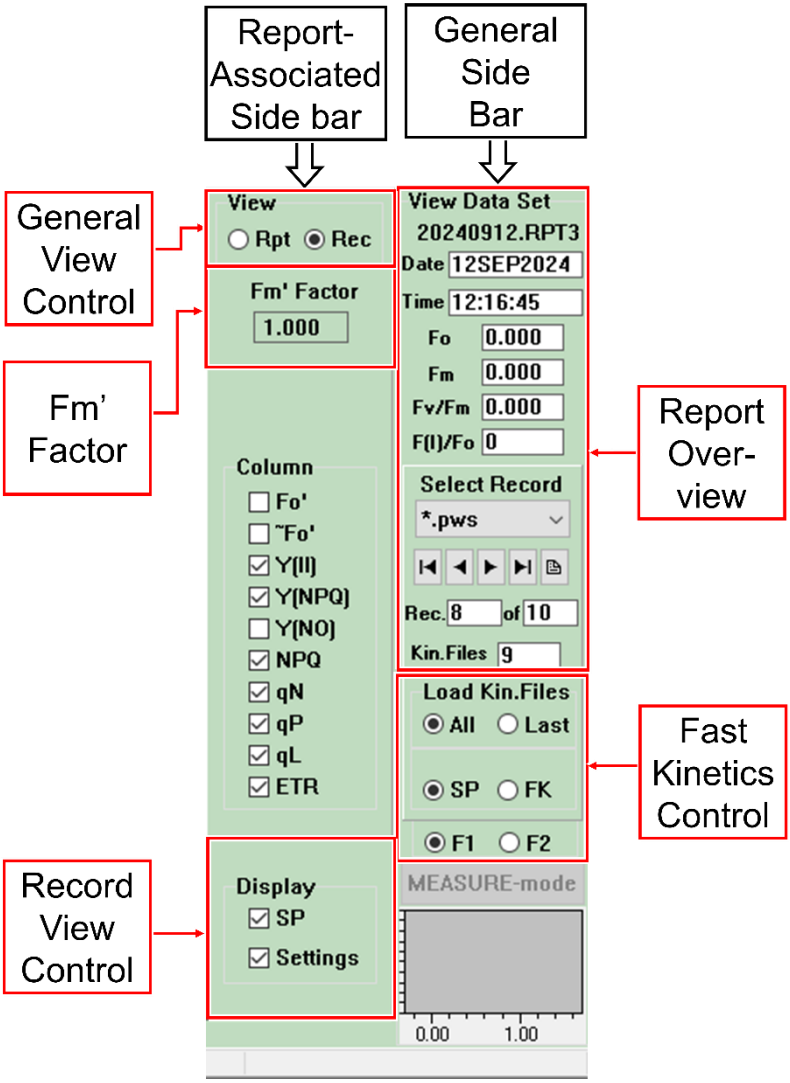


Fig. 42: Side bars in the VIEW mode.

Record View Control

This section of the side bar controls to which extent the Record data are displayed. Checking the <SP> box, the file names of saturation pulse kinetics are displayed. Checking the <Settings> box, the initial settings of the Record and changes of settings made to the Record (e.g., actinic light intensity) are displayed.

Fast Kinetics associated side bar

A feature missing in <Measurement> mode is <Alter PAR Lists>. Clicking the associated box opens the list with PAR Lists. An alternative PAR List can be selected, which remains active until the box is unchecked and the original PAR List is re-activated.

8.3.2 General Side Bar

Report Overview

This part of the “General Side Bar” provides tools for scrolling through the current Report.

The downward arrow of the text box “Select Record” opens the list of available PWS files from which a Record file can be selected. Below the text box, four arrow buttons permit selection of a Record (From left to right: first, previous, next, last Record). The

right-most icon opens a new Report file and, thus, is equivalent to the command <Open Report> in the File menu.

Below the arrow-buttons, the number of the currently selected Record and the total number of Records in the current Report are shown (e.g., Rec. 3 of 17). A double click on the number of

the field “Rec.” opens a new window. In this window the Record to be displayed next can be chosen (e.g., double click on 3 and then type in the window 15 to go to record 15 of 17).

The General Side Bar also shows the total number of Fast Kinetics files of the current Record (Kin. Files). Fast Kinetics can be picked from the drop-down list of the “Fast Kinetics” window, provided that <FK> on the “General Side Bar” is checked.

Fast Kinetics Control

This part of the side bar offers two switches. First, switching between “All” and “Last” determines whether opening of a Record occurs with loading of all fast kinetic files associated with the Record or with loading of only the last kinetic file of the Record. The latter option has advantages when computer performance limits the speed of data evaluation.

The second switch determines if fluorescence kinetics induced by saturation pulse (SP) or fast kinetics (FK) are displayed in the Fast Kinetics window. Depending on the choice made, the drop-down list of the Fast Kinetics window lists file names of SP or FK curves.

9 Definitions and Equations

9.1 Relative Fluorescence Yields

Typically, five different types of fluorescence levels are acquired by Saturation Pulse analyses. In most cases, the PAM fluorescence signal is proportionally related to the yield of chlorophyll fluorescence. Therefore, differences between these five fluorescence levels reflect variations in chlorophyll fluorescence yields.

Two of these levels (F_0 and F_M) need to be determined with a dark-acclimated sample. The three remaining levels (F_0', F, and F_M') are repeatedly measured during subsequent sample treatments (e.g., exposure to actinic light; see Fig. 43, page 167).

9.1.1 Measurements with Dark-Acclimated Samples

F_0 Minimum fluorescence level; samples excited by very low intensity measuring light to keep PS II reaction centers essentially open.

F_M Maximum fluorescence level induced by a pulse of saturating light (Saturation Pulse) which closes all PS II reaction centers.

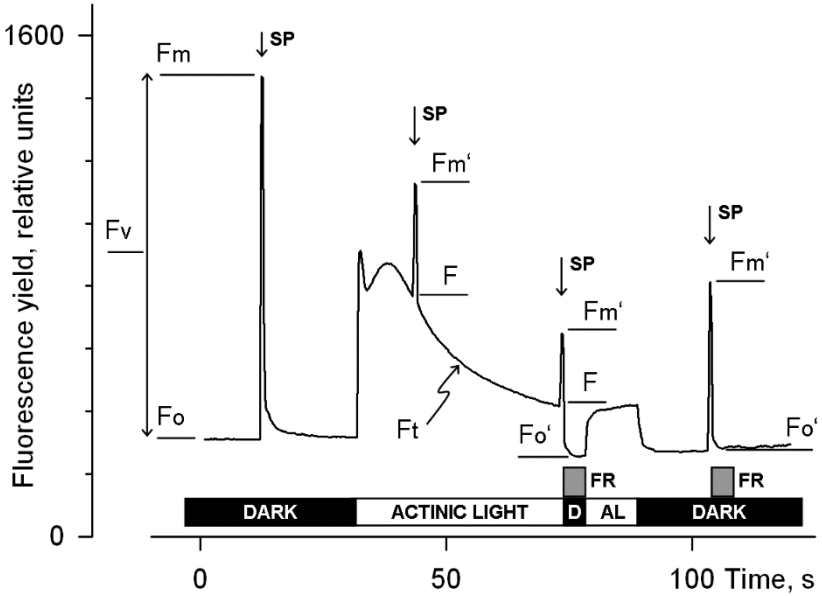


Fig. 43: **Measurements for Saturation Pulse Analysis. AL, Actinic Light; D, dark; SP, Saturation Pulse; FR, Far-red illumination.**

9.1.2 Measurements with Illuminated Samples

F_0' Minimum fluorescence level of illuminated sample, which is in most cases lowered with respect to F_0 by non-photochemical quenching. When the measuring routine for F_0' is active, the F_0' level is determined during a dark interval following the Saturation Pulse. In the dark interval, far-red light is applied to selectively drive PS I and to quickly remove electrons accumulated in the intersystem electron transport chain, thereby facilitating reopening PS II reaction centers (see Fig. 43, page 165: time point 75 s). Alternatively, the F_0' can be estimated according

to Oxborough and Baker (1997; List of references at the end of Section 9.7, page 180):

$$Fo' = \frac{1}{\frac{1}{Fo} - \frac{1}{Fm} + \frac{1}{Fm'}}$$

F_M´ Maximum fluorescence level of an illuminated sample as induced by a Saturation Pulse, which temporarily closes all PS II reactions centers. F_M´ is decreased with respect to F_M by non-photochemical quenching.

F The F corresponds to the momentary fluorescence level (Ft) of an illuminated sample measured shortly before application of a Saturation Pulse.

9.2 Fluorescence Ratio Parameters

To quantify photochemical use and non-photochemical losses of absorbed light energy, fluorescence ratio expressions have been derived which are based on the relative fluorescence yields introduced above. In Table 17 (page 169) the fluorescence ratio parameters available in PamWin-4 have been compiled. Below, these parameters will be briefly explained.

F_V/F_M and Y(II) Maximum and effective photochemical quantum yields of PS II

The parameters F_V/F_M and Y(II) are a measure for the fraction of absorbed quanta used for PS II photochemistry. F_V/F_M corresponds to the maximum Y(II). For measurements of F_V/F_M, it is important that samples are acclimated to darkness or dim light so that all reactions centers are in the open state and non-photochemical dissipation of excitation energy is minimal.

Table 17: Fluorescence Ratio Parameters.

Parameter definition and source	Equation
Maximum photochemical quantum yield of PS II (Kitajima and Butler 1975)	$\frac{F_V}{F_M} = \frac{F_M - F_0}{F_M}$
Effective photochemical quantum yield of PS II (Genty *et al.* 1989)	$Y(II) = \frac{F'_M - F}{F'_M}$
Quantum yield of light-induced non-photochemical fluorescence quenching (Genty *et al*. 1996)*	$Y(NPQ) = \frac{F}{F'_M} - \frac{F}{F_M}$
Quantum yield of non-regulated heat dissipation and fluorescence emission: this type of energy loss does not involve the action of a trans-thylakoid ΔpH and zeaxanthin (Genty *et al*. 1996)*	$Y(NO) = \frac{F}{F_M}$
Stern-Volmer type non-photochemical fluorescence quenching (Bilger and Björkman 1990)	$NPQ = \frac{F_M}{F'_M} - 1$
Coefficient of photochemical fluorescence quenching (Schreiber *et al.* 1986 as formulated by van Kooten and Snel 1990)	$q_P = \frac{F'_M - F}{F'_M - F'_0}$
Coefficient of photochemical fluorescence quenching assuming interconnected PS II antennae affecting the whole F_0 to F_M rise (Kramer *et al.* 2004)	$q_L = q_P \cdot \frac{F'_0}{F}$
Coefficient of non-photochemical fluorescence quenching (Schreiber *et al.* 1986 as formulated by van Kooten and Snel 1990)	$q_N = 1 - \frac{F'_M - F'_0}{F_M - F_0}$

* Kramer *et al.* (2004) have derived more complex equations for Y(NO) and Y(NPQ). Klughammer and Schreiber (2008) have demonstrated that the equations of Kramer *et al.* (2004) can be transformed into the simple equations of Genty *et al*. (1996) which are used by the PamWin-4 software.

In algae and cyanobacteria, the dark-acclimated state often is not showing the maximum PS II quantum yield, as the PS II acceptor pool may be reduced in the dark by stromal reductants and consequently the so-called state 2 is induced exhibiting a low(er) PS II quantum yield. In this case, pre-illumination with moderate far-red light or background illumination with low intensity far-red light should precede/accompany the determination of F_0 and F_M. The far-red pre-illuminated quasi-dark state normally serves as reference state with maximum PS II quantum yield for assessment of the functional absorption cross-section of PS II: Sigma(II).

The Y(II) value is a measure for the photochemical use of excitation energy in the light. It is lowered with respect to F_V/F_M by partial closure of PS II centers and various types of nonphotochemical energy losses induced by illumination. To derive from the Y(II) information on the overall state/activity of photosynthesis, knowledge of the absorbed PAR is essential, as a sample may e.g., have a severely inhibited Calvin-Benson cycle activity and still show a high value of Y(II) in weakly absorbed light. This aspect is particularly important in the study of algae and cyanobacteria, which display large wavelength-dependent differences in light absorption.

q_P and q_L Coefficients of photochemical fluorescence quenching.

Both parameters estimate the fraction of open PS II reaction centers. The q_P is based on the concept of separated PS II antenna units (puddle model), whereas the q_L assumes interconnected PS II antenna units (lake model), which appears to be the more realistic situation in leaves (*cf.* Kramer *et al.* 2004). Determinations of q_P and q_L do not require fluorescence

measurements of the dark-acclimated sample unless F_0' is calculated according to Oxborough and Baker (1997) and not measured.

q_N and NPQ Parameters of non-photochemical quenching

Both parameters are associated with non-photochemical quenching of excitation energy, mainly involving a low thylakoid lumen pH- and a zeaxanthin-dependent quenching mechanism. In contrast to Y(II), q_P and q_L, calculations of the q_N and the NPQ parameters always require fluorescence measurements of the sample in both the dark-acclimated and in the light-exposed states (see Table 17, page 169). Calculation of NPQ (or SV_N; Gilmore and Yamamoto 1991) corresponds to the Stern-Volmer equation for fluorescence quenching which predicts proportionality between fluorescence quenching (NPQ) and the concentration of fluorescence-quenching centers/entities in the photosynthetic antennae (e.g., zeaxanthin).

Y(NO), Y(NPQ) and Y(II) Complementary PS II quantum yields

Genty *et al.* (1996) first presented expressions based on basic fluorescence parameters that describe the partitioning of absorbed excitation energy in PS II between three fundamental pathways, which were further investigated by Klughammer and Schreiber (2004) and expressed in terms of the complementary quantum yields of

Y(NO) sum of non-regulated losses of excitation energy including heat dissipation and fluorescence emission,

Y(NPQ) regulated energy losses of excitation energy *via* heat dissipation involving ΔpH and zeaxanthin-dependent mechanisms, and

Y(II) use of excitation energy for charge separations.

The yields of photochemical energy conversion and non-photochemical losses sum up to 1:

$Y(II) + Y(NPQ) + Y(NO) = 1$

This concept of “complementary PS II quantum yields” is useful for the analysis of the partitioning of absorbed light energy in photosynthetic organisms. For instance, under high light-conditions, a much higher Y(NPQ) than Y(NO) indicates that excess excitation energy is safely dissipated at the antenna level, that is, photosynthetic energy fluxes are well-regulated. In contrast, high values of Y(NO) would signify that excess excitation energy is reaching the reaction centers, resulting in strong reduction of PS II acceptors and photodamage, e.g., via formation of reactive oxygen species.

9.3 Constant Fraction of F_0 Fluorescence ($F(I)/F_0$ – before C/F_0) not related to PS II fluorescence

The measured fluorescence signal does not only consist of PS II chlorophyll fluorescence, sensitive to photochemical and non-photochemical quenching mechanisms. It is known that PS I chlorophyll fluorescence contributes significantly to room temperature fluorescence emitted by leaves, algae and cyanobacteria. In cyanobacteria phycobilin fluorescence also has to be considered. The non-PS II fluorescence yield is supposed to be constant during illumination. In leaves, typical values of PS I contribution to total F_0 fluorescence at wavelength > 700 nm are 30% for C_3 and 50% for NADP-ME C_4 plants (e.g., maize) (Genty *et al.* 1990, Pfündel 1998, Agati *et al.* 2000, Peterson *et al.* 2001, Franck *et al.* 2002, Rappaport *et al.* 2007).

The PamWin-4 software offers a routine to estimate this contribution, which is expressed by the parameter $F(I)/F_0$. The rationale of $F(I)/F_0$ estimation is as follows:

The original Oxborough and Baker (1997) equation is

$$Fo' = \frac{Fo}{\frac{Fm - Fo}{Fm} + \frac{Fo}{Fm'}} \qquad (1)$$

which can be transformed into

$$Fo' = \frac{1}{\frac{1}{Fo} - \frac{1}{Fm} + \frac{1}{Fm'}} \qquad (2)$$

Equations (1) or (2) would determine F_0' correctly if the F_0, F_M and F_M' signals represent pure PS II fluorescence. In reality, there is always a contribution of PS I fluorescence, and often also from other signals (e.g., phycobilin fluorescence). If we assume that a constant signal, F(I), is present in all fluorescence values of the Oxborough-Baker equation, then subtracting the F(I) from all fluorescence levels results in an equation for pure PS II fluorescence:

$$Fo' - F(I) = \frac{1}{\frac{1}{Fo - F(I)} - \frac{1}{Fm - F(I)} + \frac{1}{Fm' - F(I)}} \qquad (3)$$

After rearrangement, one obtains equation (4):

$$\mathrm{F(I)/Fo} = \frac{Fo'}{Fo} - \frac{1}{\frac{1}{1 - (\mathrm{F(I)/Fo})} - \frac{1}{\frac{Fm}{Fo} - (\mathrm{F(I)/Fo})} + \frac{1}{\frac{Fm'}{Fo} - (\mathrm{F(I)/Fo})}} \qquad (4)$$

With the exception of F(I), all fluorescence terms in equations (3) and (4) are measurable. The quotient $F(I)/F_0$ can be determined iteratively or calculated after solving equation (3) for $F(I)/F_0$. PamWin-4 uses the calculation method introduced by Pfündel et al. (2013).

In practice, determination of $F(I)/F_0$ requires:

- Dark-acclimated (in algae and cyanobacteria with far-red background light) sample for accurate determination of F_0 and F_M.
- Subsequently, illumination at a light intensity that induces substantial non-photochemical quenching but does not yet cause appreciable photodamage, in order to measure F_0' and F_M'.

When $F(I)/F_0$ is known, it is possible to subtract F(I) from all measured fluorescence values, which results in more accurate F_0 values for saturation pulse analyses (except for the quenching coefficients q_P and q_N that do not require knowledge of F_0). In particular, higher Y(II) values are obtained.

9.4 Relative Electron Transfer Rate (ETR)

Relative electron transfer rates are calculated according to:

ETR = PAR · ETR-Factor · Y(II)

The basic idea of the ETR equation is to multiply Y(II), the effective photochemical quantum yield of PS II, by an estimate of the photon flux density absorbed by PS II. The latter approximation uses two parameters which are explained below:

PAR Quantum flux density of photosynthetically active radiation

Depending on the settings, the PAR values of the active PAR list or measured data are used.

ETR-Factor (Absorptance of photons by PS II)

The ETR-Factor describes the fraction of incident photons absorbed by PS II pigments, which corresponds to overall absorptance multiplied by the PS II distribution factor (P_{PS2}/P_{PPS}, see below). The default value for the ETR-Factor is 0.42 which reasonably describes the average PS II absorptance in the visible range (400-700 nm) of many green leaves. Anthocyanins, however, can reduce the availability of blue photons for photosynthesis (Pfündel *et al.* 2007) and, hence, lower the ETR-Factor.

In the case of suspensions, of course, total absorptance normally is much lower than 0.84 and for algae/cyanobacteria/etc. PS I/PS II ratios cannot be assumed to be one. Furthermore, in dilute suspensions of algae and cyanobacteria both overall absorptance and distribution of absorbed quanta to PS II display large differences at different wavelengths. For example, in cyanobacteria at equal quantum flux densities 625 nm light is about 25 times more effective at driving PS II than 480 nm light. To obtain reasonable fluorescence-based estimates of electron transport rates in suspensions, the PamWin-4 software uses the approach introduced by Schreiber *et al*. (2011) which permits calculation of quanta absorbed by PS II, PAR(II), from incident PAR and Sigma(II), the wavelength-dependent absorption cross-section of PS II.

P_{PS2}/P_{PPS} Photons absorbed by PS II relative to photons absorbed by all photosynthetic pigments.

The default value for P_{PS2}/P_{PPS} for leaves is 0.5. If the PS I/PS II ratio is unity in leaves, as often assumed, this value is reasonable if only linear electron transport is considered, that is, equal

electron transfer rates though PS I and PS II, and comparable photochemical quantum yields of PS I and PS II under strongly light-limiting conditions.

The ETR may be compared to the rate of CO_2-assimilation or of O_2-evolution. For such a comparison the following aspects are relevant:

- 4 e^- must be transported for every CO_2 assimilated or O_2 evolved.
- the value of ETR/4 is not necessarily identical to CO_2-fixation rate or O_2-evolution rate; discrepancies may e.g. arise from O_2 dependent electron flow (photorespiration, Mehler-Ascorbate-Peroxidase cycle activity, nitrite reduction) or electron cycling at PS II.
- fluorescence information primarily originates from chloroplasts in the topmost cell layers of the leaf, whereas all cell layers contribute to gas exchange activity; on the other hand, the topmost layers absorb most of the light and, hence, are also responsible for most of the gas exchange, unless photoinhibited.

The combined information that can be derived from ETR, PAR and Temperature provides insight into the photosynthetic performance of a plant. Plots of ETR versus PAR at different temperatures respond in a very sensitive manner to changes at all levels of the photosynthetic process. In the case of suspensions of algae and cyanobacteria, however, it has to be considered that the obtained values of ETR give a relative measure of photosynthetic electron transport rate only, unless for the same sample and identical color of illumination the absolute rate of quantum absorption by PS II is known. The MULTI-COLOR-PAM-II provides the means to determine the wavelength-

dependent functional absorption cross-section of PS II, Sigma(II), in dilute suspensions, based on which absolute rates of PAR(II) and ETR(II) can be calculated (see sections 5.3.2 and 13.3)

9.5 Light Curves

The light curve program of PamWin-4 exposes the sample to changing (e.g. increasing) intensities of actinic light. If the illumination time at each light intensity is too short to reach the steady state (full equilibration of photosynthetic reactions) one speaks about “Rapid Light Curves” (RLCs). Typically, RLC measurements are carried out with samples in their momentary acclimation state, that is, without intermediate acclimation period. This way, RLC data can provide information on the present acclimation state of the photosynthetic apparatus.

Measurements of RLCs should not be confused with classical photosynthetic light response curves in which photosynthetic rates under steady state conditions are plotted against the light intensity. In both cases, plotting ETR versus PAR yields light response curves which are often described by the following three cardinal points:

- α (alpha), electrons/photons: Initial slope of RLC which is related to the antenna size of photosystem II and the quantum efficiency of photosynthesis.
- ETR_{max}, µmol electrons/(m^2 · s): Maximum electron transport rate.
- I_K, µmol photons/(m^2 · s): Minimum saturating irradiance.

The EP function used by the PamWin-4 software has been derived by Eilers and Peeters (1988) using a mechanistic model

which considers the processes of photosynthesis and photoinhibition. Their final model function:

$$ETR = \frac{PAR}{a \cdot PAR^2 + b \cdot PAR + c}$$

In the above equation, a, b, and c are free parameters, which are varied by the PamWin-4 software until the best fit between theory and data is achieved. The cardinal points of a light curve are calculated according to the following equations:

$$\alpha = \frac{1}{c}$$

$$ETR_{max} = \frac{1}{b + 2 \cdot \sqrt{a \cdot c}}$$

$$I_K = \frac{c}{b + 2 \cdot \sqrt{a \cdot c}}$$

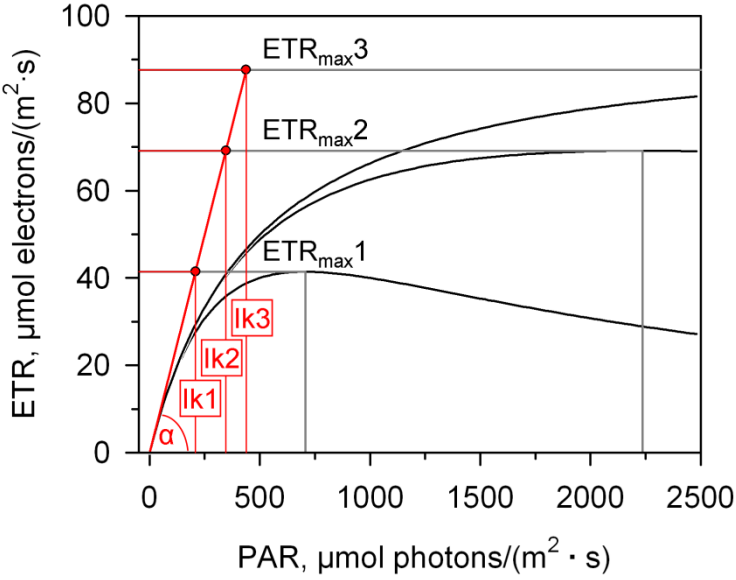
Fig. 44 illustrates the behavior of the Eilers and Peeters model function for 3 theoretical cases, which show identical values of α but, at high light intensities, different degrees of photoinhibition.

The Platt *et al.* (1980) model also considers photoinhibition. However, that equation is not based on mechanistic considerations but on the empirical finding that many light response curves can be described by the product of 2 exponentials:

$$ETR = ETR_{mPot} \cdot (1 - e^{-\frac{\alpha \cdot PAR}{ETR_{mPot}}}) \cdot e^{-\frac{\beta \cdot PAR}{ETR_{mPot}}}$$

The free parameters in this equation are ETR_{mPot}, α and β, where ETR_{mPot} is the asymptote value of the rising exponential in the equation above. The cardinal parameter α is derived

directly from the curve fit. The two remaining cardinal parameters can be obtained with the following equation (bottom next page):



Curve	Parameter					
	A	b	c	α	ETR_{max}	I_k
1	$1 \cdot 10^{-5}$	$1 \cdot 10^{-2}$	5	0.2	41	207
2	$1 \cdot 10^{-6}$	$1 \cdot 10^{-2}$	5	0.2	69	345
3	$1 \cdot 10^{-7}$	$1 \cdot 10^{-2}$	5	0.2	88	438

Fig. 44: Examples of Light Curves.

$$ETR_m = ETR_{mPot} \cdot \left(\frac{\alpha}{\alpha+\beta}\right) \cdot \left(\frac{\beta}{\alpha+\beta}\right)^{\frac{\beta}{\alpha}}$$

$$E_K = \frac{ETR_m}{\alpha}$$

The model equation of Platt *et al.* (1980) considers photoinhibition by the "photoinhibition parameter" β. Platt *et al.* (1980) introduced the "Photoinhibition Index" (I_b) to quantify photoinhibition. The authors defined I_b as the PAR value required to photoinhibit ETR_{mPot} by a factor of 1/e according to:

$$I_b = ETR_{mPot} / \beta$$

9.6 References (Light Response Curves)

Eilers PHC, Peeters JCH (1988) A model for the relationship between light intensity and the rate of photosynthesis in phytoplankton. Ecol. Model. 42: 199-215

Jassby AD, Platt T (1976) Mathematical formulation of the relationship between photosynthesis and light for phytoplankton. Limnol. Oceanogr. 21: 540-547

Platt T, Gallegos CL, Harrison WG (1980) Photoinhibition of photosynthesis in natural assemblages of marine phytoplankton. J. Mar. Res. 38: 687-701

9.7 Additional Literature Cited

Agati G, Cerovic ZG, Moya I (2000) The effect of decreasing temperature up to chilling values on the *in vivo* F685/F735 chlorophyll fluorescence ratio in *Phaseolus vulgaris* and *Pisum sativum*: the role of the Photosystem I contribution to the 735 nm fluorescence band, Photochem. Photobiol. 72: 75-84

Bilger W, Björkman O (1990) Role of the xanthophyll cycle in photoprotection elucidated by measurements of light-induced absorbance changes, fluorescence and photosynthesis in leaves of *Hedera canariensis*. Photosynth. Res. 25: 173-185

Franck F, Juneau P, Popovic R (2002) Resolution of the Photosystem I and Photosystem II contributions to chlorophyll fluorescence of intact leaves at room temperature. Biochim. Biophys. Acta 1556: 239-246

Genty B, Briantais J-M, Baker NR (1989) The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochim. Biophys. Acta 990: 87-92

Genty B, Harbinson J, Cailly AL, Rizza F (1996) Fate of excitation at PS II in leaves: the non-photochemical side. Presented at: The Third BBSRC Robert Hill Symposium on Photosynthesis, March 31 to April 3, 1996, University of Sheffield, Department of Molecular Biology and Biotechnology, Western Bank, Sheffield, UK, Abstract P28

Genty B, Wonders J, Baker NR (1990) Non-photochemical quenching of F_0 in leaves is emission wavelength dependent: consequences for quenching analysis and its interpretation. Photosynth. Res. 26: 133-139

Gilmore AM, Yamamoto HY (1991) Zeaxanthin formation and energy-dependent fluorescence quenching in pea chloroplasts under artificially mediated linear and cyclic electron transport. Plant Physiol. 96: 635-643

Kitajima M, Butler WL (1975) Quenching of chlorophyll fluorescence and primary photochemistry in chloroplasts by dibromothymoquinone. Biochim. Biophys. Acta 376: 105-115

Klughammer C, Schreiber U (2008) Complementary PS II quantum yields calculated from simple fluorescence parameters measured by PAM fluorometry and the Saturation Pulse method. PAM Application Notes 1: 27-35 (https://www.walz.com/files/downloads/pan/PAN078007.pdf)

Klughammer C, Schlosser F, Schreiber U (2024) Flash-kinetics as complementary tool in PAM fluorometry. Photosynth. Res. 161: 151-176

Kramer DM, Johnson G, Kiirats O, Edwards GE (2004) New flux parameters for the determination of Q_A redox state and excitation fluxes. Photosynth. Res. 79: 209-218

Oxborough K, Baker NR (1997) Resolving chlorophyll *a* fluorescence images of photosynthetic efficiency into photochemical and non-photochemical components - calculation of qP and Fv'/Fm' without measuring Fo'. Photosynth. Res. 54: 135-142

Peterson RB, Oja V, Laisk A (2001) Chlorophyll fluorescence at 680 and 730 nm and leaf photosynthesis. Photosynth. Res. 70: 185-196

Pfündel EE (1998) Estimating the contribution of Photosystem I to total leaf chlorophyll fluorescence. Photosynth. Res. 56: 185-195

Pfündel EE (2021) Simultaneously measuring pulse-amplitude-modulated (PAM) chlorophyll fluorescence of leaves at wavelengths shorter and longer than 700 nm. Photosynth. Res. 147: 345-358

Pfündel EE, Ben Ghozlen N, Meyer S, Cerovic ZG (2007) Investigating UV screening in leaves by two different types of portable UV fluorimeters reveals *in vivo* screening by anthocyanins and carotenoids. Photosynth. Res. 93: 205-221

Pfündel EE, Klughammer C, Meister A, Cerovic ZG (2013) Deriving fluorometer-specific values of relative PSI fluorescence intensity from quenching of F_0 fluorescence in leaves of *Arabidopsis thaliana* and *Zea mays.* Photosynth. Res. 114: 189-206

Rappaport F, Béal D, Joliot A, Joliot P (2007) On the advantages of using green light to study fluorescence yield changes in leaves. Biochim. Biophys. Acta 1767: 56-65

Schreiber U (2023) Light-induced changes of far-red excited chlorophyll fluorescence: further evidence for variable fluorescence of photosystem I *in vivo*. Photosynth. Res. 155: 247-270

Schreiber U, Klughammer C (2021) Evidence for variable chlorophyll fluorescence of photosystem I *in vivo*. Photosynth. Res. 149: 213-231

Schreiber U, Klughammer C, Schansker G (2019) Rapidly reversible chlorophyll fluorescence quenching induced by pulses of supersaturating light *in vivo*. Photosynth. Res. 142: 35-50

Schreiber U, Klughammer C, Kolbowski J (2011) High-end chlorophyll fluorescence analysis with the MULTI-COLOR-PAM. I. Various light qualities and their applications. PAN (2011) 1: 1-19 (https://www.walz.com/files/downloads/pan/PAN11001.pdf)

Schreiber U, Schliwa U, Bilger W (1986) Continuous recording of photochemical and non-photochemical chlorophyll fluorescence quenching with a new type of modulation fluorometer. Photosynth. Res. 10: 51-62

van Kooten O, Snel J (1990) The use of chlorophyll fluorescence nomenclature in plant stress physiology. Photosynth. Res. 25: 147-150

10 Some Reviews on Chlorophyll Fluorescence

Baker NR (2008) Chlorophyll fluorescence: a probe of photosynthesis *in vivo*. Annu. Rev. Plant Biol. 59: 89–113

Bernhardt K, Trissl H-W (1999) Theories for kinetics and yields of fluorescence and photochemistry: how, if at all, can different models of antenna organization be distinguished experimentally? Biochim. Biophys. Acta 1409: 125-142

Butler WL (1978) Energy distribution in the photochemical apparatus of photosynthesis. Annu. Rev. Plant Physiol. 29: 345-378

Dau H (1994) Molecular mechanisms and quantitative models of variable photosystem II fluorescence. Photochem. Photobiol. 60: 1-23

Demmig-Adams B, Adams WW, III (1992) Photoprotection and other responses of plants to high light stress. Annu. Rev. Plant Physiol. Plant Mol. Biol. 43: 599-626

Govindjee (1995) Sixty-three years since Kautsky: Chlorophyll *a* fluorescence. Aust. J. Plant Physiol. 22: 131-160

Haldrup A, Jensen PE, Lunde C, Scheller HV (2001) Balance of power: a view of the mechanism of photosynthetic state transitions. Trends Plant Sci. 6: 301-305

Harbinson J (2013) Improving the accuracy of chlorophyll fluorescence measurements. Plant Cell Environ. 36: 1751-1754

Kalaji HM, Schansker G, Ladle RJ, et al. (2014) Frequently asked questions about *in vivo* chlorophyll fluorescence: practical issues. Photosynth. Res. 122: 121-158

Kalaji HM, Schansker G, Brestic M, et al. (2017) Frequently asked questions about chlorophyll fluorescence, the sequel. Photosynth. Res. 132: 13-66

Krause GH, Weis E (1991) Chlorophyll fluorescence and photosynthesis: the basics. Annu. Rev. Plant Physiol. Plant Mol. Biol. 42: 313-349

Krause GH, Jahns P (2004) Non-photochemical energy-dissipation determined by chlorophyll fluorescence quenching: characterization and function. In: Papageorgiou GC, Govindjee (eds.) Chlorophyll *a* Fluorescence: A Signature of Photosynthesis. Springer, The Netherlands, pp. 463-495

Lazár D (1999) Chlorophyll *a* fluorescence induction. Biochim. Biophys. Acta 1412: 1-28

Logan BA, Adams III WW, Demmig-Adams B (2007) Avoiding common pitfalls of chlorophyll fluorescence analysis under field conditions. Funct. Plant Biol. 34: 853-859

Maxwell K, Johnson GN (2000) Chlorophyll fluorescence – a practical guide. J. Exp. Bot. 51: 659-668.

Mohammed GH, Binder WD, Gillies SL (1995) Chlorophyll fluorescence: A review of its practical forestry applications and instrumentations. Scand. J. Forest Res. 10: 383-410

Murchie EH, Lawson T (2013) Chlorophyll fluorescence analysis: a guide to good practice and understanding some new applications. J. Exp. Bot. 64: 3983-3998

Nedbal L, Koblížek M (2006) Chlorophyll fluorescence as a reporter on *in vivo* electron transport and regulation in plants In: Grimm B, Porra RJ, Rüdiger W, Scheer H (eds.) Advances in Photosynthesis and Respiration, Vol 25, Chlorophylls and

Bacteriochlorophylls: Biochemistry, Biophysics, Functions and Applications. Springer, The Netherlands, pp 507-519

Schreiber U (2004) Pulse-amplitude-modulation (PAM) fluorometry and saturation pulse method: an overview. In: Papageorgiou GC, Govindjee (eds.) Chlorophyll *a* Fluorescence: A Signature of Photosynthesis. Springer, The Netherlands, pp. 279-319

Schreiber U, Bilger W, Neubauer C (1994) Chlorophyll fluorescence as a non-intrusive indicator for rapid assessment of *in vivo* photosynthesis. Ecological studies: analysis and synthesis (USA) 100: 49-70

Schreiber U, Hormann H, Neubauer C, Klughammer C (1995) Assessment of Photosystem II photochemical quantum yield by chlorophyll fluorescence quenching analysis. Aust. J. Plant Physiol. 22: 209-220

11 Specifications MULTI-COLOR-PAM-II

11.1 Basic System

11.1.1 Power-and-Control-Unit MCP-II-C

General design: ARM microcontroller (480 MHz), Fast Kinetics up to 128,000 points with 14 bit resolution, ST Kinetics up to 15,000 points with 14 bit resolution, unlimited storage for Slow Kinetics

Sockets: 4 sockets for measuring light and actinic light of MCP-II-E MULTI-COLOR Emitter Head and measuring light/auxiliary detector and ST supply MCP-II-EDST, 2 sockets for signal detection by MCP-II-D1 and MCP-II-D2DST Detector Heads, charge socket or Battery Charger MINI-PAM/L, output socket for PHYTO-MS Miniature Magnetic Stirrer, 4 BNC sockets for 5 V trigger-in and trigger out signals and ST trigger in and trigger out signals, input socket for US-SQS/WB Spherical Micro Quantum Sensor or US-MQS/WB Mini Quantum Sensor, input socket for auxiliary devices, USB socket

Communication: USB 2.0, USB 3 compatible

User interface: Windows computer with PamWin-4 software

Power supply: standard: **no** sealed lead-acid battery; Battery Charger MINI-PAM/L (100 to 240 V AC)

Dimensions: 31 cm x 16 cm x 33.5 cm (W x H x D), aluminum housing with carrying handle

Power consumption: 6 W

Weight: 3.64 kg

Operating temperature: -5 to +40 °C

11.1.2 Battery Charger MINI-PAM/L

Input: 90 to 264 V AC, 47 to 63 Hz

Output: 19 V DC, 3.7 A

Operating temperature: 0 to 40 °C

Dimensions: 15 cm x 6 cm x 3 cm (L x W x H)

Weight: 300 g

11.1.3 Emitter Head MCP-II-E

Chip-on-board multi-wavelength measuring light LED emitter: 400, 440, 480, 540, 590, and 625 nm for pulse-modulated measuring light; 20 intensity settings and 14 settings of pulse frequency

Chip-on-board multi-wavelength actinic LED array: 440, 480, 540, 590, 625 and 420-640 nm (white) for continuous actinic illumination, max. 5000 µmol photons m-2 s-1; saturating single turnover flashes, max. 200000 µmol photons m-2 s-1; multiple turnover pulses, max. 12000 µmol photons m^{-2} s^{-1} PAR, adjustable between 1 and 800 ms

Far-Red LED: peak wavelength 730 nm

Dimensions: 10.5 cm x 5.5 cm x 7 cm (L x W x H)

Weight: 500 g (incl. cables, 1 m long)

11.1.4 Detector Head MCP-II-D1

Signal detection: PIN photodiode with special pulse preamplifier for measuring fluorescence changes with maximum time resolution of 10 µs

Filter box: For up to 14 mm filter thickness

Standard detector filter: long-pass filter > 650 nm (3 mm RG 665) plus short-pass filter SP 710

Dimensions: 6.9 cm x 9.8 cm x 6.4 cm (L x W x H)

Weight: 355 g (incl. cables, 1 m long)

11.1.5 Emitter-Detector Head MCP-II-EDST

Signal generation and detection: High quality 450 nm ST-flashes of variable duration and intensity up to 1 mol photons m^{-2} s^{-1}; Actinic light (450 nm); Far Red light (740 nm); STK-Detector for surface detection of direct fluorescence with sub-µs time resolution

Dimensions: 15.4 cm x 5.2 cm x 7 cm

Weight: 570 g (incl. cables, 1.2 m long)

11.1.6 Detector Head MCP-II-D2DST

STK & PAM-Detector (switchable): includes: PAM-detector equivalent to MCP-II-D1 and STK-detector for direct fluorescence with sub-µs time resolution

Filter box: for up to 14 mm filter thickness

Standard detector filter: long-pass filter >650 nm (3 mm RG 655) plus short-pass filter SP 710

Dimensions: 6.9 cm x 9.8 cm x 6.4 cm (L x H x W)

Weight: 550 g (incl. cables, 1.2 m long)

11.1.7 Optical Unit for Suspensions ED-101US/MD

Design: Black-anodized aluminum body with central 10 x 10 mm standard glass cuvette; ports for attachment of four Emitter/Detector Heads MCP-II-E, MCP-II-D1, MCP-II-EDST and MCP-II-D2DST, and Miniature Magnetic Stirrer PHYTO-MS

Weight: 750 g

11.1.8 Stand ST-101

Stand for mounting the Optical Units ED-101US/MD (suspensions) or MCP-II-BK (leaves)

11.1.9 Transport Box PHYTO-T

Design: Aluminum box with custom foam packing for MULTI-COLOR-PAM-II and accessories

Dimensions: 60 cm x 40 cm x 25 cm (L x W x H)

Weight: 5 kg

11.1.10 System Control and Data Acquisition

Software: PamWin-4 System Control and Data Acquisition Program for operation of measuring system via PC or laptop, data acquisition and analysis

Saturation Pulse Analysis.

Measured: Ft, F_0, F_M, F, F_0' (also calculated), F_M'. Fast polyphasic rise and decay kinetics (time resolution up to 10 µs). PAR using Spherical Micro Quantum Sensor US-SQS/WB or Mini Quantum Sensor US-MQS/WB. **Calculated:** F_0' (also measured), Fv/Fm and Y(II) (maximum and effective photochemical yield of PS II, respectively), q_L, q_P, q_N, NPQ, Y(NPQ), Y(NO) and ETR (electron transport rate), $F(I)/F_0$ (constant fraction of F_0 not constituting PS II chlorophyll fluorescence)

Fitting Routines

Fitting routine for fast fluorescence rise 0-I_1 to determine the functional absorption cross-section of PS II antennae (Sigma(II)) needed for the determination of PS II-specific electron transport rates. Fitting routine for exponential decay (e.g., Q_A^- reoxidation after a single turnover flash) or rise of a signal by up to three exponentials. Choice of two fitting routines for light curves (determination of the cardinal points α, I_k and ETR_{max}).

Computer Requirements

Operating system: Microsoft Windows 10 (32 and 64 bits) and 11; computers that can run these operating systems, can also work with PamWin-4.

11.2 Accessories for Suspensions

11.2.1 Temperature Control Block for Cuvette ED-101US/T

Sectioned block with central 10 x 10 mm opening to be mounted on top of the ED-101US/MD unit; to be connected to external flow-through water bath (not included), weight: 250 g

11.2.2 Temperature Control Unit US-T

Consists of Power and Control Unit US-T/DR with separate AC power supply and Peltier-Heat-Transfer Rod US-T/DS. Temperature range: from 12 K below ambient temperature to 15 K above ambient temperature. To be mounted on top of 10 x 10 mm cuvette.

11.2.3 Spherical Micro Quantum Sensor US-SQS/WB

3.7 mm diffusing Plexiglas sphere coupled to integrated PAR-sensor via 2 mm fiber, compact amplifier unit and special holder for mounting on Optical Unit ED-101US/MD; to be connected to Power-and-Control-Unit MCP-II-C.

11.2.4 Miniature Magnetic Stirrer PHYTO-MS

Type Variomag Mini, with adapter for bottom port of Optical Unit ED-101US/MD and connector to plug into Power-and-Control-Unit MCP-II-C. Control via MCP-II-C and PamWin-4 software.

11.3 Accessories for Leaves

11.3.1 Optical Unit for Leaf Measurements MCP-BK

Featuring optical ports for mounting Measuring Heads MCP-II-E and MCP-II-D1; including leaf clip holder, with opening for fixing Mini Quantum Sensor US-MQS/WB in the leaf plane. To be mounted on stand.

11.3.2 Optical Unit for Leaf Measurements MCP-II-BK

Optical unit featuring 3 optical ports for the mounting of a multi-color emitter (MCP-II-E) in the center, accompanied on both sides by two detectors (MCP-II-D1 and MCP-II-D2DST) placed under a 45° angle relative to the emitter for two-wavelength fluorescence detection. To be mounted on a stand.

11.3.3 Mini Quantum Sensor US-MQS/WB

Consisting of cosine-corrected PAR-sensor (housing: diameter 14 mm, height 16 mm; diffuser diameter 5.5 mm) with cable 3 m long (BNC connector), compact amplifier unit and adapter set. To be connected to the Power-and-Control-Unit MCP-II-C. For measuring incident PAR, when using the Optical Unit for Leaf Measurements MCP-II-BK.

Subject to change without prior notice

12 Guarantee

All products supplied by the Heinz Walz GmbH, Germany, are warranted by Heinz Walz GmbH, Germany to be free from defects in material and workmanship for two (2) year from the shipping date (date on invoice).

12.1 Manufacturer's Guarantee

Under this Manufacturer's Guarantee ("Guarantee"), subject to the Conditions and Instructions below, Heinz Walz GmbH, Germany ("Manufacturer"), guarantees (§443 BGB) to the end customer and user ("Customer") that all products supplied by it, shall substantially conform in material respects to the Specifications for 24 months from the delivery date (date on invoice). In this Guarantee, "Specifications" means the product's features (as may be amended by Manufacturer from time to time), which are set out under headings "specifications" and/or "technical specifications" within the product's respective brochure, data sheet, or respective tab on the Manufacturer's website for such product, and which may be included with the documents for the product when delivered. In case of an eligible guarantee claim, this Guarantee entitles the Customer to repair or replacement, at the Manufacturer's option, and this Guarantee does not include any other rights or remedies.

12.2 Conditions

This Guarantee shall not apply to:

- Any defects or damage directly or indirectly caused by or resulting from the use of unauthorized replacement parts and/or service performed by unauthorized personnel.

- Any product supplied by the Heinz Walz GmbH, Germany, which has been subjected to misuse, abuse, abnormal use, negligence, alteration or accident.
- Damage caused from improper packaging during shipment or any acts of God.
- Batteries, cables, calibrations, fiberoptics, fuses, gas filters, lamps (halogen, LED), thermocouples, and underwater cables.
- Defects that could reasonably have been detected upon inspection of the product when received by the Customer and not promptly noticed within ten (10) days to Heinz Walz GmbH.
- Submersible parts of the DIVING-PAM or the MONITORING-PAM have been tested to be watertight down to the maximum operating depth indicated in the respective manual. Guarantee shall not apply for diving depths exceeding the maximum operating depth. Further, guarantee shall not apply for damage resulting from improper operation of devices, in particular the failure to properly seal ports or sockets.

12.3 Instructions

- To obtain guarantee service, please follow the instructions below:
- The Walz Service Information Form available at https://www.walz.com/support/repair_service.html must be completed and returned to Heinz Walz GmbH, Germany.
- The product must be returned to Heinz Walz GmbH, Germany within 30 days after Heinz Walz GmbH, Germany has received written notice of the defect. Postage, insurance, and/or shipping costs incurred in returning equipment for guarantee service are at customer expense. Duty and taxes are covered by Walz.

- All products being returned for guarantee service must be carefully packed and sent freight prepaid.
- Heinz Walz GmbH, Germany is not responsible or liable for missing components or damage to the unit caused by handling during shipping. All claims or damage should be directed to the shipping carrier.

12.4 Applicable law

- This Guarantee is governed by German law. Place of jurisdiction is Bamberg, Germany.

13 Appendix

13.1 Practical tips for creating trigger patterns

To create a trigger pattern can initially be very frustrating since the changes made work rarely in the intended way.

Adding a new trigger event for example or moving an existing one along the time-axis. It is important to realize that only one change is executed each time. Changing the on/off times of a particular trigger event at the same time does not work.

The choice of time points depends on the rate. If the rate is 20 µs, it is not allowed to choose the time point 10 µs; the system will not accept it.

Take single turnover flashes. These are difficult to shift, but it is easy to add extra flashes. The best approach is to design this trigger pattern each time new by clicking on the “All Low” icon (). This yields a flat yellow line. Then select the ST width, e.g., 50 µs, enter the start time and press enter. The program will automatically add the end time 50 µs later. To add a second flash, again enter a start time and press enter, etc.

To avoid interference of the flashes with the measurement, S&H is turned off around the flash (that means the detector is switched temporarily off to avoid signal overload and blinding). If the flash is given at time = 0, S&H can be turned off at -10 µs. Enter -10 and push enter. Then enter the endpoint of this event, e.g., 80 µs, and press again enter.

To move the event to longer times, first change the end point time to for example 100080 µs and press enter (gives an event that starts at -10 µs and ends at 100080 µs) and then change the start point for example to 99990 µs.

To add a new event at longer times, first enter a new start time, e.g., 99990 µs, and then press enter. This gives a new event that starts at 99990 µs and ends at the end of the window. Then an endpoint can be entered, e.g., 100080, and a new event has been created.

If you want to add a new event at shorter times, first enter a new endpoint, e.g., 80 µs, and press enter and then add a new start point, e.g., -10 µs, and then press enter.

It works the same way for the other trigger parameters.

To work efficiently with trigger files, it is possible to modify similar, existing trigger files. Alternatively, it is possible to write down a protocol with the exact timing in order to work from left to right or from right to left. Introducing a new event between two existing events is difficult or maybe even impossible.

13.2 Writing scripts

In Chapter 7 the basic principles of writing scripts are discussed, and the different script commands explained. In the present section suggestions are made for the creation of new scripts.

Defining the initial conditions

At the beginning of a script file the starting conditions are defined. The first command to add to the script can be: ***New Record***. However, if you want to keep a series of measurements together in one record file this command is not a good idea. In that case it is better to start new records manually, making use of the New Record button in the bottom panel of all windows.

The second command should then be ***Analysis Mode*** where a choice is made between ***SP-Analysis*** and ***Fast Acquisition*** mode. This defines the type of measurements that can be carried out. It is possible to define a saturation pulse experiment under

fast acquisition mode conditions. However, in that case no quenching analysis is carried out and the associated parameters are not determined/calculated automatically.

When working with green algae with a rather high chlororespiratory activity (causes non-photochemical reduction of the PQ-pool) it may be an idea to work with low intensity far-red background light (FR1):

FR-int = 1

FR ON

It may further be an idea to activate “autoMF_H”; this means that the measuring frequency switches from low to high when the actinic light is switched on. This can also be defined in a trigger file.

autoMF_H On

It is also possible to define the wavelength used for the measurements:

ML Color = 625nm (5)

AL Color = 625nm (5)

In the MULTI-COLOR-PAM-II the used wavelength can also be selected manually in the “Multi Color” window. However, when in a single script all wavelengths are used (for example in the case of the determination of the wavelength dependence of Sigma(II)) the wavelengths should be defined at the appropriate places within a script.

If single turnover flashes will be included in the script, it is a good idea to check <Multi Color ST>:

<Multi Color ST> On

This means that the actinic light LEDs of all colors (= wavelengths) will be used to generate the ST.

Other parameters (if relevant) that can be defined here are the ***Gain***, ***F ML int.***, ***SP int.*** and ***AL int***. This does not turn those light sources on; it only defines their intensity once they are triggered.

In the ***SP-Analysis*** mode, using a low measuring frequency, the measuring light *F ML* can be on at all times. However, in the case of fast kinetic measurements (***fast acquisition*** mode), where a high measuring frequency is essential to achieve a sufficiently high time resolution, or in the case where even minor actinic effects of low frequency measuring light are unwanted, it may be better to switch the measuring light only on during a measurement.

To make sure that the measuring light is not accidently left on, a command can be included:

F ML Off

If the user wants to restrict the use of measuring light, the easiest method is to define the measuring light characteristics in the trigger file(s) to be used. It is for example possible to define that the *ML* should be switched on 100 µs before the start of the measurement and the measurement should switch to high frequency measuring light (***MF-max***) at the same time as the actinic light (or in this case the measuring light) is switched on. The switch from low to high measuring frequency can also be made in the script, as mentioned above, by adding the command ***autoMF _H On*** at the beginning of the script. In that case the script switches automatically from low to high measuring frequency each time the actinic light is turned on.

Interaction between script and settings

It is important to be aware of the fact that interactions between the script and settings elsewhere in the software are possible.

For example, if the command ***FR On*** is added to the script, one might think that the far-red light remains on until the command ***FR Off*** is given. However, this is only true if the Width (s) of the PS I light in the General Settings window is set to 0 (= manual). If it is set to 10 s, the far-red light will be turned off 10 s after the ***FR On*** command was executed.

Data recording

It is also important to be aware of the fact that in fast kinetics mode it is possible to trigger an ST or MT, but that that does not mean the signal induced by this ST or MT will be recorded. For that, it is necessary to define this ST or MT in a trigger file. To execute a trigger file in a script takes the following form (for trigger file ST.FTM):

Open Fast Kin. Trig. File ST.FTM

Sat-Pulse/Fast kin.

Comment = 1 ST

Paste to Fast Chart Comment Line

In the first line the fast kinetic trigger file (ST.FTM) is opened. In the second line the trigger file is executed. The third line defines a comment characterizing the experiment and the fourth line tells the software that the comment should be added to the comment line of the fast kinetics window.

It is also possible to define an experiment consisting of two flashes. For that an extension of the script is needed:

Open Fast Kin. Trig. File ST.FTM

Sat-Pulse/Fast kin.

Comment = 1 ST

Paste to Fast Chart Comment Line

Sat-Pulse/Fast kin.

Comment = 1 ST

Paste to Fast Chart Comment Line

It is not necessary to open the trigger file again, so only the last three lines have to be copied and pasted.

Flash interval

It is electronically not a problem to give STs spaced less than 100 ms apart. However, the reproducibility of flash intensities following short time intervals is not 100%. In practice, the time interval if not defined in the script is ~1 s (1 Hz). However, with the new powerful flash lamp of the MULTI-COLOR-PAM-II it is possible to give two identical flashes spaced 1 µs apart.

TimeStep(s) vs Wait(s)

It is also important to be aware of the fact that the ***TimeStep(s)*** and ***Wait(s)*** commands differ in precision. When a ***Wait(s)*** command is added to a script (e.g., 10 s) the program will execute the different commands in the program and when it comes across ***Wait(s)*** it will simply wait 10 s before continuing with the rest of the script. A ***TimeStep(s)*** command defines the time between two events and the time all the different commands need to be executed during this step is ignored. This is more precise. However, for general dark-times or times between measurements, illumination with far-red light, high precision may be irrelevant and the ***Wait(s)*** function will be a suitable tool.

Measurement repetition and averaging

Despite the sensitivity of the MULTI-COLOR-PAM-II, there are certain measurements where an accumulation and averaging of several measurements is a good idea. For example, for the determination of the re-oxidation kinetics of Q_A^- following an ST. To

make such repetitive measurements the most primitive (but effective) approach would be to copy and paste the relevant lines of script several times. These measurements can then be averaged manually, making use of the “+”-function in the fast kinetics window (see 6.1, page 81).

The next level of complexity is to create a subprogram out of the commands that have to be repeated and Call this subprogram using the <Call> command each time it is needed.

The script finally offers a third possibility. It is also possible to put these lines of script between the commands *Begin of Repetition Block* and *End of Repetition Block; Loops = 10* (or any other number). Before the start of the Repetition Block *Fast Kinetic Averaging* has to be turned on and the number of *Target Averages* has to be indicated (should equate number of Loops of the Repetition Block).

Open Fast Kin. Trig. File ST.FTM

Fast Kin Averaging On

Target Averages = 10

Begin of Repetition Block ST av

Sat-Pulse/Fast kin.

Wait(s) = 30

End of Repetition Block; Loops = 10

Comment = 1 ST

Paste to Fast Chart Comment Line

It is possible to create a script with a sequence of several of such repetition blocks. See for example the script in Fig. 22, page 129.

Conditional commands

It required some creativity to find applications for the conditional commands. Photosynthesis is temperature dependent and if the user would want to make a temperature dependence, coupling the progress of the measurement to the heating up/cooling down of a sample by a water bath, it would be possible to more or less automate such a measurement.

With respect to an unstirred sample the user may want to stop the measurement if the cells start to settle, and the Ft value falls below a certain threshold. I can imagine though, that most users will never make use of these conditional commands.

13.3 Making Light Curves

Short light curves

If leaves are kept under controlled gas conditions and each light step lasts at least 5-10 min, the starting conditions of the experiment are not that important. However, most researchers do not use a gas-controlled environment for their light curves, and they also do not want to spend so much time on them. Which factors have to be considered when making short light curves.

A Light Curve protocol

Fluorescence-based light curves have contradictory requirements. On the one hand, for the determination of qN, respectively NPQ a dark acclimated sample with an inactive Calvin-Benson cycle/inactive PS I acceptor side is needed. On the other hand, for the light curve itself, an active Calvin-Benson cycle is needed.

This problem can be solved in two different ways. The user can use the first two steps of the light curve for the determination of Fo,Fm followed by a 3 min e.g. 50 µmol photons m^{-2} s^{-1} pre-illumination (step 1), followed by a 1-2 min dark period (step 2) to start subsequently during step 3 with the light curve.

Alternatively, an Fo,Fm determination can be made followed by a manual 3 min illumination with 50 µmol photons m^{-2} s^{-1} and a 2 min dark acclimation after which the light curve can then be started.

For leaves the 50 µmol photons m^{-2} s^{-1} light intensity is high enough to activate the Calvin-Benson cycle but at the same time so low that probably almost no NPQ will be induced. If algae grown at low at relatively low light intensities are measured it may be a good idea to choose an even lower light intensity.

Thioredoxin system

However, chloroplasts try to keep the Calvin-Benson cycle and electron transport rate in balance making use of the thioredoxin system. Each time the light intensity is changed, that is on each light step a new balance has to be found and this takes some time. To avoid interference between light curve and balancing, it may be needed to have light steps of at least 40 s to allow this balancing process to complete.

What happens if you stepwise increase the light intensity but do not pre-activate the Calvin-Benson cycle? If you do activate the Calvin-Benson cycle the initial slopes of the ETR-curves are independent of the step length. If you don't, they become sensitive to the step length.

Each time the light intensity is increased, the thioredoxin system will increase the Calvin-Benson cycle capacity by activating more of the enzymes that are under its control. Initially the electron transport chain will become more reduced because the Calvin-Benson cycle cannot yet cope with the increased electron flow, but once a new equilibrium has been determined this effect will disappear. If you measure too early, there is no steady state, and the still more-reduced electron transport chain will yield ETR-values that

are too low. We observed that between a 10 s and 90 s step length the difference may be as much as a factor 2.

Hysteresis

Another term that is associated with light curves is hysteresis. It is for example possible to measure from low to high light intensities and subsequently from high to low light intensities (or the opposite). The term hysteresis is used for the mismatch/difference between these two measurement series. On going from low to high regulatory mechanisms are gradually induced and the capacity equilibrium has to be established each time, whereas in the opposite direction there is always enough capacity, whereas the regulatory mechanisms have to be reversed.

Therefore, it is not surprising that the two light curves are not identical.

Light intensity range

Further, it may be an idea to limit the highest light intensities to approx. 4 times the growth light intensity. Unless the user wants to induce photoinhibition, there is not much point to go higher than that. In most light curves the majority of kinetic changes is observed below 200 µmol photons m^{-2} s^{-1}.

Modifications of the light intensity

Something else that may be useful is a modification of the light intensities. If you open the light curve protocol, you find 20 potential light steps. This number can be reduced by including a step length 0. This will stop the light curve. If a sample saturates at low light intensities the user may want to increase the available number of low light intensities. The protocol also contains a column with current values. These can vary between 0 and 256 units. Double clicking on such a current cell it is possible to change the current value, and this reduces the light intensity. Changing the current does not

automatically change the PAR value. For that a new light list has to be determined. The new PAR-value can also be estimated by plotting the PAR versus current values of the measured PAR list. To get a linear relationship, the PAR values should be plotted on a logarithmic timescale. By interpolation the new current-PAR value pair(s) can then be determined.

13.4 Practical considerations of O-I_1 fits

Sigma(II)

Sigma(II) is a parameter introduced by Ley and Mauzerall (1982, 1986) to define the effective cross section of PS II of a sample. If connectivity between PS II antennae exists, the Sigma(II) value will increase as more PS II reaction centers close. By determining Sigma(II), it is possible to define activity on a per PS II basis.
This way it is possible to determine absolute electron transport rates instead of relative ones and for this reason Sigma(II) represents an attractive parameter for investigators.

Ley and Mauzerall studied Sigma(II) mainly on the basis of the oxygen signal. Working with the MULTI-COLOR-PAM-II, Sigma(II) is determined on the basis of the fluorescence signal. Sigma(II) is related to the initial slope of the fluorescence rise following a dark-to-light transition. To derive Sigma(II) from the fluorescence signal requires a fit of the first phase – the photochemical phase – of the fluorescence rise: the O-I_1 phase. The script for the Sigma(II)-determination consists of 2.3 ms of actinic light with a single turnover flash after 1 ms to obtain the I_1-max value.

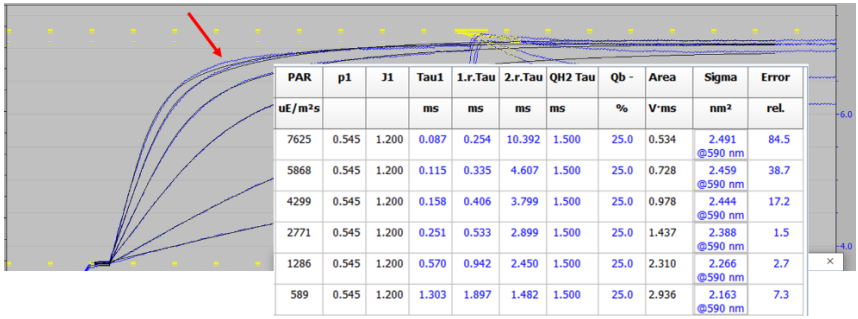
The simple fit

To derive Sigma(II) from the O-I_1 rise, Klughammer and Schreiber (2015) introduced a relatively simple fit model that did not consider the effect of the S-states on the O-I_1 rise. Slow S-state donation may lead to longer lived $P680^+$ states, and $P680^+$ is a strong fluorescence quencher. This may lead to quenching of the I_1-level and thereby affect the reference amplitude for the initial slope.
For dark acclimated samples, starting out in the S_1 state, this effect is minimal because the electron donation by the S_1 state to $Tyr_Z^+/P680^+$ is fast compared to the PS II acceptor side kinetics. If at the beginning of the measurement mixed S-state and acceptor side conditions exist, a model considering the S-states could take the presence of $P680^+$ into account.
The fit model considers: the connectivity parameter J, electron flow from Q_A^- to Q_B (1.r.tau), electron flow from Q_A^- to Q_B^-, the fraction Q_B^- at the start of the measurement and the slope (tau) and the exchange rate Q_BH_2 for Q_B can also be defined. Assuming that after 1 ms a maximum of 2 charge separations has taken place, the exchange parameter will probably not play a role if the sample was in the dark-acclimated state at the beginning of the measurement.
Sigma(II) is light intensity independent. If the fitting routine gives very similar Sigma(II) values at different light intensities, it is an indication that the value is correctly determined. Another criterion that should be considered is the I_1-value. The O-I_1 amplitude should be approximately the same at different light intensities. Lower O-I_1 values may point to a quenching process.
Of these parameters, the sigmoidicity parameter J (where J = p/(1-p) and p is Joliot's connectivity parameter) (Schreiber et al. 2012) is the most controversial. Ley and Mauzerall (1986) proposed that J is low and that there is very little connectivity. Laisk and Oja (2013) concluded that the non-linearity indicating connectivity is only observed during a dark-to-light transition and not in continuous light. Joliot and Joliot (1964) calculated a p-value of ~0.55 which

equates a J-value of ~1.2. For *Chlorella vulgaris* cells this value gave reasonable fit parameters.

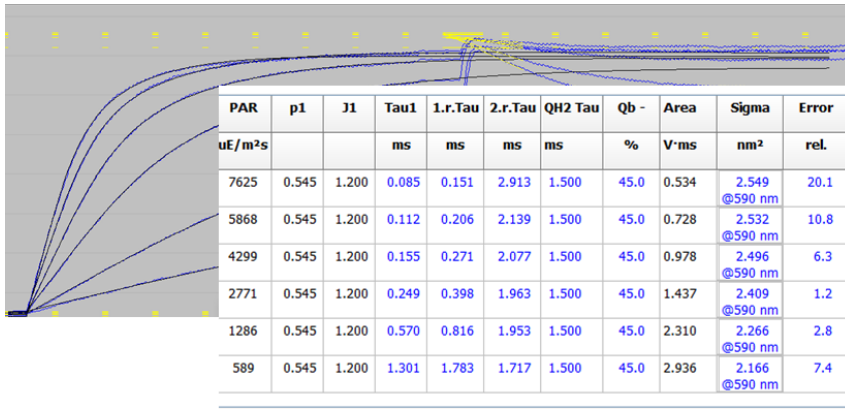
The fit routine allows the user to make J a fit parameter. However, it is important to fix the J-value, because it interacts with Sigma(II). Higher J-values lead to higher Sigma(II) values. Variation in J is, as a consequence, an important source of variability in Sigma(II). For a particular state of an organism, J should have a fixed value. As noted above, J = 1.2 gave good results with *Chlorella*, but the user may want to test a few J-values when working with other organisms.

With the combination Ctrl A, it is possible to increase the number of parameters that can be modified. It is for example possible to reduce the exchange rate at the Q_B site to 1.5 ms and to increase the fraction Q_B^- to 0.45. As shown in the next two figures, the fraction Q_B^- at the start of the measurement may improve the fit near 1 ms.



PAR	p1	J1	Tau1	1.r.Tau	2.r.Tau	QH2 Tau	Qb -	Area	Sigma	Error
uE/m²s			ms	ms	ms	ms	%	V·ms	nm²	rel.
7625	0.545	1.200	0.087	0.254	10.392	1.500	25.0	0.534	2.491 @590 nm	84.5
5868	0.545	1.200	0.115	0.335	4.607	1.500	25.0	0.728	2.459 @590 nm	38.7
4299	0.545	1.200	0.158	0.406	3.799	1.500	25.0	0.978	2.444 @590 nm	17.2
2771	0.545	1.200	0.251	0.533	2.899	1.500	25.0	1.437	2.388 @590 nm	1.5
1286	0.545	1.200	0.570	0.942	2.450	1.500	25.0	2.310	2.266 @590 nm	2.7
589	0.545	1.200	1.303	1.897	1.482	1.500	25.0	2.936	2.163 @590 nm	7.3

Fig. 45: Increasing the fraction Q_B^- from 25 to 45% improved the fit around 0.5 ms (red arrow; compare with Fig. 47).



PAR	p1	J1	Tau1	1.r.Tau	2.r.Tau	QH2 Tau	Qb -	Area	Sigma	Error
uE/m²s			ms	ms	ms	ms	%	V·ms	nm²	rel.
7625	0.545	1.200	0.085	0.151	2.913	1.500	45.0	0.534	2.549 @590 nm	20.1
5868	0.545	1.200	0.112	0.206	2.139	1.500	45.0	0.728	2.532 @590 nm	10.8
4299	0.545	1.200	0.155	0.271	2.077	1.500	45.0	0.978	2.496 @590 nm	6.3
2771	0.545	1.200	0.249	0.398	1.963	1.500	45.0	1.437	2.409 @590 nm	1.2
1286	0.545	1.200	0.570	0.816	1.953	1.500	45.0	2.310	2.266 @590 nm	2.8
589	0.545	1.200	1.301	1.783	1.717	1.500	45.0	2.936	2.166 @590 nm	7.4

Fig. 46: Dataset of O-I1 transients measured at 590 nm and different light intensities. Good fits and stable Sigma(II) values could be obtained.

When fitting a set of measurements made at different light intensities, there is another source of variability that has to be considered and that is the time point at which the fluorescence starts to increase. This may at high light intensities occur at shorter times than at lower light intensities.

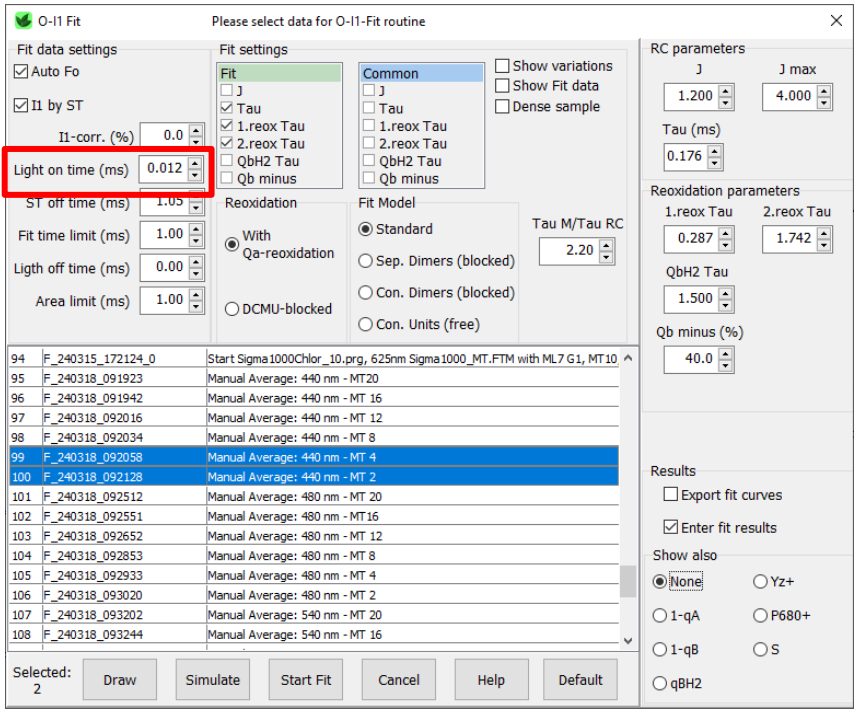


Fig. 47: The O-I$_1$ fit window. The two selected measurements are plotted and analyzed in Fig. 51.

In the fit window the parameter light on time (ms) can be modified. At high light intensities with steep slopes a value of 0.012 may be appropriate, whereas at lower light intensities with relatively gentle slopes a value of 0.017 or 0.020 may work better. Given that for Sigma(II) the initial slope is very important, an incorrect starting point for the fit is another source of variability.

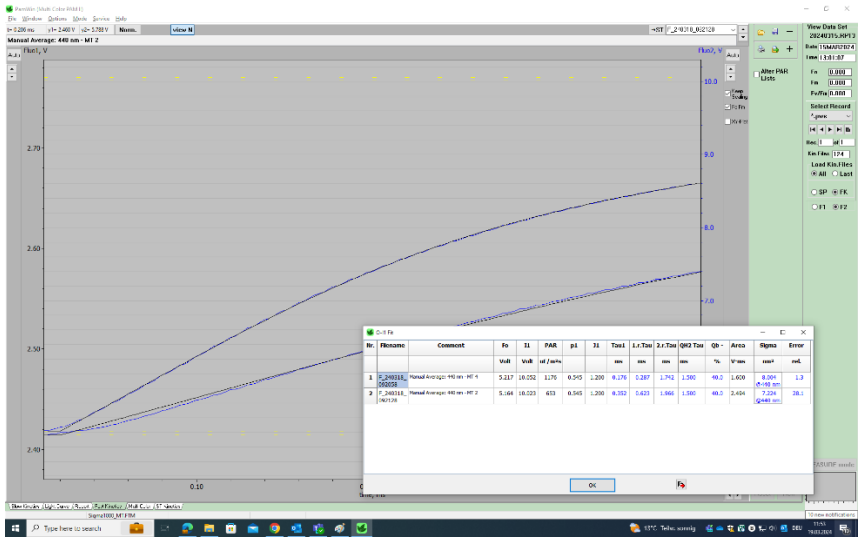


Fig. 48: The timing of the fluorescence rise shows some variability, which can be compensated for by slightly modifying the 'light on time'.

In the example given, a value of 0.020 works well for the steepest of the two measurements, but probably is still too low for the other measurement.

However, it may be important to establish if these relatively flat curves, where forward electron transport plays a relatively large role, contain enough kinetic information for a reliable fit.

Looking at the literature typical values for the parameters 1.r.tau (Q_A^- to Q_B transfer) are between 100 and 400 µs and for 2.r.tau (Q_A^- to Q_B^- transfer) between 600 µs and 800 µs. The fit routine gives often considerably higher values for 2.r.tau. In this context, it may be noted that at high light intensities and steep slopes, the contribution of forward electron transfer to the fluorescence rise strongly decreases. The question is then how much this affects the determination of these values.

Users often only consider the error. However, it is also important to consider the values of the different fit parameters. Are they reasonable? If the fit yields extreme values (e.g. J = 0 or 1.r.tau or 2.r.tau

is 20 or 40 ms) it is better to change the starting fit parameters and try again.

As noted above, for a given sample, the Sigma(II) value should be independent of the light intensity. That means that a determination of the Sigma(II) light intensity dependences for the different wavelengths should yield straight lines. As the figure below shows this is more or less achieved for *Chlorella vulgaris* and J = 1.2.

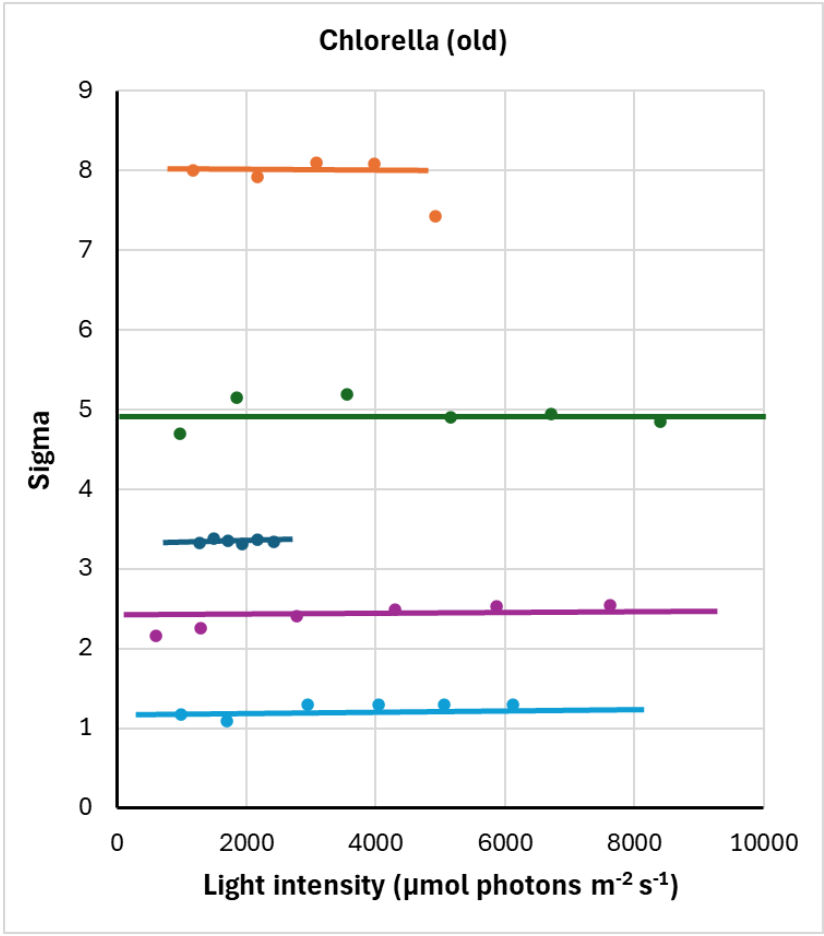


Fig. 49: The light intensity independence of the Sigma(II) values when derived from O-I_1 transients measured at high light intensities (yielding well-defined O-I_1 transients).

The points in the figure are averages of each time three measurements.

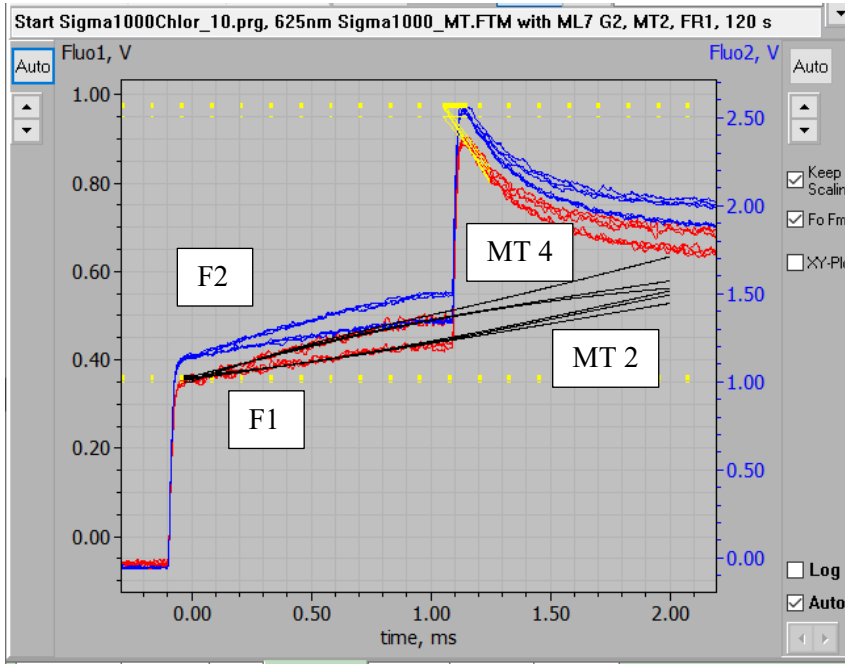


Fig. 50: Low intensity pulses, yielding relatively flat, not well-defined O-I_1 transiens and a relatively high variability in the obtained fits.

In this respect, it is important to obtain curves with well-defined rise kinetics. Above, an example of two relatively flat measurements, where very similar curves may yield quite different fits. Below, an example from a 480 nm measurement (MT12 and MT8) containing much more kinetic information and much more consistent fits.

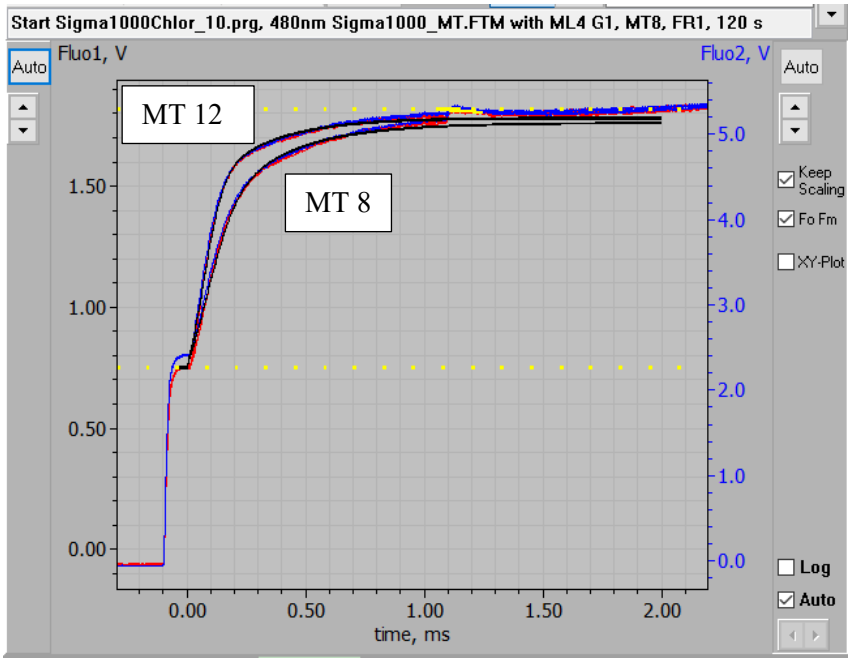


Fig. 51: High intensity MT-pulses yielding well defined O-I_1 transients and reproducible fits.

On the basis of these figures, it is possible to propose a light intensity around 2000 µmol photons m^{-2} s^{-1} for 440 and 480 nm and a light intensity of 3000-4000 µmol photons m^{-2} s^{-1} for 540, 590 and 625 nm. *Chlorella* is a green alga and for, e.g., diatoms showing a different wavelength dependence the conclusion may be different.
To get good results with the simple fit model discussed above, it is important that the samples return between measurements to the S_1 state. In the experiments shown here, a time interval of 120 s between measurements was used. It was not tested if shorter time intervals would also have been sufficient.
With a simple protocol, the reproducibility of the measurements during a series can be tested. The standard Sigma(II) protocol consists of 6 measurements. To test the reproducibility of the measurements, the user can change the protocol, making all the pulses

the same. For example, 6 times MT 20 at 625 nm. Clicking <Calc>, the fit window pops up. In the fit window the user can select the 6 measured transients and click <Draw>. If the 6 measurements lay perfectly on top of each other, the chosen time interval was sufficient (the samples each time returned to the same state). If the first measurement differs from the next 5, it is an indication that the dark time interval was not long enough to allow the samples to return to their initial state.

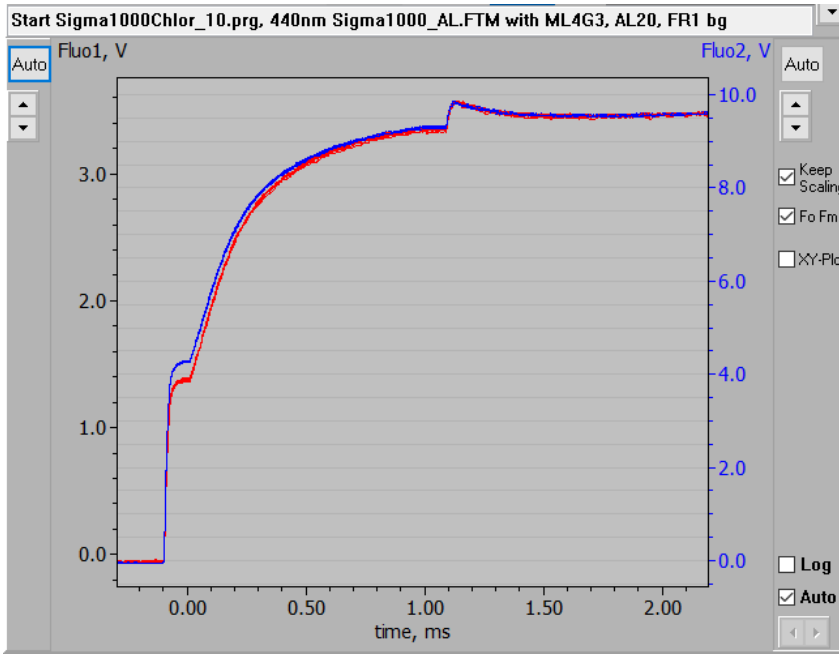


Fig. 52: Six O-I_1 measurements on *Chlorella vulgaris* cells spaced 120 s apart. For each measurements the identical starting state and rise kinetics was obtained.

The Figure shows a sequence of 6 pulses spaced 120 s apart for fluorescence channel F1 (red) and fluorescence channel F2 (blue). The different measurements are essentially identical.

If the first measurement is different, it suggests that the next five had a different PS II acceptor/donor side configuration. To get a successful fit under such conditions, a fit model taking the S-state

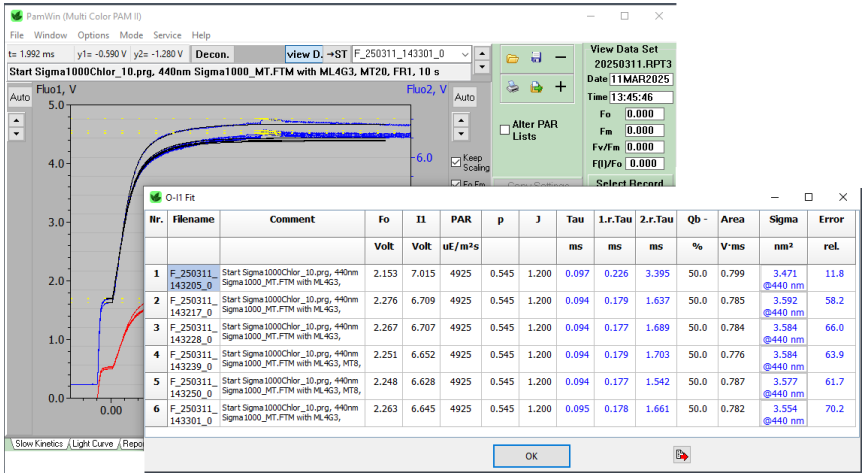
contributions into account should be used. Klughammer and Schreiber are working on a 48-state model which can do this. In that case, S-state related I_1-level quenching and potential S-state related effects on the initial slope can be taken into account.
In summary, to get good and reproducible Sigma(II) values several things should be considered:
Preferentially, a kinetically well-defined transient is needed, containing enough kinetic information for the fit routine to work with. For this, MT-pulses are needed. In my hands, there is an optimum. 480 nm transients determined at the highest light intensity possible, gave sub-optimal fits. Suggested light intensities for *Chlorella vulgaris*: ~2000 µmol photons m^{-2} s^{-1} at 440 and 480 nm and 3000-4000 µmol photons m^{-2} s^{-1} at 540, 590 and 625 nm. For cyanobacteria absorbing light less efficiently in the blue than green algae, the user may want to use the highest light intensities possible at 440 and 480 nm.
Above, the O-I_1-measurements of the dark-adapted state were discussed. This is already a relatively complicated situation where at least 5 processes play a role (Sigma(II), J, 1.r.tau, 2.r.tau and fraction Q_B^-). Starting with a mix of states, the S-states should be considered as well. Allowing the samples to return to their dark state between measurements takes time. Maybe more time than users are willing to invest. In that case it may be an idea to determine what the effect of an S-state mix on the Sigma(II) value is and to correct Sigma(II) values on that basis.
The J-value should be fixed. Sigma(II) and J interact, higher J values yield higher Sigma(II) values. This is an avoidable source of variation.
The light-on time should be checked and if necessary corrected. To make deviations visible, the logarithmic-scale-option for the O-I_1 transient may be used.

The fraction Q_B^- should maybe be increased to ~45% (default is 25%) although the observed improvement of the fit (around 1 ms) does not necessarily affect the Sigma(II) values.

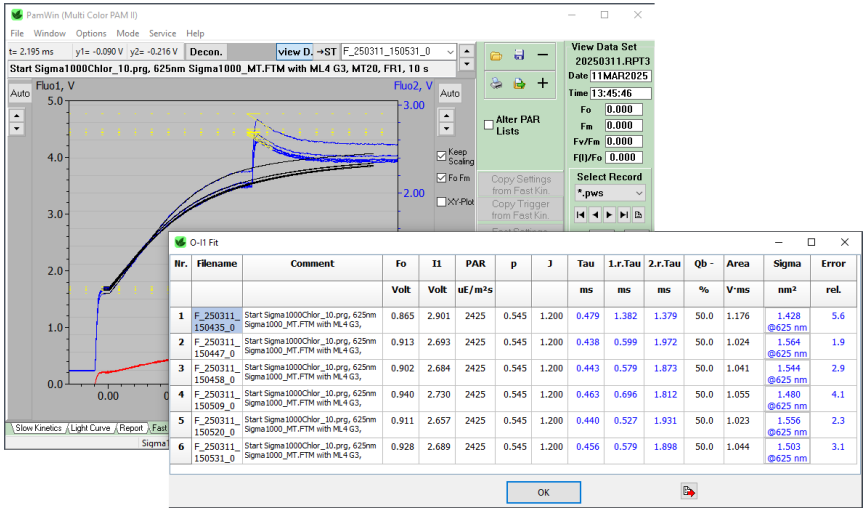
Using 10 s intervals between O-I_1 determinations

If the user wants to measure O-I_1 curves with only 10 s time intervals, there is a methodological issue that should be considered. For the first measurement, in essence all PS II reaction centers are, at time 0, in the S1 state. The subsequent measurements contain at time 0 considerable levels of higher S states which lead to a quenching of the I_1 level. This quenching effect affects the Sigma(II) determination. The extent of quenching is for measurements 2-6 quite similar. This quenching effect is not described by the standard fit algorithm, but since the Sigma(II) value is defined by the initial part of the transient, the effect on the Sigma(II) value may be limited.

To quantify the size of this effect, the following experiment can be made. Repeat 6 times the same O-I_1 measurement for all 5 wavelengths, and determine the ratio between the average value of the Sigma(II)s of measurements 2-6 and Sigma(II) determined for the first measurement. This approach is illustrated in the next few figures.



Nr.	Filename	Comment	Fo	I1	PAR	p	J	Tau	1.r.Tau	2.r.Tau	Qb -	Area	Sigma	Error
			Volt	Volt	uE/m²s			ms	ms	ms	%	V·ms	nm²	rel.
1	F_250311_143205_0	Start Sigma1000Chlor_10.prg, 440nm Sigma1000_MT.FTM with ML4G3,	2.153	7.015	4925	0.545	1.200	0.097	0.226	3.395	50.0	0.799	3.471 @440 nm	11.8
2	F_250311_143217_0	Start Sigma1000Chlor_10.prg, 440nm Sigma1000_MT.FTM with ML4G3,	2.276	6.709	4925	0.545	1.200	0.094	0.179	1.637	50.0	0.785	3.592 @440 nm	58.2
3	F_250311_143228_0	Start Sigma1000Chlor_10.prg, 440nm Sigma1000_MT.FTM with ML4G3,	2.267	6.707	4925	0.545	1.200	0.094	0.177	1.689	50.0	0.784	3.584 @440 nm	66.0
4	F_250311_143239_0	Start Sigma1000Chlor_10.prg, 440nm Sigma1000_MT.FTM with ML4G3, MT8,	2.251	6.652	4925	0.545	1.200	0.094	0.179	1.703	50.0	0.776	3.584 @440 nm	63.9
5	F_250311_143250_0	Start Sigma1000Chlor_10.prg, 440nm Sigma1000_MT.FTM with ML4G3, MT8,	2.248	6.628	4925	0.545	1.200	0.094	0.177	1.542	50.0	0.787	3.577 @440 nm	61.7
6	F_250311_143301_0	Start Sigma1000Chlor_10.prg, 440nm Sigma1000_MT.FTM with ML4G3,	2.263	6.645	4925	0.545	1.200	0.095	0.178	1.661	50.0	0.782	3.554 @440 nm	70.2



Nr.	Filename	Comment	Fo	I1	PAR	p	J	Tau	1.r.Tau	2.r.Tau	Qb -	Area	Sigma	Error
			Volt	Volt	uE/m²s			ms	ms	ms	%	V·ms	nm²	rel.
1	F_250311_150435_0	Start Sigma1000Chlor_10.prg, 625nm Sigma1000_MT.FTM with ML4 G3,	0.865	2.901	2425	0.545	1.200	0.479	1.382	1.379	50.0	1.176	1.428 @625 nm	5.6
2	F_250311_150447_0	Start Sigma1000Chlor_10.prg, 625nm Sigma1000_MT.FTM with ML4 G3,	0.913	2.693	2425	0.545	1.200	0.438	0.599	1.972	50.0	1.024	1.564 @625 nm	1.9
3	F_250311_150458_0	Start Sigma1000Chlor_10.prg, 625nm Sigma1000_MT.FTM with ML4 G3,	0.902	2.684	2425	0.545	1.200	0.443	0.579	1.873	50.0	1.041	1.544 @625 nm	2.9
4	F_250311_150509_0	Start Sigma1000Chlor_10.prg, 625nm Sigma1000_MT.FTM with ML4 G3,	0.940	2.730	2425	0.545	1.200	0.463	0.696	1.812	50.0	1.055	1.480 @625 nm	4.1
5	F_250311_150520_0	Start Sigma1000Chlor_10.prg, 625nm Sigma1000_MT.FTM with ML4 G3,	0.911	2.657	2425	0.545	1.200	0.440	0.527	1.931	50.0	1.023	1.556 @625 nm	2.3
6	F_250311_150531_0	Start Sigma1000Chlor_10.prg, 625nm Sigma1000_MT.FTM with ML4 G3,	0.928	2.689	2425	0.545	1.200	0.456	0.579	1.898	50.0	1.044	1.503 @625 nm	3.1

Examples of a set of 6 identical O-I_1 measurements spaced 10 s apart at respectively 440 and 625 nm for a Chlorella culture. These measurements at the five wavelengths were fit using a connectivity parameter J = 1.2 and 50% Q_B^-. This led to the following dataset:

440 nm
1 – 3.471 – 11.8
2 – 3.592 – 58.2
3 – 3.584 – 66.0
4 – 3.584 – 63.9
5 – 3.577 – 61.7
6 – 3.554 – 70.2 – 3.578 – 1.031
av. 2-6 – deviation

480 nm
1 – 2.473 – 33.6
2 – 2.621 – 46.6
3 – 2.598 – 42.4
4 – 2.600 – 39.7
5 – 2.604 – 41.4
6 – 2.611 – 40.6 – 2.607 – 1.054

540 nm
1 – 0.594 – 19.5
2 – 0.633 – 8.4
3 – 0.636 – 7.1
4 – 0.609 – 10.7
5 – 0.623 – 14.1
6 – 0.655 – 3.5 – 0.631 – 1.063

590 nm
1 – 1.131 – 66.4
2 – 1.211 – 38.9
3 – 1.197 – 28.8
4 – 1.197 – 40.6
5 – 1.185 – 47.4
6 – 1.209 – 53.4 – 1.200 – 1.061

625 nm
1 – 1.428 – 5.6
2 – 1.564 – 1.9
3 – 1.544 – 2.9
4 – 1.480 – 4.1
5 – 1.556 – 2.3
6 – 1.503 – 3.1 – 1.529 – 1.071

The data give a 3.1% too high Sigma(II)-value (taking the first O-I_1 measurement as a reference) at 440 nm, 5.4% at 480 nm, 6.3% at 540 nm, 6.1% at 590 nm and 7.1% at 625 nm.
The same experiment can also be used to determine the Sigma(II)-deviation in the case of other organisms or mixtures of organisms.

Literature

Joliot A and Joliot P (1964) Étude cinétique de la reaction photochimique libérant l'oxygène au cours de la photosynthèse. C R Acad Sci Paris 258: 4622-4625

Klughammer C, Schreiber U (2015) Apparent PS II absorption cross-section and estimation of mean PAR in optically thin and dense suspensions of *Chlorella*. Photosynth Res 123: 77-92

Laisk A, Oja V (2013) Thermal phase and excitonic connectivity in fluorescence induction. Photosynth Res 117: 431-448

Ley AC, Mauzerall DC (1982) Absolute absorption cross-sections for photosystem II and the minimum quantum requirement for photosynthesis in *Chlorella vulgaris*. Biochim Biophys Acta 680: 95-106

Ley AC, Mauzerall DC (1986) The extent of energy transfer among Photosystem II reaction centers in Chlorella. Biochim Biophys Acta 850: 234-248

Schreiber U, Klughammer U, Kolbowski J (2012) Assessment of wavelength-dependent parameters of photosynthetic electron transport with a new type of multi-color PAM chlorophyll fluorometer. Photosynth Res 113: 127-144

13.5 Single turnover flashes and measurement of period-4 oscillations

Single turnover flashes

The purpose of a single turnover flash is to induce one charge separation in all reaction centers before forward electron transfer from Q_A to Q_B can occur. The complete configuration offers for these measurements two types of flashes. The multi-color emitter can either produce flashes of the different wavelengths available or a multi-color flash where the light emitted by the different LEDs is pooled. The only way to change the intensity of these flashes is to

modify their length between 5 and 50 µs with steps of 5 µs. The new strong flash lamp of the MULTI-COLOR-PAM-II produces only flashes of 450 nm light. Flash intensities of more than 1 mol photons m^{-2} s^{-1} are possible and the intensity can be varied by changing the voltage between ~800 and 3300 mV. At voltages below ~800 mV, flashes are no longer generated. With this flash lamp it is possible to induce a charge separation in all reaction centers with a 1 µs flash. However, the fluorescence induced by such a flash will stay far away from the maximum fluorescence intensity due to the presence of simultaneously induced $P680^+$, a strong fluorescence quencher and HIQ (Car triplets) which also quenches fluorescence. To allow most of the $P680^+$ to become re-reduced again and to reach the maximum attainable fluorescence intensity (which still is affected by HIQ) much longer flashes are needed.

Period-4 oscillations

Fluorescence is in the first place associated with the redox state of Q_A. $P680^+$ is also a strong quencher of fluorescence, but due to its short lifetime it only modulates the fluorescence intensity. At F_0 and F_M by default its concentration is zero.
At the end of the 1960s it was discovered that the oxygen release induced by a train of single turnover flashes is characterized by a period-4 oscillation in the oxygen signal with a peak on the third flash. Shortly after, it was discovered that the same period-4 oscillations could be observed in the F_0 and F_M levels as well. However, the period-4 oscillations in the F_0 and F_M level are not the same, if anything, they are opposite (see Delosme and Joliot 2002 and references therein; Klughammer et al. 2024).

Assay for single turnoverness

With the MULTI-COLOR-PAM-I it was already possible to measure period-4 oscillations. In the new MULTI-COLOR-PAM-II, the

improved flash quality and intensity as well as the ability to measure the fluorescence rise kinetics gives many new options. Both for the multi-color flashes and the STKs, the flash length can be varied. It is important to determine when a flash is sub-saturating and which length leads to more than 1 charge separation. The single turnoverness of the single turnover flash is, therefore, important. The more single turnover a flash, the more flashes will be needed before the period-4 oscillations have completely disappeared. By varying the flash length/intensity, it is possible to determine which flash length/intensity gives the best period-4 oscillations and thereby comes closest to being single turnover.

S-states

Period-4 oscillations can be used to probe the S-state of a sample, that is the redox state of the Mn-cluster of a sample. In response to a charge separation (for example induced by a single turnover flash) an electron is extracted from the Mn-cluster. Such an oxidation reaction increases the S-state by 1. If the dark-adapted state is the S_1 state, then a charge separation creates the S_2 state. After two more charge separations and two more electron extractions the S_4 state is reached, which has an oxidation potential that is high enough to split two water molecules into an oxygen molecule, 4 protons and 4 electrons. These 4 electrons change the S-state of the Mn-cluster back to S_0. Further charge separations and electron extractions then start to build-up the oxidation potential again for the next water splitting event.

Tyr_D

In experimental terms the system has one peculiarity. Tyr_Z, which is located in the D1 protein of PS II donates electrons to $P680^+$ and accepts electrons from the Mn-cluster. In the D2 protein that forms with D1 the heterodimer of the reaction center of PS II, there is also

a tyrosine residue which is called Tyr_D. In a fraction of the PS II reaction centers Tyr_D is in the reduced state following dark adaptation. In thylakoid membranes a value of 25% reduced has been found, but in PS II membranes the observed percentage is lower. Between the first and the second flash of a flash train Tyr_D can donate its electron to the Mn-cluster and thereby revert an S_2 state to an S_1 state. From an analysis point of view this gives the impression that a fraction of the S_0 state was present. In quite a few publications it is still claimed that the dark-adapted state contains a fraction S_0 even though we know already since the 1980s that this is not the case.

Probing the PS II donor side redox state

Applying a flash train of 15-20 single turnover flashes to a dark-adapted photosynthetic sample gives a particular pattern of period-4 oscillations with the highs and lows at particular flash numbers. If the sample had been in the S_0 state at the start of the flash train, all these lows and highs would have occurred one flash later. Had the sample been in the S_2 state, they would have occurred one flash earlier. In other words, a difference in S-states causes a shift in the pattern. The more synchronized the PS II reaction centers in the sample are, the sharper the period-4 oscillations. Due to misses (mostly due to recombination reactions between flashes) and double hits (a flash induces two charge separations) the period-4 oscillation will start to disappear, having disappeared completely once a mix of 25% of each S-state is reached.

Kinetic measurements

Using a pump probe technique, it is also possible to determine the S-state decay kinetics. Giving 1 or 2 pre-flashes, either the S_2 or S_3 state can be induced. Over time these states will decay back in darkness to the S_1 state. By giving a flash train Δt after the pre-

flash(es) snapshots of this decay process can be obtained. The S-state decay can be followed by fitting the period-4 oscillations. It is, however, also possible to force every fourth flash value to either 0 or 1 and subsequently plot the oscillation amplitude of an in between flash as a function of Δt. The time resolution of this measurement is determined by the time interval between the flashes in the flash train. For fluorescence measurements with the MULTI-COLOR-PAM-II a flash rate of 10 Hz is possible, giving a time resolution of 100 ms.

Processes responsible for period-4 oscillations

The period-4 oscillations in F_M-value are determined to a large extent by the presence and fluorescence quenching effect of $P680^+$, especially the $P680^+$ due to the equilibrium with Tyr_Z^+ and this equilibrium is S-state dependent because the electron donation rate of the Mn-cluster to Tyr_Z^+ is S-state dependent. The mechanistic basis for the period-4 oscillations in the F_0 values is still unknown. Experiments have shown that the size of these oscillations is sensitive to antenna size and sample quality.

Period-2 oscillations and Sample and Hold

The MULTI-COLOR-PAM-I did not record the fluorescence during a flash. During the flash the detector was turned off to avoid blinding by the energy overload caused by the very intense flash-light intensity. This function is called ‘Sample & Hold’. The fluorescence detector was turned on again a few µs after the flash (this time delay is defined in the script). At this point in time electron transfer from Q_A^- to Q_B has already started and this causes so-called period-2 oscillations. Electron transfer from Q_A^- to Q_B has a tau-value of about 150/300 µs, whereas electron transfer from Q_A^- to Q_B^- has a tau-value of about 600/800 µs, which means that in the Q_B^- state the re-oxidation rate of Q_A^- is somewhat slower than in the Q_B state.

In principle, these period-2 oscillations can be eliminated by extrapolating the detected F_M back to 'all Q_A still reduced'. An alternative approach that works for the period-4 oscillations in the F_0 level is making a gliding average of each time two flash values.

Potential applications

This type of measurements can be used to characterize site directed mutants of amino acid residues at the donor side of PS II or to characterize the effects of a deletion of one or more of the extrinsic proteins on the redox state of the Mn-cluster, whereby the extrinsic proteins can be removed due to a mutation or chemically by a high salt-wash. It is also possible to look at the reactivity of the S-states towards external reactants like hydroxylamine or nitric oxide. The period-4 oscillations can also be used as a functional assay of the PS II donor side in response to e.g., Ca^{2+} depletion/substitution or Cl^- depletion substitution.
Crofts and co-workers in Urbana-Champaign have shown that also the study of the period-2 oscillations can yield meaningful information about the pH-dependence of electron transfer and the Q_A-Q_B equilibrium at the acceptor side of PS II.

Advantages of fluorescence measurements over oxygen measurements

Making use of period-4 oscillations of the F_0 and F_M signal has several advantages over the flash oxygen signal. For fluorescence measurements low chlorophyll concentrations can be used. The cells can be measured instantaneously, no settling of the sample on the bare oxygen electrode is needed. The time resolution can be much higher (10-20 Hz is no problem, whereas for flash oxygen measurement 1 Hz is a more typical flash frequency). This means that it allows a much higher time resolution. Finally, quartz cuvettes are less sensitive to the effects of corrosive reagents compared

with the bare oxygen electron. A disadvantage is that fluorescence is much more complex signal and, therefore, the interpretation is less straightforward.

13.6 Things not to forget!

Working with the MULTI-COLOR-PAM(-II), I came across a few points that I felt other users should be aware of as well.

It is important to be aware of the fact that the hardware stirrer switch only puts the stirrer in standby mode. Stirring is controlled by the software and the “Stirrer on” box should be checked in the “Multi Color” window. Only then, the LED of the hardware switch is turned on and at the same time stirring of the sample starts.

In several languages (for example German or Dutch) decimals are preceded by a comma instead of a point. Make sure that in the “Windows System Settings” the decimal point is set. Decimal commas cause problems with the data analysis of the PamWin-4 software. Go to Start, choose Control Panel and then Regional and Language Options. Then click on Additional Settings. Finally, choose a point for the Decimal Symbol.

It also took me a while before I discovered where to find the pulse width window. This window can be opened by a right mouse click on either the <MT>, <ST> or <TR pulse> button. It is particularly important for the settings of (series of) STs.

In the MULTI-COLOR-PAM-II it is possible to overlay several measurements. The option is a bit hidden. To view a set of measurements overlaid on top of each other click the “Calc” button, then choose, for example, O-I1 fit. In the O-I1 fit window measurements can be chosen. Finally choose “Draw” to get the wished for graph.