PAM-2500

Portable
Chlorophyll Fluorometer

Handbook of Operation

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Contents

1 Safety Instructions

1.1 General Safety Instructions

a) Read the safety instructions and the operating instructions prior to operation of the device.

b) Pay attention to all the safety warnings.

c) Keep the device away from water or high moisture areas.

d) Keep the device away from dust, sand and dirt.

e) Do not put the device near sources of heat.

f) Always ensure there is sufficient ventilation.

g) 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

h) Clean the device only according to the manufacturer's recommendations.

i) Ensure that neither liquids nor other foreign bodies can get inside the device.

j) The device should only be repaired by qualified personnel.

1.2 Special Safety Instructions

The PAM-2500 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 PAM-2500 can emit very strong light! In order to avoid harm to your eyes, never look directly at the fiberoptics end, or at open light ports on the front side of the control unit.

Further, the Special Fiberoptics 2010-F must not be kinked, bent sharply, or pinched.

2 Introduction

The PAM-2500 Portable Chlorophyll Fluorometer is the follow-up model of the well-known PAM-2000/2100 instruments which were introduced in the 1990s as the first portable PAM fluorometers and since then have been successfully applied worldwide by numerous scientists. In the development of the PAM-2500, particular care was taken to maintain all properties appreciated by PAM-2000/2100 users and, at the same time, to take account of the recent technical progress.

Essentially, the hardware and optical system are thoroughly modernized. Also, while continuing basic elements of the graphical user interface, instrument operation is based on the newly developed PamWin-3 software. The program permits operation under Windows operating systems on normal personal computers, but also on touch screen tablet computers.

Major points of progress of the PAM-2500 with respect to its predecessors are:

- Use of LEDs (light emitting diodes) for all internal light sources including Saturation Pulses and Actinic Light.
- Blue and Red Actinic internal light sources.
- Single turn-over and multiple turn-over saturating flashes.
- Time resolution down to 10 µs.
- Easily updateable firmware.
- Optional touch-screen tablet computer.

2.1 Intention of this Handbook

The Portable Fluorometer PAM-2500 displays a high degree of flexibility in measuring and analyzing fluorescence. This does not mean,

however, that all features of this multifunctional instrument must be understood before measurements can be started. Actually, due to the "intelligent" central control of all functions by the special PamWin-3 software, serious operational mistakes harming the instrument are highly unlikely. Also, at first, there is no need to care about the numerous settings of instrument parameters, because these are pre-adjusted for standard measurements. Hence, even the inexperienced user can start measuring with a minimum of background knowledge and will be gradually guided to deeper understanding and more complex applications.

This handbook tries to cover all of the numerous features and applications of the PAM-2500 Fluorometer, some of which probably are not of immediate interest to many users, but probably will become relevant, as new questions arise on the basis of the results obtained. The best way to become acquainted with all features of the PAM-2500 Fluorometer is to read this handbook section by section, trying out all described functions. On the other hand, in order to get a quick start, it will suffice to read Section 4.2.

2.2 Important points

Help:

All program levels of the PamWin-3 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 until a small tag ("Tooltip") appears.
- A "Tooltip" remains visible for 2.5 seconds. During this interval, pressing the <**@**> or <**F1**> key results in the display of the relevant Help-text.

PAR sensor:

The continuous Reading of PAR sensor data can be switched off on the <General Settings> window in the Advanced Mode of PamWin-3. This should not be forgotten when running a Light Curve protocol! In that case the instrument will use the ambient light intensity for the light intensity axis of the Light Curve!

Battery:

Volt Battery voltage. A completely charged battery shows voltages up to 13.7 Volts. At Voltages below 10.5 Volts, the PAM-2500 operates unreliably, particularly during application of Saturation Pulses which require high current flows.

- Note: to prevent deep discharge of the battery, the PAM-2500 shuts off when the battery voltage drops below 9.4 Volts.
- Note: at battery voltages below 11.8 Volts, the PAM-2500 cannot be switched on.

3 Components and Setup

3.1 Basic System Components

The basic system consists of the following components:

a) PAM-2500 Control Unit

b) Special Fiberoptics 2010-F

c) Distance Clip 60° 2010-A

d) Battery Charger MINI-PAM/L

e) MINI-PAM/AK cable

f) Special USB cable PAM-2500/K1

g) Fluorescence Standard Foil

h) Spare Fuse

i) Carrier Bag

j) Transport Box 2040-T

k) Software. PamWin-3 System Control and Data Acquisition

3.2 Basic System Setup

Note:	Great caution should be exercised to prevent any dirt or foreign matter from entering the ports for the fiberoptics pins.

To set up the PAM-2500 fluorometer:

- Connect fiberoptics to the control unit using the three-pin optical connector on the front side of the unit).

Note: Do not force a plug into the wrong socket. Orient each plug so that the red dot on the plug coincides with the red dot of the socket. Do not try to disconnect a plug by pulling at the cable. Disconnect plug by pulling at the rippled metal part of the plug.

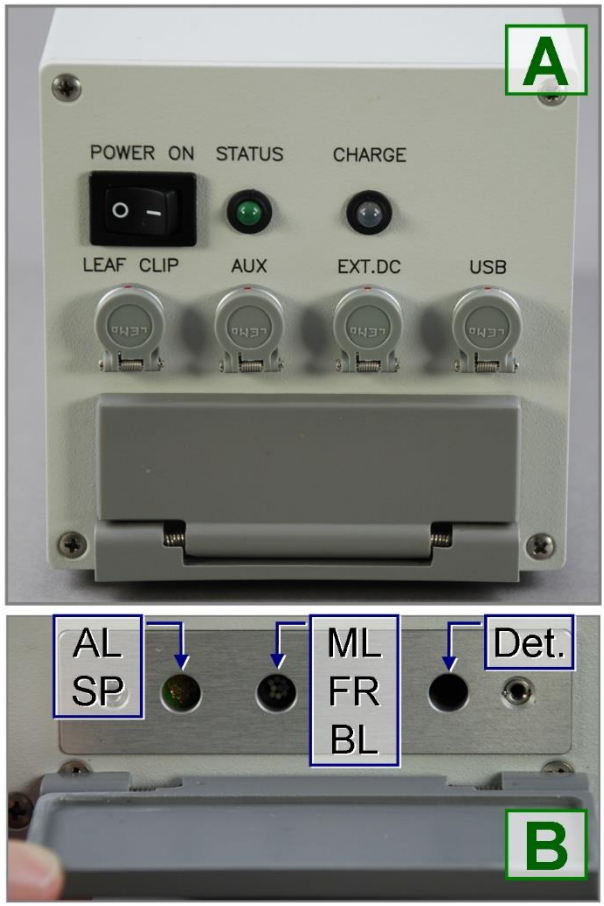


Fig. 1: PAM-2500 Control Unit.
Front. (AL: Red Actinic Light, SP: Saturation Pulse, ML: Measuring Light, FR: Far-Red light, BL: Blue Actinic Light, Det.: Detector)

- Connect the battery charger cable to the <EXT.DC> socket (see Fig. 1 A), and to line power: thereafter, a green <CHARGE> LED (Fig. 1 A) indicates that the internal battery is charged but yellow LED light signifies that the battery is currently charging. Please note that an external 12 V battery cannot recharge the internal battery; but it can provide power (*via* the MINI-PAM/AK cable) when the internal battery is empty.

- Connect computer and control unit (USB socket, Fig. 1 A) by the PAM-2500 special USB cable.

- Switch on PAM-2500 using the toggle switch labeled POWER ON (Fig. 1 A). Flashing of the <Status LED> indicates that the system is ready for communication. The LED will give continuous light once communication with the computer has been established. Constant light from the <Status LED> in the absence of computer communication indicates malfunction of the PAM-2500. Usually, switching power OFF and, after a couple of seconds, ON again will restore normal operation.

3.3 PamWin-3 Software Installation

The PAM-2500 software works, at the moment, under Windows 7/8/10.

Depending on the type of CD-ROM delivered, start with **a)** or **b)**.

a) CD contains **only the setup file** (e.g. PamWin_setup.exe) and the PDF copy of the present manual.

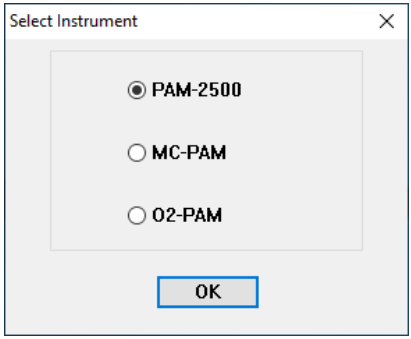
Close all programs, double click on the setup file and follow instructions. The setup routine will create in the C: root directory the folder PamWin_3 containing the PAM_WIN.exe program and accessory files. Further, an icon representing a shortcut to PamWin-3 is placed on the computer desktop, and

a program group called “PamWin-3” is created in the program list available in the start menu. The PamWin-3 program group includes the uninstall command for PamWin-3.

b) Your CD is entitled “Software & Manuals” and contains a **complete collection of Walz Software & Manuals**. The CD in the CD drive automatically starts the default internet browser of your computer. If automatic browser start fails, double-click on the file “index.html” in the root directory of the CD.

The browser loads the Walz homepage from the CD. Click “All Products” in the title bar of the homepage, select PAM-2500, MC-PAM or O2-PAM (the same software controls all three devices).

Close all programs and install PamWin-3 by clicking on “EXE-File”. Alternatively, you can copy the PamWin-3 software to any storage medium and use the copied file for installation. During the installation process, icons and shortcuts are created as described above.



c) Connect PAM-2500 to computer via USB-cable, switch Power on and with minimal delay of about 5 s start the PamWin-3 software by clicking on the “PamWin_3” icon. If the fluorometer is not switched on or connected, PamWin-3 will ask for the offline mode of operation. To view and analyze data acquired by the PAM-2500, select “PAM-2500” on the “Select Instrument” window.

3.3.1 Software Update.

The PamWin-3 software is continuously improved. Therefore, check if on the Walz web site a newer version of the software is available than that delivered with your instrument. PamWin-3 software for the PAM-2500 is available at:

https://www.walz.com/products/chl_p700/pam-2500/downloads.html

3.4 Accessories

See Chapter 7 for specifications of accessories.

3.4.1 Field Combo (optional)

The add-ons summarized as <Field Combo> convert the PAM-2500 chlorophyll fluorometer into a highly mobile field station, equipped with the options that a normal PC offers and allows working for hours independent of line current.

3.4.1.1 Tablet-PC

3.4.1.2 External Battery 000160101314

3.4.1.3 Automatic Charger 0001X

To assemble the <Field Combo> proceed as shown in Fig. 2. and described subsequently:

- Piece together <External Battery> and rectangular rubber foam filler (Fig. 2 A) and place in PAM-2500 carrier bag as shown in (Fig. 2 B and C).

- Slide-in computer box below elastic belt (Fig. 2 C and D). Note that the computer box is designed to accept an additional high-capacity battery to increase the time during which the instrument can be used independently.
- Insert the Tablet Computer (the model used changes rather frequently; Fig. 2 E).

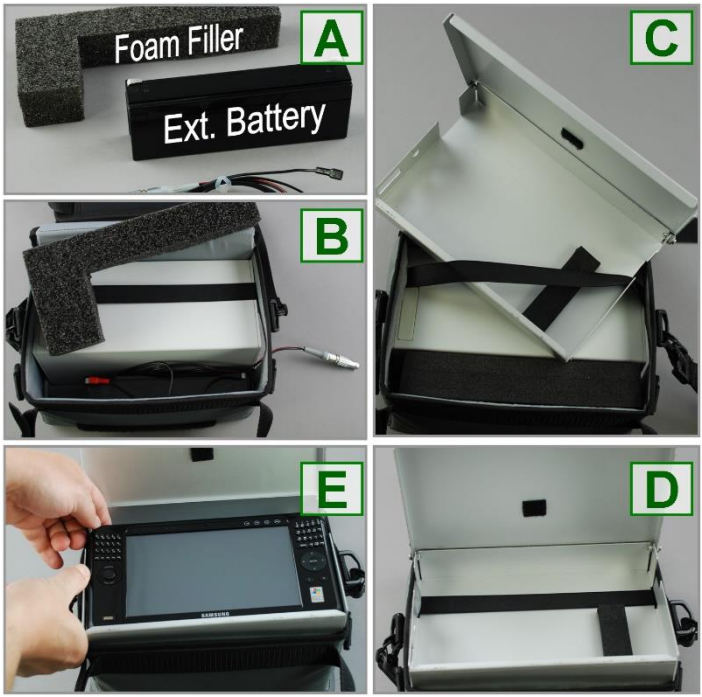


Fig. 2: PAM-2500 Field Setup

3.4.2 Additional Components

3.4.2.1 Special Fiberoptics 2010-F

The Special Fiberoptics 2010-F are connected to the front side of the PAM-2500 control unit with the help of a special plug that resembles an electrical connector. There are three “fiber pins” with different

optical cross-sections, which fit into the corresponding holes at the front side of the PAM-2500 housing, where they interface with the various light sources and the photodiode detector. Within the "interface plug" the three fiber branches are joined with a common fiber bundle and randomized *via* a 100 cm mixing pathway.

Note: The fiberoptics should be handled with care. Excessive bending, in particular close to the connector plug, should be avoided, as it would lead to fiber breakage resulting in a loss of signal amplitude. The fibers are protected by a steel-spiral and plastic mantle, which provide a natural resistance to strong bending.

3.4.2.2 Distance Clip 60° 2010-A

A Distance Clip is provided with the fiberoptics for convenient positioning of the fiberoptics end-piece relative to the sample. The axis of the end-piece is positioned at a 60° angle relative to the sample plane (angle of incidence, 30°). Shading of the sample is minimized when the tip of the fiberoptics points towards the sample at the side opposite to incident light. Two spacer rings may be used to define fixed distances.

The sample may be placed either below the hole of the leaf clip (e. g. thick leaves, lichens and mosses) or, preferentially, with normal leaves, above the hole (compare **Fig. 3**). In the latter case, the leaf can be kept in place by the folded part of the clip.

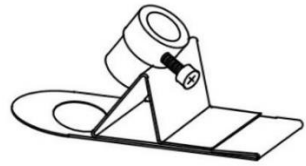


Fig. 3: Distance Clip (to position leaf with respect to fiberoptics)

The distance between the fiberoptics exit plane and sample has considerable influence on signal amplitude and effective light intensity (Fig. 4**Fehler! Verweisquelle konnte nicht gefunden werden.**). Clearly, with a 60° angle between sample plane and fiberoptics, the distance between fiber optics tip and leaf surface varies from one side of the measured area to the other side, and, hence, the leaf surface is exposed to a slight light gradient. A much more pronounced intensity gradient exists inside the leaf, because photons are lost by absorption as the light penetrates into the leaf reducing the effective light intensity. In essence, the measured signal will be dominated by the cell layers of the leaf closest to the fiberoptics, which are exposed to the maximum light intensity and thereby absorb the bulk of the light. These cell layers also absorb the largest fraction of the Measuring Light and emit most of the fluorescence which is received by the fiberoptics.

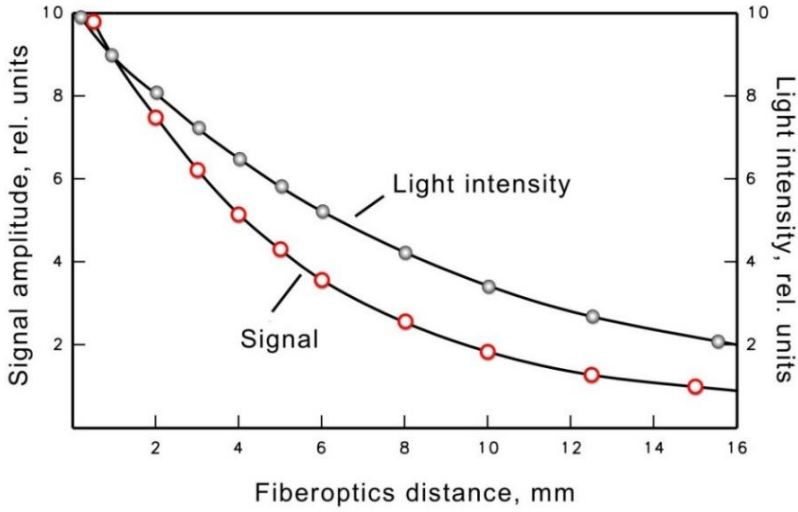


Fig. 4: Relationship between signal amplitude/light intensity and distance between fiberoptics exit plane and sample

3.4.2.3 Leaf-Clip Holder 2030-B (optional)

The Leaf-Clip Holder 2030-B is connected to the <LEAF CLIP> socket (Fig. 1 A) recording PAR and temperature parallel to chlorophyll fluorescence.

The Leaf-Clip Holder 2030-B is almost indispensable for field investigations, where ambient light and temperature conditions may vary considerably. It substitutes for the standard 'Distance Clip' as a device for defined positioning of the fiberoptics relative to the leaf plane. It features special mini-quantum and temperature sensors, the readings of which are transferred to the PAM-2500 with every Saturation Pulse measurement.

Placed in the holder, the leaf is resting on a Perspex tube with broadened upper side, which can be vertically adjusted, to adapt the holder to different leaf thicknesses. The fiberoptics axis forms a 60° angle with the leaf plane. Optionally, a 90° fiberoptics adapter (2030-B90) is available. The distance between fiberoptics and leaf can be varied. Standard distances are defined by spacer rings. The illuminated leaf area is limited by a steel ring with 10 mm ∅ opening.

At the bottom of the Leaf-Clip Holder 2030-B, a tripod mounting hole has been built in. Mounting the device on a tripod (e. g. Compact Tripod ST-2101) facilitates long measurements with the same plant.

The handle of the Leaf-Clip Holder 2030-B features a red push-button for remote control of the PAM-2500. Pressing this button initiates a Saturation Pulse.

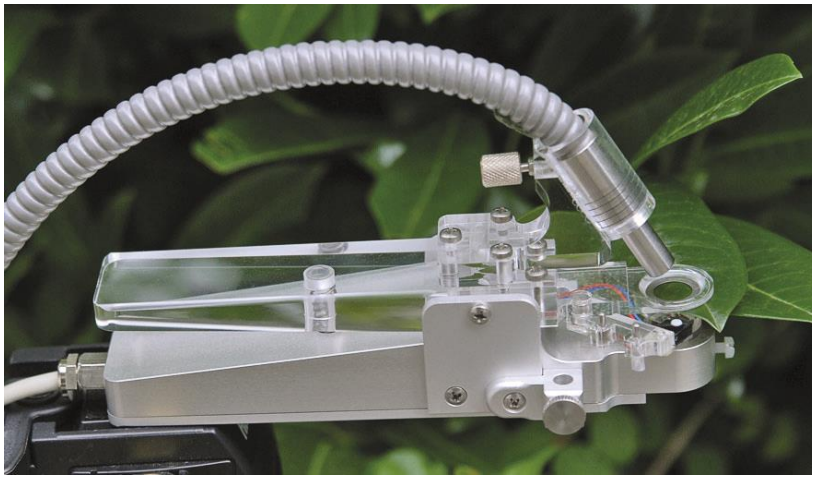


Fig. 5: Leaf-Clip Holder 2030-B with Fiberoptics 2010- F.

3.4.2.4 Mini-Quantum-Sensor

A mini-quantum sensor is integrated into the Leaf-Clip Holder 2030-B to monitor the photosynthetic active radiation (PAR) to which the sample is exposed. The mini-quantum-sensor measures incident PAR in µmol photons/(m^2·s), i.e. in units of flux density. Hence, the measured parameter PAR is identical to PPFD (photosynthetic photon flux density).

Essential optoelectronic elements of this mini-quantum-sensor are:

- a 1.5 mm ∅ diffusing disk
- a 0.5 mm diameter fiber, guiding the scattered light to the detector
- a filter combination selecting the photosynthetic active wavelength range between 380 and 710 nm
- a blue-enhanced silicon photodiode (detector with additional sensitivity in the 350-550 nm range)

The sensor is factory calibrated against a standard lamp. Radiation of the standard lamp fell perpendicularly on the sensor surface, that is, at an angle of incidence of 0°. However, the 2030-B sensor can measure with adequate accuracy both collimated (parallel) light incident at low angles (approx. perpendicular) as well as completely diffuse radiation coming from all directions. The angular response of the sensor, however, deviates from the ideal cosine behavior: to be exact, the sensor overestimates collimated light approaching at greater angles of incidence. When the PAM-2500 internal Actinic Light sources are applied *via* the fiberoptics (Fig. 5), e.g. for recording of light response curves, the sensor should be switched off *via* the software (see Advanced Level, General Settings, Section 4.3.1) and the PAR should be derived from previously defined/determined PAR-lists (see Section 4.3.1.5).

For the reliability of the calibration it is critical to keep the diffuser clean. It is advisable to check calibration regularly by comparison with a standard quantum sensor. Any deviation can be corrected by entering a recalibration factor in the Options menu of the PamWin-3 program (see Section 4.3.1.1). A substantial increase of the calibration factor from its original value of 1.000 indicates dirt-deposition on the diffuser, which may be reversed by gentle cleaning using a cotton tip applicator, moistened with some ethanol.

Thermocouple Monitoring Leaf Temperature

A NiCr-Ni thermocouple is mounted in the Perspex tube on which the investigated leaf area is resting. Its tip is forming a loop that gently presses against the lower surface of the leaf. In this way there is effective temperature equilibration and the thermocouple is protected from direct sun radiation. The reference couple is located on the circuit board, in close proximity to the thermovoltage amplifier, enclosed in the bottom part of the holder. The relationship between thermovoltage and temperature is almost linear. With decreasing temperatures there is a small decrease of $\Delta V/°C$. Calibration was performed at 25 °C. At

0 °C or –15 °C the deviation amounts to 0.5 and 0.8 °C, respectively. An offset value can be entered in the PamWin-3 program (see Section 4.3.1.1).

The temperature, as well as the PAR data, are automatically stored in the Report-file after every saturation pulse, together with the online calculated quenching parameters.

Fig. 6: Fiberoptics holder for Surfaces 2060-A (top) and Arabidopsis Leaf Clip 2060 (bottom) in combination with the mini-quantum/temp. sensor 2060-M.

3.4.2.5 Mini-Quantum/Temp.-Sensor 2060-M (optional)

The Mini-Quantum/Temp.-Sensor 2060-M essentially has the same features as outlined above for the Leaf-Clip Holder 2030-B, except that the mini-sensors of PAR and temperature are not mounted in a leaf-clip. This device is rather designed for experiments with objects which are not leaf-shaped, like crustose lichens and cushions of moss. The two miniature sensors can be attached to the site where fluorescence is monitored without interfering with the actual measurement. The 2060-M can for example be used in combination with the Arabidopsis Leaf Clip 2060 or the Fiberoptics holder for Surfaces 2060-A (see Fig. 6).

3.4.2.6 Dark Leaf Clip DLC-8 (optional)

The Dark Leaf Clip DLC-8 weighs approx. 4 g (Fig. 7) and, hence, can be attached to most types of leaves without any detrimental effects. It is equipped with a miniature sliding shutter which prevents light access to the leaf during a dark-adaptation period. This shutter is opened for the actual measurement only. The clip prevents exposure to external light. Proper dark-acclimation is essential for determination of the maximum quantum yield Fv/Fm and for recording of dark-to-light induction kinetics.

Using the Dark Leaf Clip DLC-8, the fiberoptics is positioned at a right angle with respect to the leaf surface at the relatively short distance of 7 mm. As a consequence, signal amplitude is distinctly higher than when the Leaf-Clip Holder 2030-B with 60° fiberoptics angle is used. In order to avoid signal saturation, the settings of Measuring Light Intensity and Gain have to be correspondingly lowered with respect to the standard settings (see Section 4.2).

When the leaf clip shutter is closed and the Measuring Light is on, an artefactual Ft signal is observed. This signal is due to a small fraction of the Measuring Light which is reflected by the closed shutter and reaches the photodetector. This background signal can be ignored as the reflection is much smaller when the shutter is opened, and the Measuring Light hits the strongly absorbing leaf instead of the metal surface of the shutter that acts like a mirror.

Fig. 7: Dark Leaf Clip

3.4.2.7 Suspension Cuvette KS-2500 (optional)

The PAM-2500 can also be used for the measurement of suspensions. For that purpose, the suspension Cuvette KS-2500 was developed, which can be connected to a water bath to control the sample temperature and an additional accessory is a magnetic stirrer with fiberoptics holder (MKS-2500) with which the fiber can be stabilized and settling of the cells/chloroplasts/thylakoid membranes can be prevented (Fig. 8).

Fig. 8: Suspension Cuvette KS-2500 (left) and magnetic stirrer with fiberoptics holder MKS-2500 (right).

4 PAM-2500 Operation

4.1 PamWin-3 Help

All program levels of the PamWin-3 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 until a small tag ("Tooltip") appears.
- A "Tooltip" remains visible for 2.5 seconds. During this interval, pressing the <**@**> or <**F1**> key results in the display of the relevant Help-text.

For getting acquainted with the instrument and its software, it is strongly recommended to make frequent use of this function.

4.2 Field Screen

The <Field Screen> of the PamWin-3 software has been developed for outdoor operation of the PAM-2500 fluorometer where ease and simplicity of instrument control is important. Conveniently, the elements of the <Field Screen> are accessible *via* the display of a touch screen PC. The <Field Screen> is divided into three areas containing (1) alphanumeric fields, (2) program and Script control, and (3) monitoring graphs, respectively (see Fig. 9).

The alphanumeric area contains the switches for light control and data derived from Saturation Pulse analysis. It also includes the **Zoom In** button: the <Zoom In> command results in the full screen display of the alphanumeric area. This can be reversed by clicking on the **Zoom Out** button on the expanded alphanumeric area.

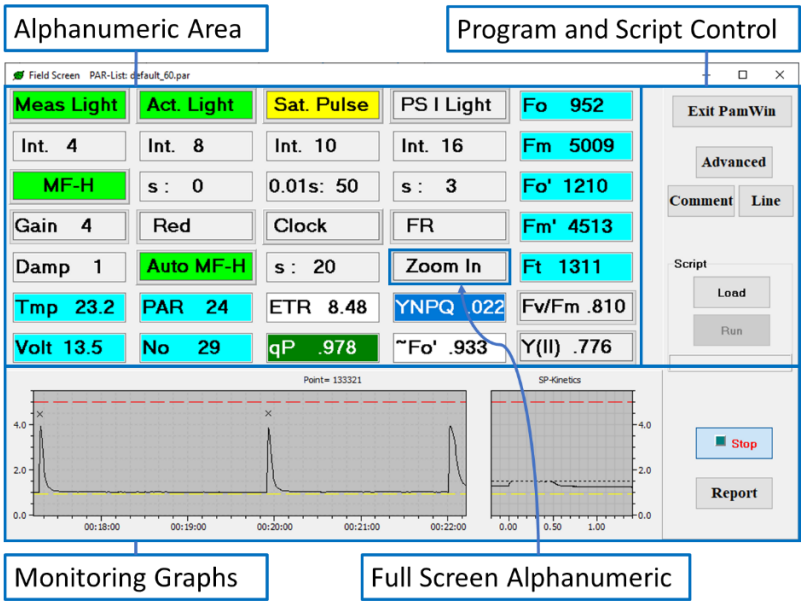


Fig. 9: Field Screen Overview.

4.2.1 Monitoring Graphs

The <Field Screen> provides 2 monitoring graphs (Fig. 9): the left graph records slow fluorescence changes, and also depicts Fm and Fm′ values as small crosses. Generally, only those Saturation Pulse analyses which result in graphical display of Fm or Fm′ values are entered in the Report file - Saturation Pulse analyses carried out with stopped monitoring screen are not reported. To view the Report file click on the **Report** icon. Editing of the Report is restricted to the <Advanced> level of the PamWin-3 program.

Monitoring of the fluorescence level (slow kinetics) begins automatically with PamWin-3 program start. The slow kinetics monitoring graph is restarted by an <Fo, Fm> determination clicking the **Fv/Fm** button (see below) or *via* the **Stop**/**Start** button. An

Fv/Fm determination automatically adapts the Y axis range of both monitoring screens to the signal amplitude. The actual signal amplitude (in Volts) can be read from the Y-axes. In both screens, the Fo and Fm levels are displayed as yellow and red dashed lines, respectively (Fig. 9). The time axis of the slow kinetics monitoring screen covers always an interval of 5 minutes, which is automatically shifted to the left for monitoring times > 5 minutes.

The right monitoring graph (shows a 1.6 seconds time interval) displays the fast fluorescence kinetics induced by the last Saturation Pulse. In addition to the Fo and Fm lines, the determined Fm′ level is displayed as a dashed black line (Fig. 10).

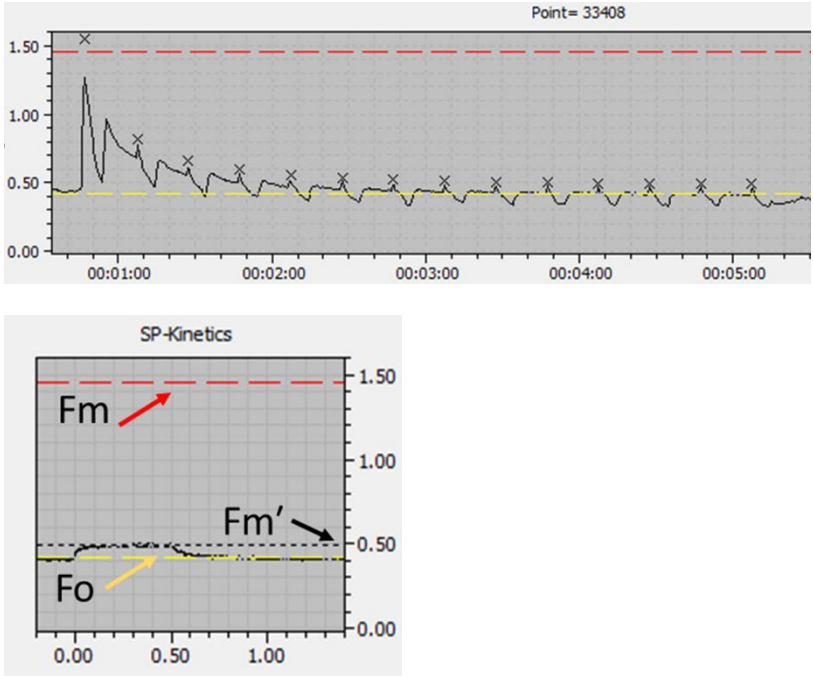


Fig. 10: The yellow, red and black dashed lines of the two <Field Screen> graph areas representing Fo, Fm and Fm', respectively.

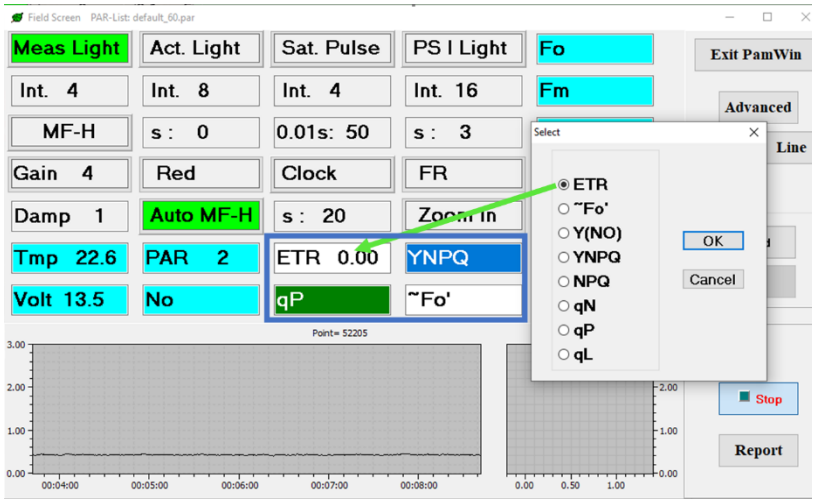


Fig. 11: Field Screen. The blue square surrounds the four fields for the selectable parameters. On clicking on one of these four fields a menu pops up that allows the selection of a parameter for this field.

4.2.2 Program and Script Control

The Program and Script Control section (Fig. 9) includes buttons to quit the program (**Exit PamWin**), to access the full range of PamWin-3 capabilities (**Advanced**), and to load Script files which carry out automatically pre-programmed experimental protocols (Script: **Load** and **Run**). Two additional options are available on this side bar: **Comment** and **Line**.

Comment and Line

After clicking either of the buttons, a pop-up text window appears. The **Comment** button and the **Show record comment file** button on top of the Report window (🗋) available in the Advanced mode are equal. The text entered in the **Comment** input window

forms the headline of the current Record. The **Line** button allows the annotation of Fv/Fm and Y(II).

The typical procedure is to start a Report by Fv/Fm determination, add text in the comment window and add a line comment after each saturation pulse analysis (Fv/Fm or Y(II)). Enter line comments only after the saturation pulse analysis has been finished.

4.2.3 Alphanumeric Area

The elements of the alphanumeric area are divided into four groups: (**1**) light control, (**2**) primary fluorescence data, (**3**) fluorescence ratio parameters (see Fig. 11 for the selection of these parameters), and (**4**) additional data like PAR and temperature (Fig. 12). **Set** buttons provide access to settings of parameters (e.g. light intensity or clock interval). Settings can be adjusted in different ways: (1) single-click with left mouse key on the button and turn the mouse wheel, (2) double-click with left mouse key on the button and enter the value, or (3), when a tablet is used, tap on the button.

Group 1 (Light Control, see Fig. 12) includes "status" and "set" buttons. A **Status** button either turns on or off a function (e.g. the Measuring Light) or it changes the light color (e.g. red to blue Actinic Light). To change a status, click on the **Status** button with the left mouse key. **Status** buttons with on/off-switching function display a green background when the associated function is active. The **Status** buttons controlling light color, display the selected type of light as text information.

All parameter fields of **groups 2 to 4** (Fig. 12) display information related to the current Saturation Pulse measurement. The "Fluorescence Ratio Parameters" (**group 3**) also contain two fields to trigger a Saturation Pulse analysis: the **Fv/Fm** (for dark-adapted leaves:

reference values Fo and Fm) and **Y(II)** (for light acclimated leaves: F or Fo′ and Fm′) buttons (see 4.2.3.2). The **Fv/Fm** button (equates the **Fo,Fm** button in the Advanced window) and the **Y(II)** button equates the **Sat. Pulse** button on the Field Screen (i.e. these two buttons represent a redundancy) and the **Sat-Pulse** button in the Advanced window.

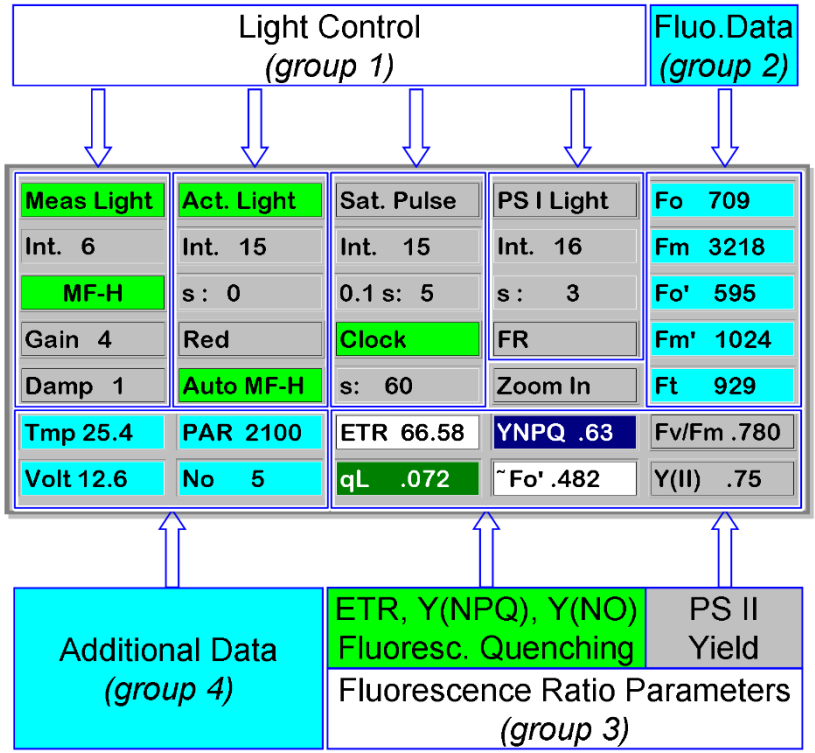


Fig. 12: Alphanumeric Area of Field Screen.

4.2.3.1 Light Control

First Column

Meas Light The **Meas Light** button is the on-off switch for the Measuring Light.

Int. Measuring Light intensity is set by changing the level in the <Int.> field: 20 different intensity levels are available. The Measuring Light intensity varies nearly linearly with the level number. While at low frequencies the actinic effect of the Measuring Light can be neglected, its integrated intensity can be appreciable at high frequencies. When PAR is not recorded by an external sensor, the Measuring Light intensity is derived from the currently active internal light intensity list (see 4.3.1.1 AL Current/PAR Lists).

MF-H Clicking on the **MF-H** button switches between low and high Measuring Light frequency. Grey background color indicates low frequency (e.g., for Fo determination) and green background color indicates high frequency (e.g., during actinic illumination). "Measuring Light" frequencies can be modified at the Advanced Level of WinControl-3.

Gain Ten different levels for electronic signal amplification are provided: at Gain 10 the signal is amplified 7-fold compared to Gain 1.

Damp Ten different levels of electronic signal damping are provided ranging from damping switched off (setting 1, t(½) = 10 µsec) to maximum damping (setting 8, t(½) = 4 ms). Note that at low Measuring Light frequencies, the response time is determined by the current sampling rate and, hence, much lower than the damping levels specified above.

Second Column

Act. Light Clicking on this button switches Actinic Light on or off.

Int. Actinic light intensity is set as described above. For different optical geometries (60° or 90° relative to leaf plane), the PAR produced by the red and blue internal light sources are provided as internal light lists which are available in the Options menu at the Advanced Level (see 4.3.1.1 AL Current/PAR Lists).

s: The time interval in seconds of Actinic illumination is defined *via* the **s:** button. For <s: 0>, both, turning on and off the Actinic Light, is carried out manually by clicking on the **Act. Light** button.

Red / **Blue** The button toggles between red and blue actinic illumination.

Auto MF-H When the <Auto MF-H> function is activated, switching on Actinic Light automatically increases the Measuring Light frequency from low to high. The status of <Auto MF-H> does not affect the Measuring Light frequency during Saturation Pulses, which is always 100 kHz.

Third Column

Sat. Pulse Clicking on the **Sat. Pulse** button triggers a Saturation Pulse. Its function equates that of the **Y(II)** button.

Int. The **Int.** button allows the setting of the intensity of the Saturation Pulses. Typical PAR values for settings 1 and 20 are 910 and 16500 µmol photons/(m^2·s), respectively. Level and PAR value are quasi-linearly related. (these PAR values were measured by a MQS-B Quantum Sensor or a Universal Light Meter (ULM, Walz) at standard distance and 60° optical geometry of a 2030-B Leaf-Clip Holder.)

0.1 s: Using this button permits the setting of the duration of a Saturation Pulse in steps of 100 ms, with 1 = 100 ms and 8 = 800 ms.

Clock Activation of the <Clock> function initiates the repetitive triggering of Saturation Pulses.

s: The button allows the setting of the time interval (in seconds) between 2 Saturation Pulses in a pulse sequence (see above).

Fourth Column

PS I Light Clicking on the **PS I Light** button switches the alternative light source on. When Actinic Light is set to red, <PS I Light> is either far-red radiation (LED emission peak at 740 nm) or blue radiation (with maximum emission at 460 nm). Far-red preferentially excites PS I in plants and many eukaryotic algae but the blue is used to specifically excite PS I in cyanobacteria. The PS IIs of bluegreens, the nick name of cyanobacteria, absorb both blue and green light badly. With red as the Actinic Light source, choosing blue in the <PS I light> section will turn these LEDs into a second Actinic Light source for non-cyanobacterial algae and plants.

Int. Setting of <PS I Light> intensity works as described for other light sources. Typical blue light intensities are reported together with red Actinic Light on the <Advanced> level of PamWin-3 (see Section 4.3.1.1 AL Current/PAR Lists). We do not report far-red light intensities. Far-red is mainly there to oxidize the electron transport chain or to keep it oxidized. The choice of far-red intensities will be mainly determined by the wish to minimize side effects. It should be kept in mind that far-red light is slightly actinic. This is mainly relevant for the O-I1 rise of fluorescence induction experiments and experiments with e.g. DCMU inhibited samples. When working with algae, using FR1 background light is recommended to keep the PQ-pool oxidized and the samples in State 1.

s: The time interval in seconds of <PS I light> is defined *via* the **s:** button. For <s: 0>, both, turning on and off of the PS I light, is carried out manually by clicking on the **PS I Light** button.

FR / **Blue** The button toggles between far-red and blue illumination as the second light source. With blue as the second light source, the Actinic Light is always red.

4.2.3.2 Fluorescence Data

Dark-acclimated sample

With a dark-acclimated sample, clicking on **Fv/Fm** records two types of Saturation Pulse data (see "Fluo.Data" in Fig. 12):

Fo Minimum chlorophyll fluorescence yield when all photosystem II reaction centers are open, recorded with low Measuring Light intensities (i.e. low Measuring Light frequencies).

Fm Maximum chlorophyll fluorescence yield when all photosystem II reaction centers are closed by a Saturation Pulse.

The length of the Fv/Fm pulse is set in the general settings window of the Advanced Mode: SP-Width (Fo,Fm), between a minimum value 1 (= 10 ms) and 80 (= 800 ms). For the determination of Fo and Fm, a much lower light intensity is enough compared with the Fo′ and Fm′ determination. The software allows, therefore, to set width and intensity for Fo and Fm and Fo′ and Fm′ separately.

Light-exposed sample

In response to a click on the **Y(II)** button, three types of Saturation Pulse data are recorded with a light-exposed sample: Fo′, Fm′, and F, which is the Ft value shortly before a Saturation Pulse:

Fo′ Minimum chlorophyll fluorescence yield in the state of open photosystem II reaction centers. The Fo′ is measured in the presence of far-red illumination with Actinic Light switched off. To activate the Fo′ measuring mode, go to <Advanced Level> and <General Settings>. When the Fo′ mode is inactive, the <Fo′> field displays the value of 0. In the presence of non-photochemical quenching, the Fo′ is (often, but not always) lowered with respect to Fo (Fo quenching).

Fm′ Maximal chlorophyll fluorescence yield when photosystem II reaction centers are closed by a strong light pulse. The Fm′ is lowered with respect to Fm by non-photochemical quenching.

Ft Ft denotes the continuously recorded fluorescence. The value of Ft measured shortly before a Saturation Pulse with light-exposed samples is denoted "F". Unlike the previous fluorescence levels, the value of F is only displayed on the screen during the measurement.

4.2.3.3 Fluorescence Ratio Parameters

PS II yield

Two fluorescence ratio parameters are calculated to estimate the efficiency of photosystem II to use excitation energy for photochemistry (see Fig. 12, PS II Yield):

Fv/Fm = (Fm-Fo)/Fm = Y(II)max; maximum photochemical quantum yield of photosystem II, normally observed after dark-acclimation (*cf.* Section 4.2.3.2). Secondly,

Y(II) = (Fm′-F)/Fm′; effective photochemical quantum yield of photosystem II. The Y(II) is lowered with respect to Y(II)max by non-photochemical down-regulation and reaction center closure.

ETR, Y(NPQ), Y(NO), Fluoresc. Quenching

In addition to PS II yield data, seven fluorescence ratio parameters and the relative electron transport rate (ETR) are evaluated. These data are shown against parameter specific background colors (*cf.* Fig. 12). The background colors match the symbol colors used in the graphs at the <Advanced> level of PamWin-3. For all measured and calculated data, definitions are provided in Chapter 5: Definitions and Equations.

Only four of the possible eight parameters can be displayed simultaneously. Therefore, for each of the 4 display fields (see: ETR, Y(NPQ), Y(NO), Fluoresc. Quenching, Fig. 12), one parameter can be selected from a list which appears after a left-click on a parameter field.

When Fo′ is measured for a light-exposed sample during post-pulse illumination with far-red light, two of the eight parameters (qP and qL, see Chapter 5, Table 8) can be calculated without fluorescence data from the dark-acclimated sample, that is without Fo and Fm. Calculations of qP and qL, however, require Fo and Fm measurements when the Fo′ is derived from Fo, Fm and Fm′ data according to Oxborough and Baker (1997) (see Chapter 5). The eight parameters are (see Chapter 5, Table 8 for definitions):

ETR – Electron transport rate in µmol electrons/(m^2•s) derived from Y(II) and PAR.

~Fo′ – Calculated minimum chlorophyll fluorescence yield of a light acclimated/non-dark-adapted leaf when all reaction centers are open.

NPQ – Non-photochemical fluorescence quenching: quantification of non-photochemical quenching (alternative for qN). The NPQ-value has been suggested to be proportional to the number of quenching centers in the light-harvesting antenna. Its value can be larger than 1.

qN – Coefficient of non-photochemical fluorescence quenching. Its value ranges between 0 (in the dark-acclimated state) and 1.

Y(NO) – Quantum yield of non-photochemical energy dissipation in PS II other than that caused by down-regulation of the light-harvesting function and/or an increase of the rate constant for heat dissipation.

YNPQ – Quantum yield of non-photochemical energy dissipation in PS II due to down-regulation of the light-harvesting function and/or an increase of the rate constant for heat dissipation.

Note: Y(II) + Y(NPQ) + Y(NO) = 1 (complementary quantum yields)

qP – Coefficient of photochemical fluorescence quenching. Its value ranges between 0 (upon application of a Saturation Pulse) and 1 (in the dark-acclimated state); the parameter is based on a separate photosynthetic units (puddle) model.

qL – Coefficient of photochemical fluorescence quenching assuming that all reaction centers share their light-harvesting antenna (lake model) and assuming that connectivity affects the whole fluorescence rise. This in contrast to the parameter qP, which is based on a model of separate photosynthetic units (puddle model) as noted above.

4.2.3.4 Additional Data

Tmp – With the Leaf-Clip Holder 2030-B connected to the Control Unit, the temperature (°C) of the lower leaf side is displayed.

PAR – With an external PAR sensor connected to the Control Unit, the measured photosynthetic active radiation in µmol photons/(m^2·s) is displayed. The continuous Reading of PAR sensor data can be switched off in the <General Settings> window in the <Advanced Mode> of PamWin-3. This should not be forgotten when running a Light Curve protocol! In that case the instrument will use the ambient light intensity for the light intensity axis of the Light Curve! When the

external PAR sensor is turned off, PAR is derived from the currently active internal PAR list (see Section 4.3.1.1 AL Current/PAR Lists).

Note that the PAR sensor of the Leaf-Clip Holder 2030-B does not give correct readings for Actinic Light applied *via* the fiberoptics, unless properly calibrated for this purpose (*cf.* Section 3.4.2.3). It is, therefore, recommended to derive PAR values from the internal PAR lists when an Actinic Light source of the PAM-2500 is used.

Volt – Battery voltage. A completely charged battery shows voltages up to 13.7 Volts. At Voltages below 10.5 Volts, the PAM-2500 operates unreliably, particularly during application of Saturation Pulses which require high current flows.

- Note: to prevent deep discharge of the battery, the PAM-2500 shuts off when the battery voltage drops below 9.4 Volts.
- Note: at battery voltages below 11.8 Volts, the PAM-2500 cannot be switched on.

No – The <No> field displays the number of Yield-determinations by the Saturation Pulse method in the current Record. Counting is reset by the **Start/Stop** button of the field screen.

4.2.4 First Measurements Using the Field Screen

This section introduces two basic chlorophyll fluorescence measurements using the Field Screen.

In the first place, make sure that the Measuring Light is switched on. As long as there is no chlorophyll containing object, the Ft parameter field shows values close to 0. When you approach a leaf with the fiberoptics (measuring light (and actinic light) on), the light passing through the fiberoptics excites the chlorophyll molecules in the leaf; a small part of the absorbed light is re-emitted as fluorescence, which in turn is guided *via* the fiberoptics to the detector system. Depending on

the distance, more, or less Ft will be measured. For reproducible measurements the distance between the tip of the fiberoptics and the leaf should be constant. For this purpose, leaf and fiber tip need to be fixed, e.g. by using the small Distance Clip 60° 2010-A or the Leaf-Clip Holder 2030-B.

Information on photosynthesis is obtained when the yields of fluorescence induced under different illumination conditions are compared. For this purpose, the PAM-2500 has various built-in light sources. When you click the **Act. Light** key, you will see that the leaf is illuminated by relatively strong red light. At the same time the Ft-value quickly rises and then slowly decreases again. This is the so-called "Kautsky-effect". Clicking on the **Act. Light** key again turns off the Actinic red light resulting in an Ft decrease.

With far-red radiation selected, clicking on the **PS I Light** key immediately after shutting-off the Actinic Light, will start a far-red illumination for the set time interval (typically 3 s). Far-red light, speeds up the decline of Ft (compared to the kinetics without far-red), with Ft approaching the fluorescence level before Actinic Light exposure (or lower: Fo quenching). The effect of far-red light on the fluorescence yield can be explained in the framework of the so-called Z-scheme of photosynthesis and by the theory of fluorescence quenching. Far-red light is preferentially absorbed by PS I, which, in the absence of actinic illumination, pumps electrons out of the intersystem electron chain. In the process, re-oxidized PQ molecules can bind to the Q_B-site re-opening PS II reaction centers by allowing the re-oxidation of Q_A^-.

The minimum fluorescence yield, called **Fo**, is observed when all PS II reaction centers are open, which is the case after dark-acclimation when working with higher plants. The maximum fluorescence yield, called **Fm**, is observed when all PS II centers are closed. Full closure of reaction centers and subsequent Fm-determination is achieved by a Saturation Pulse that is triggered by clicking the **Fv/Fm** key.

Actually, this command determines Fo and Fm quickly, one after the other. At the same time, the value of **Fv/Fm** is calculated and entered into the <Fv/Fm> field. The latter parameter corresponds to the ratio (Fm-Fo)/Fm, which gives information on the photochemical quantum yield of open PS II reaction centers. With a healthy and dark-adapted leaf, Fm is about five-six times higher than Fo, and, hence, Fv/Fm is approx. 0.80-0.85.

Saturation Pulses are also triggered *via* the **Y(II)** key. Then, a new value is entered in the Y(II) field. As long as the Actinic Light is off, these values will be very close to the Fv/Fm value. However, as soon as actinic illumination is turned on, you will see that the values of Y(II) first decrease, then rise again, and eventually assume a constant value that is characteristic for the photosynthetic performance of the given leaf sample at the applied/ambient actinic light intensity.

The Y(II)-determination can be repetitively triggered using the <Clock> function: the Saturation Pulses now will be applied at time intervals defined in the <s:>-time field below the **Clock** key. The fluorescence data measured during Saturation Pulse events are automatically stored in the Report file, which can be accessed *via* the **Report** key.

4.3 PamWin-3: Advanced Level

The <Advanced> level of the PamWin-3 software includes Saturation Pulse analysis, fluorescence kinetics (ranging from fast changes in the µsec domain to slow changes over many seconds, minutes or even hours), and a wide range of graphical and analytical features. The <General Settings> screen is the start window for the user interface of the <Advanced> level. Under <General Settings>, the mode of operation of the PAM-2500 is selected (either <SP-Analysis> or <Fast Acquisition>) and the PAM-2500 settings are adjusted. The icons and display fields to the right of and below the <General Settings> window are retained when switching to other windows of the PamWin-3 software. The details of PAM-2500 operation on the Advanced level will be given below.

4.3.1 General Settings

Fig. 13 shows the <General Settings> window. Parameter fields showing the same information as already outlined for the <Field Screen>, or buttons having similar functions as corresponding buttons on the <Field Screen>, are grayed out and will not be considered in the following sub-sections. (Note that the selection of 4 out of 8 fluorescence parameters for numerical display works as described previously: see Section 4.2.3.3).

4.3.1.1 Menu Bar

The menu bar of the <General Settings> window (see Fig. 13) is the scaffold for 6 menus. The list of menu items may vary if you switch from the <General Settings> window to another PamWin-3 window. To access a menu item, click sequentially on <menu title> and <item>. Also, menu items can be accessed by Windows shortcuts: press down

and hold the <Alt> key, and sequentially type the first (underlined) letter of the menu title and the first (underlined) letter of the menu item. Underlining itself is switched on and off by the Alt key.

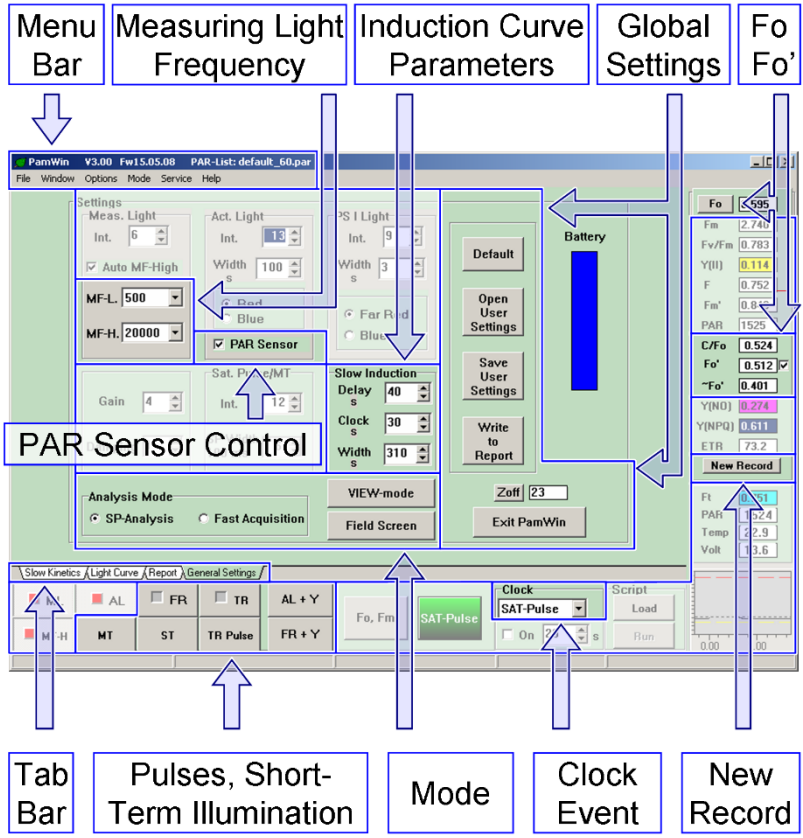


Fig. 13: General Settings.

File

The file menu includes items for loading and execution of PamWin-3 Script files which are used to automatically perform measuring routines. Also, printer selection, printing and program closure can be carried out using the <File> menu.

Window

The <Window> menu is equivalent to the <Tab Bar> (see Fig. 13). The <Window> menu has tabs for the 4 available windows in the <SP-Analysis> mode; in the <Fast Acquisition> mode, there are only 3 tabs for 3 windows.

Options

L Curve Fit Parameters

The menu item <Light Curve Fit Parameters> opens a window, in which 4 parameters are displayed that were derived by fitting a theoretical Light Response Curve to a measured Light Curve (ETR versus PAR data points) (see also Sections 4.4.2, Light Curve, and 5.5 for theoretical information). These parameters are:

Fv/Fm x ETR factor/2

	Maximum yield of electron-transport calculated from Fv/Fm	electrons/photons
alpha	Initial slope of the light curve, related to maximum yield of photosynthesis	electrons/photons
ETRmax	Maximum electron transport rate	µmol electrons/(m^2·s)

Ik	PAR value of the point of intersection between a horizontal line ETRmax and the extrapolated initial slope.	µmol photons/(m^2·s)

Light Calibration

Here, a factor can be entered to calibrate a quantum sensor connected to the PAM-2500 (Factor range: 0.2 to 5). For normal operation of the quantum sensor of the Leaf Clip Holder 2030-B, the default factory value of 1.000 applies.

Light Offset

The PAR sensor of the 2030-B leaf clip holder works within two ranges of signal amplification. The **Auto Zero** function establishes automatically the correct offset values for both ranges: the determination of the zero offset requires that the PAR sensor is kept in the dark. Offset values can also be entered manually.

Temperature Offset

<Temperature Offset> can be used to set an offset value for an external temperature sensor (offset range is -30 to +30). Normally, this function is not used. It allows the correction of a discrepancy between a temperature measured by the external sensor and a known temperature.

ETR Factor

The ETR-Factor corresponds to the fraction of incident photons absorbed by photosynthetic pigments that are photosynthetically active. The factor is required to estimate the electron transport rates on the basis of the effective quantum yield of PS II, Y(II) (see Section 5.5). The default value for the ETR Factor is 0.84 which reasonably matches the average fraction of absorbed light in the visible range (400-700 nm) of many green leaves.

AL Current/PAR Lists

All light sources of the PAM-2500 are LEDs which show a very reproducible relationship between LED current and light output. Therefore, illumination conditions with PAM-2500 light sources are well-defined for a particular optical geometry between sample and fiberoptics tip (60° or 90° angle between fiber and leaf plane). For frequently used optical geometries, a set of Current/PAR lists is provided (default_60.par and default_90.par). This greatly facilitates the use of PAR values for the calculation of ETR values, particularly when online measurement *via* a mini-quantum sensor is problematic.

Each PAR list consists of three columns: <AL> gives the levels (1-20) of the Actinic Light intensity, <Current> represents relative values for the LED current with the value of 255 corresponding to the maximum current, and <PAR> lists measured values of PAR in µmol photons/(m^2·s). Current values and red Actinic Light intensity are linearly related; current values and blue Actinic Light intensity are quasi-linearly related.

Default PAR lists show an approximately exponential relationship between PAR and level (Fig. 14). Each default PAR list comes with information on the optical geometry used which can be viewed by clicking on the comment icon: [ ]. To modify the current or PAR data, click once on the field to be edited, and enter the new value. New PAR lists can be commented (icon: [ ]), saved (icon: [ ]), and recalled (icon: [ ]).

Note that in all default PAR lists, the PAR value at AL setting <0> (Actinic Light off) represents the integrated Measuring Light intensity at ML intensity setting 10 and a 100 000 Hz measuring pulse frequency. An "internal PAR value" corresponds to the sum of the Actinic Light intensity plus the integrated Measuring Light intensity. The latter is derived from the base value at AL level <0> by taking into account the frequency and intensity setting of the Measuring Light.

During setup of PamWin_3, the <default_60.par> file is automatically placed in the <C:\PamWin-3\Data_2500> directory together with additional PAR lists as specified in Table 1. The <default_60.par> data are automatically loaded during the first start of the PamWin-3 software. The <default_60.par> list corresponds to the situation at sample level of a 2030-B Leaf-Clip Holder with the fiber optics fully inserted in the 60° port.

When the file <default_60.par> is not present in the <C:\PamWin-3\Data_2500> directory, factory values are used. Thereafter, at program start, the most recently used PAR list is loaded.

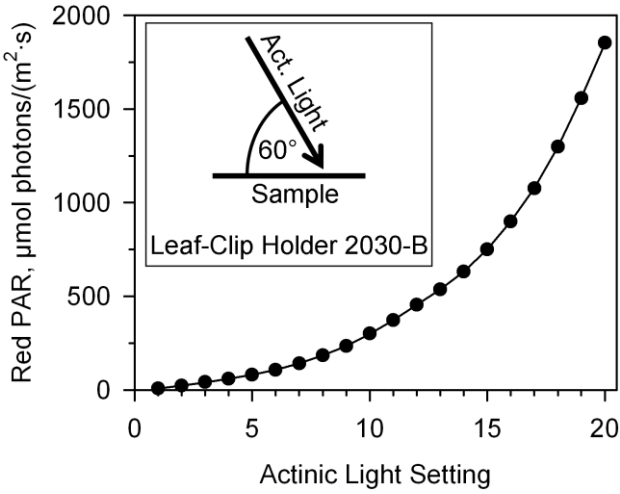


Fig. 14: Red Actinic Light Intensities at Sample Level of a Leaf-Clip Holder 2030-B.

Table 1: Default Red and Blue PAR Lists: Intensities at Sample Level

File Name	Sample Holder	Optical Geometry*
default_60.par	Leaf-Clip Holder 2030-B	60°
	Distance Clip 2010-A	60°, 2 mm distance ring
	Arabidopsis Leaf Clip 2060-B	60°, 6 mm distance ring
default_90.par	Leaf-Clip Holder 2030-B	90°, 4 mm distance ring
default_90_2060.par	Arabidopsis Leaf Clip 2060-B	90°, 2 mm distance ring
default_DLC.par	Dark-Leaf-Clip DLC-8	90°
default_KS.par	KS-2500 Suspension Cuvette	90° and 4 mm

*Detailed descriptions: see <Comment Files> attached to each PAR list.

Mode

In the <Mode> menu, the <VIEW> command switches to off-line operation: clicking on the **MEASURE-mode** button reverses the latter action. The <VIEW> and <MEASURE> commands in the <Mode> window are equivalent to the **VIEW-mode** key in the <General Settings> Window (Fig. 13) and the **MEASURE-mode** key which is available in the off-line mode.

Service

The <Service> menu is required for firmware updates (the software residing on instrument processors is called firmware). New firmware is provided by the Walz Company on a regular basis. To determine your firmware version, click on the menu item <Read Firmware Version>.

Note: to avoid interruption of communication during firmware update, a stable power supply is mandatory: power failure can lead to malfunction of the processors, which then need to be re-programmed at the Walz Company.

To start a firmware update, click **Controller Service**. The PAM-2500 contains 2 consecutively programmable processors: RISC and TINY (a tiny RISC processor).

a) RISC

Sequentially click on **Program RISC** → **Read HexFile** → **Download HexFile**. Then, the actual process of programming is initiated by clicking on the **Program RISC** key (this process requires some time). Finally, terminate the procedure by a mouse click on **OK** in the <Program RISC> window, confirm with **OK** the information on program restart, and once again click **OK** in the <PAM-2500 Controller Service> window.

Before proceeding, close and restart the PamWin-3 program!

b) TINY

Programming the TINY processor is similar to the procedure explained for the RISC processor. Start by clicking on **Program TINY**.Then, click **Read HexFile** → **Download HexFile** → **Program RISC**. Finalize TINY programming by an **OK** in the <Program RISC> window, the (program restart) information window, and the <PAM-2500 Controller Service> window. Close and restart the PamWin-3 program.

Trigger out with SP

A somewhat unexpected item on the Service menu is <Trigger out with SP>. Checking this function puts out a 5 Volt trigger pulse with each Saturation Pulse. See Section 4.3.1.8 and Fig. 15 for a description of the trigger pins at the AUX connector and configuration of the trigger pulse.

Help

In the Help menu, checking the option **Tooltips** activates the PamWin-3 tooltips function described earlier (Section 4.1). Clicking on **Info** displays information on the current PamWin-3 version.

4.3.1.2 Measuring Light Frequency

Measuring Light frequencies are set in the <General Settings> window (see Fig. 13): icons and settings are summarized in Table 2.

Table 2: Measuring Light Frequency

Icon		Range
MF-L.	Low Measuring Light frequency	10 - 5 000 Hz
MF-H.	High Measuring Light frequency	10000 - 100000 Hz*

* The instrument's maximum frequency of 200 000 Hz is effective in the <Fast Acquisition Analysis Mode> in triggered Fast Kinetics only.

4.3.1.3 Induction Curve Parameters

PamWin-3 defines the time course of fluorescence Induction Curves (Kautsky events) by three Induction Curve parameters (Fig. 13):

Delay, seconds: determines the dark interval between Fv/Fm determination and onset of actinic illumination. Default interval is 40 s.

Clock, seconds: defines the interval between Saturation Pulse analyses during actinic illumination.

Width, seconds: specifies the length of time of Actinic illumination.

All three time-intervals can be set using upward and downward arrow keys, or by double click on the interval number followed by manually

entering the new value. The AL-Width is automatically adjusted to equate a multiple of clock intervals plus 10 seconds. The extra 10 seconds are added to the initial and last Saturation Pulse applied during actinic illumination. Example: for a clock interval of 50 s, possible illumination times (<Width>) are 60 s, 110 s, 160 s …

4.3.1.4 Global Settings

The PamWin-3 software allows the adjustment of numerous instrumental settings, so that an almost infinite number of setting combinations is possible. Therefore, PamWin-3 offers the possibility to load a default set of standard settings and to save any particular set of settings which can be recalled later to carry out the same experiment with identical settings.

Four buttons are involved in the PAM-2500 <Settings> management (see Global Settings in Fig. 13).

Default	Installs default settings (File: <Walz2500.DEF>).
Open User Settings	Loads previously stored settings except the Zoff which is set to zero when new settings are loaded.
Save User Settings	Saves the current settings to a file with format <filename.DEF> except the Zoff.
Write To Report	Writes the settings to the current Report (see 4.4.3.1 for abbreviations used in a Report).

Other Commands

Zoff The <Zoff> (Zero Offset) serves to suppress a "background signal" which does not originate from the investigated sample. It is subtracted from all fluorescence signals. Background signals can arise from traces of scattered Measuring Light reaching the photo-detector. While this normally does not play a role in experiments with leaves, it may matter with suspensions at low chlorophyll content. In this case, the Zoff is determined with the cuvette containing pure suspension medium by pressing **Zoff**. The <Zoff> can also be entered manually.

Exit PamWin with this button PamWin-3 is closed down.

4.3.1.5 PAR Sensor

Checking the <PAR Sensor> results in the reading of data from an external PAR sensor integrated in the measuring head. When <PAR Sensor> is unchecked, PAR values are derived from the active internal PAR list. Internal Actinic Light arriving laterally at the entrance optics of the mini-quantum sensor of the Leaf-Clip Holder 2030-B is not properly measured (Section 3.4.2.3). Therefore, when this sensor is connected, it should be disabled unchecking <PAR Sensor> for applications involving actinic illumination by internal light sources (e.g. recording of Light Curves).

4.3.1.6 Fo, Fo′

Clicking the **Fo** icon (determination of Fo; see Fig. 13) takes the present Ft fluorescence level as Fo level fluorescence corresponding to the minimum fluorescence of a dark-acclimated sample. Hence, an Fo is set without subsequent Fm determination, i.e., without exposing the sample to a Saturation Pulse.

The **C/Fo** value is a measure for the contribution of non-PS II fluorescence to Fo. This is likely constant PS I fluorescence although other fluorescence sources like free LHCII aggregates cannot be excluded. The assessment of C/Fo requires that the **Fo′** mode is activated and that non-photochemical fluorescence quenching decreases the Fo′ below the Fo level (see Section 5.3 for details).

Fo′ is the measured Fo′ level fluorescence. Checking the Fo′ box (Fig. 13) activates the Fo′ measuring mode. This means that a 5 seconds interval succeeds each Saturation Pulse during which the Actinic Light is switched off and <PS I light> is turned on. The PS I light quickly oxidizes the PQ pool and, hence, facilitates the opening of the PS II reaction centers so that photochemical fluorescence quenching is maximized. The PS I light intensity can be set in the <General Settings> window but the post-pulse time interval for far-red illumination is fixed.

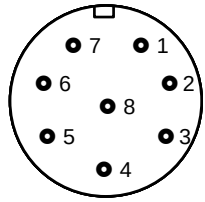
˜Fo′ represents the calculated Fo′ level fluorescence. ˜Fo′ is derived from Fo, Fm, and Fm′ data according to Oxborough and Baker (1997; for details see Section 5.1.2).

4.3.1.7 Tabs (<Tab Bar>)

The windows that can be selected in the PamWin-3 software are arranged as a deck of cards with Tabs on the bottom side. Clicking on one of the Tabs (in the <Tab Bar>) will bring the associated tab-card to the front.

4.3.1.8 Pulses, Short Term Illumination

The advanced level offers additional (user-designed) ways of sample illumination but also the possibility to generate a trigger signal to control an external device (Fig. 13).



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

Fig. 15: AUX Socket.

Short-term events ranging from a few µs to 1000 ms are:

ST – Single turn-over flash with a flash length between 5 µs and 50 µs, and a PAR intensity of about 125 000 µmol photons/(m^2·s).

MT – Multiple turn-over pulse with a pulse length between 1 ms and 300 ms, and PAR intensity of up to 25 000 µmol photons/(m^2·s).

TR Pulse – 5 Volt trigger pulses delivered to the AUX socket to trigger an external device connected to the AUX socket. Pulse times range from 10 µs to 1000 ms (Fig. 15).

To access time settings of short-term illumination, open the <Pulse Widths> window by a click with the right mouse key on **ST**, **MT**, or **TR Pulse**.

When in the <Pulse Widths> window **S+H off** is checked, the sample-and-hold amplifier of the PAM-2500 is switched off for the duration of the ST flash. The deactivation of the sample-and-hold amplifier prevents artefactual signals arising from the extremely high light intensity of the flash. The S+H off time can be extended beyond the ST flash time by selection of an additional time from the drop down list **Extended S+H off time**.

TR – The key permits manual control of the trigger out signal.

The following two buttons are used for illumination in the seconds range in combinations with Saturation Pulse analysis.

AL + Y – The command illuminates the sample with Actinic Light as defined in <General Settings> and thereafter carries out a Saturation Pulse analysis. This command is available for Actinic Light widths of 3 s or longer. This option equates a single light step in a (R)LC experiment. With an appropriate choice of AL Intensity and Width, AL+Y measurements can be a very compressed version of a Light Curve providing a reference state for the screening of mutants/deficiencies and for monitoring the development of abiotic stress conditions.

FR + Y – This command works similarly as the <AL + Y> command except that the Actinic Light is replaced by PS I light. This represents a more exotic option that allows the study of far-red illumination effects on the state and functionality of the photosynthetic electron transport chain.

4.3.1.9 Analysis Mode

The Analysis Mode area in the <General Settings> window (Fig. 13) represents a hub allowing a choice between two different program levels, that is, the two PamWin-3 measuring modes (<SP-Analysis> and <Fast Acquisition>). Two other choices: PamWin-3 <View> mode for offline data evaluation and the <Field Screen> mode for measurements in the field can be found at the bottom of the right-hand side parameter bar and below the battery bar, respectively. In the <Fast Acquisition> mode, emphasis is on the recording of kinetic changes. While the same multiple turnover saturating light pulses (MT) can be applied as in the <SP-Analysis> mode, there is no automatic determination of fluorescence parameters. An exception is the <Fo, Fm> determination, which initiates the calculation of Fv/Fm.

4.3.1.10 Clock Event

In total, 6 different events can be repetitively triggered by the clock function. All events are defined under <General Settings> except for the light curve, which is defined in the <Light Curve> window. Theoretically, the interval time between events can range from 3 to 900 seconds. Practically, the minimum interval needs to be longer than the event time plus some extra time to allow for data processing. The clock-triggered events are:

SAT-Pulse: Saturation Pulse with quenching analysis.

AL: Illumination with Actinic Light for a time period defined under General Settings/Act.Light/Width.

AL + Y: Illumination with Actinic Light for a time period defined under General Settings/Act.Light/Width, followed by a Saturation Pulse with quenching analysis.

FR + Y: Illumination with PS I light for a time period defined under General Settings/PS I Light/Width, followed by a Saturation Pulse with quenching analysis.

Light Curve: Light Curve defined in the Light Curve Edit window.

Slow Induc.: Fluorescence Induction curve (Kautsky-effect), as defined under General Settings/Slow Induction.

4.3.1.11 New Record

In the <SP-Analysis> Mode, data are grouped as <Records>. A new <Record> is started by clicking on the **New Record** button, which also is activated by the first <Fo, Fm> determination after program start. Starting a new <Record> does not interrupt continuous recording in the <Slow Kinetics> graph.

Usually, a <Record> starts with an <Fo, Fm> determination using a dark-adapted sample. The Fo and Fm fluorescence levels play a role in the calculations of several fluorescence ratio parameters (see Chapter 5). It is, therefore, important to carry out proper <Fo, Fm> determinations and to correctly organize the recording of Saturation Pulse data (i.e. assigning one <Record> to each sample).

4.4 SP-Analysis Mode

4.4.1 Slow Kinetics Window

The primary function of the <Slow Kinetics> window is the real-time display of fluorescence kinetics, Ft, and of fluorescence ratio parameters derived from Saturation Pulse analyses.

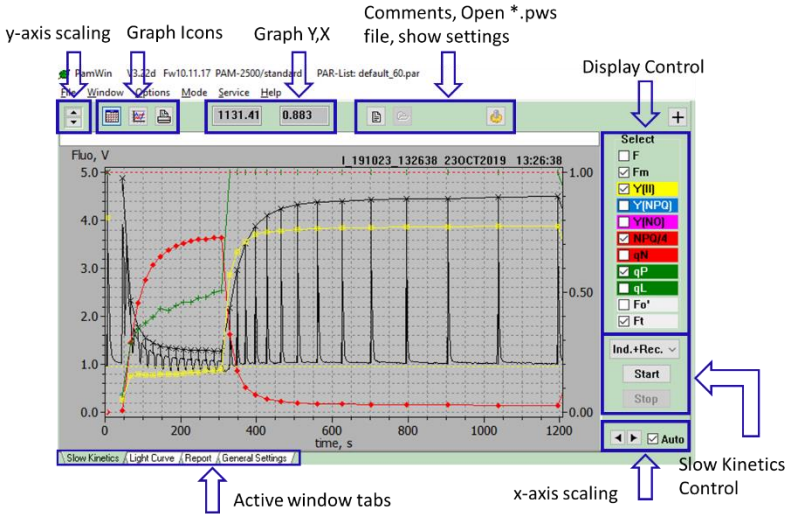


Fig. 16: Slow Kinetics Window

The new graphical elements of the <Slow Kinetics> window are displayed in Fig. 16. The figure does not show the elements already introduced in the previous section (<General Settings>). The <Menu

Bar> of the <Slow Kinetics> and <General Settings> windows are similar. A difference is that the <File> menu contains as additional commands <Print Graph> and <Open pws-file>, i.e., a "**P**am**W**in **S**low Kinetic" format (*.pws file). In <View> mode, the Ft file can be opened and converted into text format to be imported by other programs.

4.4.1.1 Graph Icons

The three graph icons in Fig. 16 control display of the graph grid (left icon), automatic scaling of both graph axes (central icon), and printing of the current graph (right icon).

4.4.1.2 Graph Y, X

To the right of the three graph icons there are two numerical fields (Fig. 16) representing the x (time in s) and y (signal intensity in relative units) coordinates, respectively, of the cursor position in the graphics field.

4.4.1.3 Save Ft

To save Ft traces use the disk icon ( , see Fig. 16). The document icon () opens the comment file associated with the most recently saved Ft fluorescence trace.

4.4.1.4 Display Control

Display of the various fluorescence parameters in the graphics field is controlled by checkboxes (see <Display Control>, Fig. 16). The colors of the data points and the background colors of the corresponding checkbox labels are identical.

4.4.1.5 X-Axis Scaling/Zoom In

Clicking on the left arrow (◀, see Fig. 16) doubles the total x-axis time range, whereas the total x-axis time range is halved, when the right arrow (▶) is clicked.

In addition to the x-axis scaling buttons, both, x- and y-scaling can be modified to allow magnification of a particular part of the graph: to <Zoom in>, click with the left mouse button on the left upper border of the target area, move the mouse with the left button pressed down to the right lower border of target area (the area that will be shown after releasing the mouse is shown as a rectangle in the graphics field), and release the mouse button (see Fig. 16). A right mouse click anywhere on the graph field restores the original display.

The currently displayed graph can be moved vertically by pressing <Shift> key and gripping the graph with the left mouse button. With the <Ctrl> button depressed, the graph can be moved horizontally with the mouse.

4.4.1.6 Slow Kinetics Control Area

The drop-down menu in the <Slow Kinetics Control> area (Fig. 16) allows the user to choose between a manually controlled recording (**Manual**), automated recording of dark-to-light fluorescence induction curves (**Ind.Curv.**) during which the induction of non-photochemical quenching is monitored, or dark-to-light induction curves followed by an extended dark recovery time, during which the relaxation of non-photochemical quenching is monitored (**Ind.+Rec.**).

The **Start** and **Stop** buttons in the <Slow Kinetics Control> area start and end a <Record>. Several <Records> can be stored in one <Report>.

Recordings of **Ind.Curv.** and **Ind.+Rec.** are always initiated by automated <Fo,Fm> measurements and the Fm-value is used to scale the Ft signal between 0 and 1 (ordinate scale corresponding to Ft/Fm). The same scale also applies to the fluorescence ratio parameters, except for NPQ, which can assume values above 1. Therefore, NPQ/4 values are plotted.

Manual recordings can be carried out without <Fo,Fm> measurements. In this case, the Ft signal is recorded using the original voltage scale. For a full quenching analysis, however, an <Fo,Fm> determination is required (*cf.* Chapter 5).

4.4.2 Light Curve

The <Light Curve> window allows exposure of samples to a series of illuminations which may differ in duration as well as in intensity. At the end of each illumination step, a Saturation Pulse analysis is carried out. The <Light Curve> chart plots Saturation Pulse analysis data against PAR. Often, such illumination programs consist of light steps with identical time intervals and increasing intensities. The definition is not precise, but roughly 10-30 s per light intensity can be considered a Rapid Light Curve (for which a light adapted leaf is needed to avoid artefacts related to activation of the photosynthetic apparatus) and if intervals >120 s are chosen it goes in the direction of a normal Light Curve (possible to start out with dark adapted leaves and a determination of the Fo and Fm values). The graphical representation of the results is called a (Rapid) Light Curve. For more information on Rapid Light Curves see Section 5.5.

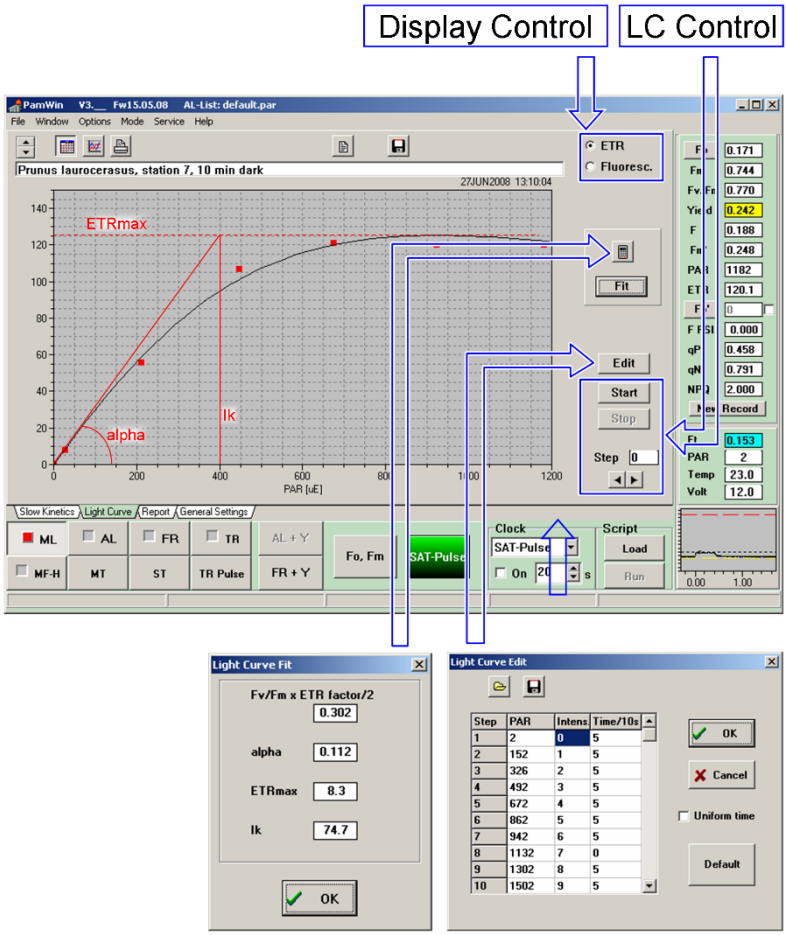


Fig. 17: Light Curve Window. In the present version of the software, below the Fit button, either the EP (Eilers an Peeters fit model) or the Platt et al. fit model can be selected.

4.4.2.1 Display Control

Light Curves are plotted with PAR (in µmol photons/(m^2·s)) as the abscissa parameter. Ordinate data are selected by checking **ETR** or **Fluoresc.** in the Display Control field (see Fig. 17): when

Fluoresc. is selected, the user can select several parameters to be displayed: fluorescence ratio parameter Fv′/Fm′ (Y(II)), Y(NPQ), Y(NO), NPQ/4 (divided by 4 to keep it on scale), qN, qP and/or qL. With **ETR** checked, the relative electron transport rate derived from Y(II) and the flux of photons absorbed by PS II is displayed (equation: Section 5.4) as a function of PAR. A plot of photosynthetic fluxes versus light intensity is called light response curve (LC). Compared to traditional light curves, which require steady state conditions for each light step, the ETR versus PAR plots of Rapid Light Curves often employ much shorter intervals per light step (see 5.5.1 for papers on light curves).

4.4.2.2 LC (Light Curve) Control

The **Start** and **Stop** buttons initiate and manually terminate a Light Curve recording. Normally a Light Curve recording is terminated automatically following application of the last pre-programmed Saturation Pulse.

4.4.2.3 Light Curve Edit

Setting of the Light Curve parameters is done in the <Light Curve Edit> window (see Fig. 17). Up to 20 illumination steps can be defined. A particular Light Curve illumination program can be saved as **L**ight **C**urve **P**rogram and loaded again at any later time using the icons at the top of the <Light Curve Edit> window. The default light curve file is denoted <default.**lcp**>.

Programmable parameters are the PAM-2500 Actinic Light intensity setting (1 to 20 in the **Intens.** column) and the time intervals of a light intensity step (in 10 seconds intervals, **Time/10s**). To change a setting, click in the target field once and enter the new number. The change becomes effective upon the next click. To set the same illumination interval for all steps, check <**Uniform time**>, before entering the desired time and terminating with another click on for example the

next Time/10 s cell. A zero in the **Time/10s** column ends the list of light intensities that will be executed (e.g., when "0 seconds" is attributed to step 6, only steps 1 to 5 will be executed.) When the 0 is entered, <Uniform time> should be disabled. The light intensities used as x-values correspond to those of the selected internal PAR list. It is not recommended to have PAR online activated (see Section 3.4.2.3). If the Leaf-Clip 2030-B is connected, the PAR sensor should be disabled under General settings unchecking PAR Sensor.

4.4.2.4 Light Curve Fit

The equation derived by Eilers and Peeters (*Ecological Modelling* 42 (1988) 199-215) is used to describe rapid light curves. From the results of curve fitting, three cardinal parameters are calculated (see Fig. 17):

alpha: The initial slope of the rapid light curve which is related to the maximal quantum yield of PS II electron transport under light limited conditions (unit: electron/photon).

ETRmax: The maximal relative electron transport rate reached during Light Curve recording (unit: µmol electrons/(m^2·s)), which reflects the light saturated capacity of the sample.

Ik: The light intensity at which the alpha and ETRmax lines intersect (unit: µmol photons/(m^2·s)), which is considered a proxy for the PAR intensity where saturation sets in.

In the Light Curve Fit window, besides the three cardinal parameters obtained from the fitting procedure, also the **Fv/Fm x ETR factor/2** is displayed. This is meant to remind the user that Fv/Fm corresponds to the maximum quantum yield of charge separation in PS II reaction centers, from which the parameter alpha can be predicted, when the ETR factor (normally 0.84) and the fraction of quanta distributed to PS II (normally 0.5) are considered. The **Fv/Fm x ETR factor/2** tends

to be somewhat larger than alpha, due to a small decrease of Y(II) at low light intensities.

4.4.3 Report

Generally, a Report file consists of a list of data measured by Saturation Pulse analyses. The data are organized by date/time and columns of the various measured parameters. The user may add comments with additional information on the particular measurements. A Report file can contain several Records, each of which normally represents a series of Saturation Pulse analyses with the same sample.

4.4.3.1 Report Data Management

A Report file can contain up to 1600 Saturation Pulse analyses. The percentage of file space used is indicated numerically and graphically (see <Report Data Management> in Fig. 18). The window area <Report Data Management> also provides icons to access the current comment text ( ), to open a previously saved Report ( ), and to save the current Report ( ).

The <Save Report> command saves the current Report data in PamWin-3 format using the extension <rpt> (e.g., filename.rpt). File name and location can be selected. Default directory is C:\PamWin_3\PamWin-3\Data_2500. The <Open Report> command activates <View Mode> in which previously saved files can be opened and processed. Switching from <Measure Mode> to <View Mode> automatically saves the current Report data in the file <PamWin.RPT>. The data of <PamWin.RPT> are loaded when returning to <Measure Mode>, or after program start.

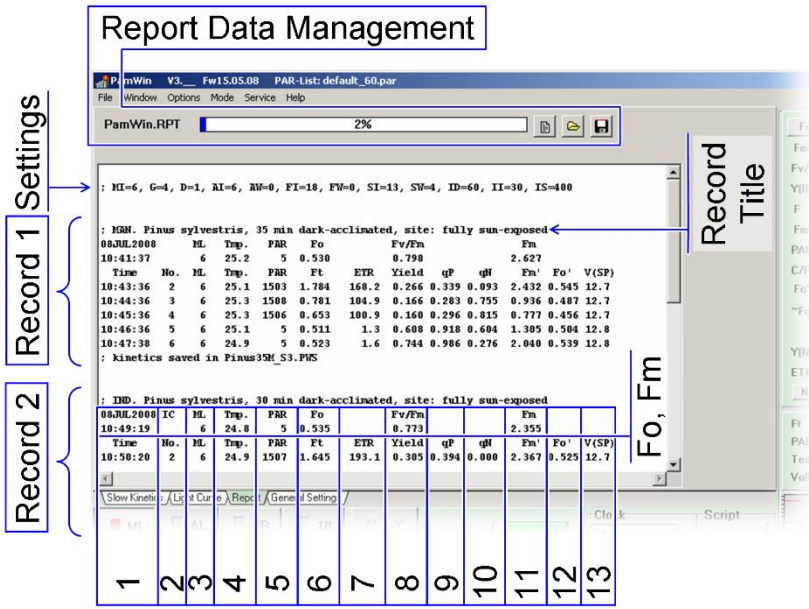


Fig. 18: Report Window.

The <Save Report> and <Open Report> commands are also available in the <File> menu which also provides the <Clear Report> command to delete the current Report data. The <Clear Report> command also initiates a new Report by writing a PAM-2500 <Settings> line at the top of the new Report: the PAM-2500 <Settings> line consists of 16 parameters (see Table 3). Similarly, the <Write to Report> command in the <General Settings> window creates a PAM-2500 <Settings> line.

Table 3: Report of Instrument Settings

Abbreviation	Parameter
AI	Actinic Light Intensity
AW	Actinic Light Width
BI	Blue Light Intensity
BW	Blue Light Width
D	Damping
FI	Far-red Light Intensity
FW	Far-red Light Width
G	Gain
H	High Measuring Frequency
ID	Slow Induction Delay
II	Slow Induction Clock
IS	Slow Induction Actinic Light Width
L	Low Measuring Frequency
MI	Measuring Light Intensity
SI	Saturating Pulse Intensity
SW	Saturating Pulse Width

4.4.3.2 Record Header and Last Line

First Line: Record Title

The first line of a Record contains the Record title. A Record title is written automatically, if entered in the text field above the graphics chart in the <Slow Kinetics> or <Light Curve> window. It can also be written manually. In this case, just like with any other text comment line, the text must begin with a semicolon (;) to distinguish it from data lines.

Second and Third Line: Fo,Fm

The second and third Record lines contain data related to an <Fo,Fm> determination, which normally should be carried out at start of a new Record. The abbreviations <IC> and <IC+> in the second line indicate that the measuring programs <Induction Curve> and <Induction Curve and Recovery>, respectively, have been started.

Notes: (1) when the <Fo,Fm> determination is not the first Saturation Pulse analysis of a Record, and when the graphics text field is left blank, lines 1 to 3 are reduced to the date of the experiment, (2) an <Fo,Fm> determination does not start a new Record, (3) a <Fo,Fm> determination during a Record is not indicated in the Record, but the Fo and Fm values are used for calculations of fluorescence ratio parameters.

Last Line: Saving of Slow Kinetics file

If the Slow Kinetics measured during the Record were saved, the corresponding file name is documented in the last line.

4.4.3.3 Record Columns

In <Measuring> mode, a Record chart contains 12 columns. More data are available in <View> mode. The subsequent comments on Report columns use the column numbering depicted in Fig. 18.

1 Date and time.
2 Sequential numbering of Saturation Pulses. The number <1> is omitted if the first Saturation Pulse was applied to determine <Fo,Fm>.
3 Setting of Measuring Light intensity
4 Temperature (°C)
5 Actinic light intensity (PAR, µmol photons/(m^2·s)). Light intensity (PAR, µmol photons/(m^2·s).
6 Fo (header lines) and F (subsequent data lines) in Volts.

7 Electron transport rate (ETR, µmol electrons/(m^2·s))
8 Maximum (header lines) and effective photochemical quantum yield (subsequent data lines) of PS II
9 Coefficient of photochemical fluorescence quenching (qP). Set to 1.000 in the absence of appropriate Fo and Fm data.
10 Coefficient of non-photochemical fluorescence quenching. Set to 0.000 in the absence of appropriate Fo and Fm data.
11 Fm (header lines) and Fm′ (subsequent data lines) in Volts.
12 Fo′ data as determined with the help of far-red illumination. If the Fo′-mode is not selected, column 12 stays blank.
13 Battery voltage (Volts).

4.5 Fast Acquisition Mode

The <Fast Acquisition Mode> of PamWin-3 allows the study of fluorescence kinetics in the sub-msec to sec range using a maximum time resolution of 10 µs. This mode is specifically used for well-defined triggered illumination by red, blue or far-red light. The measured fluorescence changes can be averaged, and, in this way, an excellent signal-to-noise ratio can be obtained at high time resolution even with weakly fluorescent samples. Apart from calculation of Fv/Fm, no SP analysis is carried out. Switching the analysis mode, the three windows present in the SP-analysis mode (<Slow Kinetics>, <Light Curve>, and <Report>) are replaced by the <Fast Kinetics> and <Fast Settings> windows. The new features of these windows will be introduced next.

4.5.1 Fast Settings

The <Fast Settings> window represents the user interface for program timing and light conditions of fast kinetics recordings. In this window, the various tools required to design particular fast kinetics trigger programs are grouped in different window areas according to their functionality (Fig. 19).

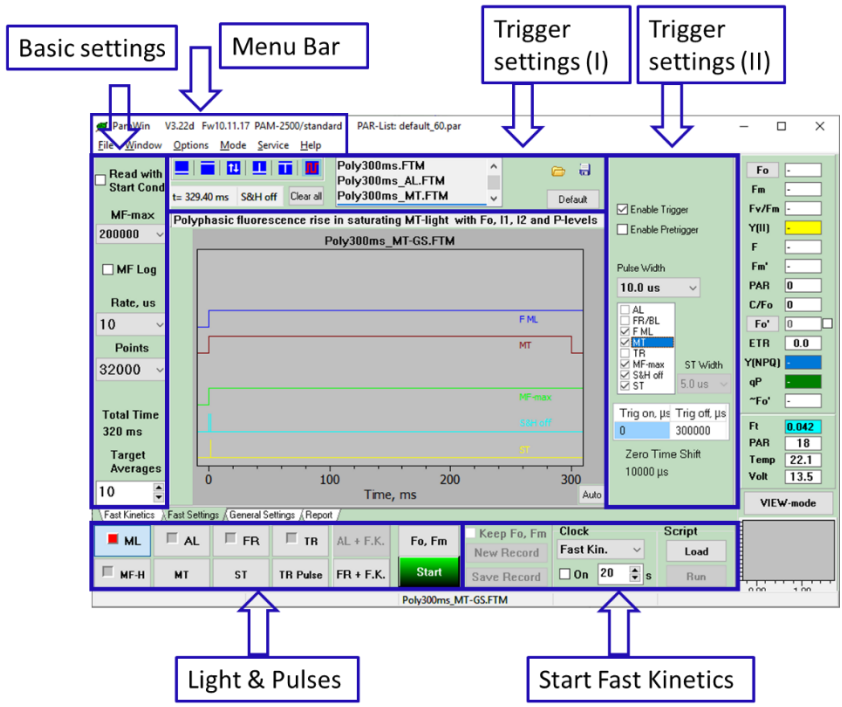


Fig. 19: Fast Settings Window.

4.5.1.1 Basic Settings

Read with Start Conditions – 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.

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 this MF-max trigger.

MF-log – The MF-log setting decreases 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, which is particularly important during fluorescence relaxation kinetics following a single turnover flash (ST).

Rate/Points – The sampling rate corresponds to the time interval between two consecutive ML pulses. The rate thus determines time resolution. The total time interval of a <Fast Kinetics> recording is determined by the product of the sampling rate and the number of points. A trigger signal cannot be programmed at times exceeding 32 000 points, while the overall <Fast Kinetics> may comprise up to 128 000 points. For example, <Fast Kinetics> can be composed of an initial fluorescence rise, induced by triggered illumination, which is followed by a fluorescence decay curve.

Target Averages – <Target Averages> corresponds to the number of Fast Kinetics recordings that will be averaged, when Averaging is enabled *via* the <Averaging> checkbox in the <Fast Kinetics> window. Consecutive recordings can be either triggered manually (**Fast Kin.** button / **Start** button) or via the Clock.

4.5.1.2 Menu Bar

The composition of the <Menu Bar> in the <Fast Kinetics> and the <SP-Analysis> mode is largely identical (Section 4.3.1.1).

4.5.1.3 Trigger Settings (I) and (II)

To configure trigger signals, 6 different tool icons are provided (see Fig. 19 and Fig. 20). The 3 left-most icons in Fig. 20 represent commands which affect the entire trigger signal and the three icons on the right hand side can be used to configure signal changes of various lengths which are shorter than the total trigger interval.

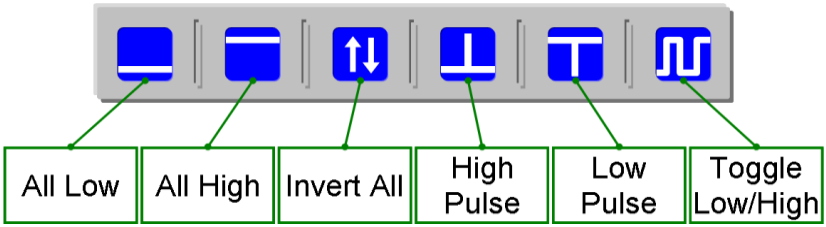


Fig. 20: Trigger Tool Icons.

Eight different events can be triggered (see Trigger Settings (II) and Table 4). Among these events, the ST (single turn-over flash) needs to be short enough to induce only a single charge separation in all PS II reaction centers. Only the **High Pulse** and **All Low** buttons are available for configuring the ST.

Table 4: Trigger Chart: Checkbox Panel

Check-box Label	Action	Comments
AL(Red)	Red Actinic Light	Is intensity defined for <Act. Light> in <General Settings>
FR/BL	Far-red light/Blue light	FR/Blue selection: <General Settings>. Intensity is defined by <PS I Light> in <General Settings>
F ML	Measuring Light	Frequencies (MF-L and MF-H) and intensity: <General Settings>. Measuring Light settings at start kinetic measurements are controlled by **ML** and **MF-H** buttons.
MT	Multiple turn-over pulse	Is intensity defined for <Sat. Pulse/MT> in <General Settings>
TR	Trigger out	See Section 4.3.1.7
MF-max	Maximum frequency of Measuring Light	Is defined in <Fast Settings> window (MF-max)
S&H off	Sample and hold circuit off	Time interval during which no signal is measured to avoid artefacts caused by extremely strong and fast intensity changes of non-modulated light and associated saturation of the detector
ST	Single turn-over flash	Always about 125 000 µmol photons/(m^2·s)

All other events can be triggered by pulses and by signal low/high shifts. To set a pulse:

a) Select an event by clicking on its checkbox label (Fig. 19. Trigger Setting (II)). The event is selected when its label is typed white on blue background.

b) Activate event by clicking the box associated with the label.

c) Click on the **High Pulse** icon.

d) Select time interval in drop-down menu. Note that the <ST Width> menu applies for ST only; pulse lengths of the other events must be selected in the <Pulse Width> menu. Also, the minimum pulse width depends on the sampling rate selected (Fig. 19. Basic Settings).

e) Click in the field below <Trig on, µs> (see Fig. 19. Trigger Setting (II)) and type in the time point of pulse start in µs. Clicking the **Enter** key results in the digital display of the pulse end time in the neighboring data field (calculated with the interval selected above), and in the graphical display in the <Trigger Graph>.

Repeating step e) places an additional pulse on the same trigger line. For the same event, pulse intervals may vary: simply visit step d) before proceeding to e). To move to the next event, simply select and activate the event (steps a) and b), respectively).

Longer-term triggering is achieved by using the <Toggle Low/High> tool (Fig. 20). Basically, the procedure is identical to the setting of trigger pulses but in step c) the <Toggle Low/High> icon must be selected and, in step e), the setting of the <Trig on, µs> time point does not automatically result in an entry in the <Trig off, µs> field. Consequently, the trigger signal can be

- switched only from low to high (no entry in the <Trig off, µs> field)
- switched only from high to low (no entry in the <Trig off, µs> field followed by the <Invert All> command. Fig. 20), or
- switched from low to high and then back at the time points entered in the <Trig on, µs> and <Trig off, µs> fields, respectively.

Table 5: Fast Settings - Trigger Program Tools*

Action	Active Icon	Key Combination	Parameters
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 (click on checkbox label)	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 events, <Pulse Width> applies except the ST interval which is determined by <ST Width>. Time points in µseconds of trigger on and off events are numerically displayed (see Fig. 19, Trigger Settings (II)).

****** Trigger setting *via* these shortcuts does not require selection of a trigger event in the checkbox panel.

Additionally, a trigger pattern can be created by directly manipulating the <Trigger Chart> using the mouse pointer as outlined in Table 5. The graphical editing of trigger lines is facilitated by the numerical displays of the mouse cursor position (numerical displays below <Trigger Tool Icons>).

For accurate setting of time points, the x-axis can be enlarged as outlined in Table 6, where also comments on how to shift the x-axis after enlargement can be found.

Trigger files can be saved and recalled using the appropriate icons in the <Trigger Settings (I)> field: [📂] and [💾], respectively. Clicking the **Default** button in <Trigger Settings (I)> opens the <Poly300ms.FTM> which is one of the predefined trigger files of PamWin-3.

When **Enable Pretrigger** is checked (<Trigger Settings (II)>, Fig. 19), a display field appears below the checkbox, which is associated with a drop down list of pre-trigger time intervals. Also, a small chart, entitled <Pre>, to the left of the <Trigger Graph> appears. In the <Pre> chart, a trigger signal can be toggled between low and high by left-clicking on that trigger line. A pre-triggered event is active for the pre-trigger time interval selected. The pre-trigger interval is immediately followed by the fast kinetics. Note that, different to the main trigger pattern, the pre-trigger settings are not set <All Low> by the **Clear All** button.

Zero Time Shift (<Trigger Settings (II)>, Fig. 19) right-shifts the zero point of the time axis so that recording of a fast kinetics starts at negative time values but triggered action can be initiated at zero or positive time points. Zero time shift is advantageous when fluorescence data are plotted against a logarithmic time scale. To adjust the

zero time shift, double-click on the number displayed below the phrase <Zero Time Shift> and enter the new time value.

Table 6: Fast Settings - Chart Manipulation

Action	Key Combination
Enlarge x-axis scale	Click with <left mouse key> at lower x limit, while keeping the mouse key depressed move cursor to the upper x limit, release key.
Switch between enlarge x-axis and shift x-axis	Single <right mouse key> on trigger chart
Shift x-axis	Click on chart with <left mouse key>, hold mouse key down and move cursor horizontally
Full x-axis scale	<Auto button>

4.5.1.4 Light & Pulses

In the <fast acquisition> mode, two buttons are available which first start an illumination with Actinic or far-red light and then initiate the execution of a fast kinetics (F.K.) (Fig. 19):

The **AL + F.K.** command starts an Actinic illumination and then executes the current trigger pattern.

The **FR + F.K.** is similar to the previous command except that far-red illumination is given instead of Actinic Light.

Note: the illumination regimes of **AL + F.K.** / **FR + F.K.** differ from the pre-trigger illumination (see previous Section) because the Actinic/far-red light stays on during the fast kinetics. The time interval

for Actinic/far-red light must be defined in <General Settings>; the fast kinetics will be performed 2 seconds before the time interval ends.

4.5.1.5 Start Fast Kinetics

Fo,Fm Clicking the **Fo,Fm** button starts determinations of Fo and Fm values, calculation of the Fv/Fm and entry of the data in the current Report as described in Section 4.4.3.3.

Start The command starts immediate execution of the current trigger pattern.

Clock Three different events can be triggered by the <Clock> function in the <Fast Kinetics> mode: fast kinetics, fast kinetics in the presence of illumination with Actinic Light, and fast kinetics in the presence of illumination with far-red light. The three events are equivalent to the commands <Start>, <AL + F.K.>, and <FR + F.K.> (see above). The clock interval can be set using the up and down arrows, but the interval can also be entered manually after double-clicking on the displayed time interval.

4.5.2 Fast Kinetics

The data acquired in the <Fast Kinetics> mode are displayed in the <Fast Kinetics> window. Most options available for axis control in the <Slow Kinetics> Window (Section 4.4.1) also work with the <Fast Kinetics> window. For optimal illumination of the leaves, fast kinetics measurements are best carried out under a 90° angle, for example using the leaf clips. The new features of the <Fast Kinetics> graphics display are:

- Control of y-axis scaling by up (▲)/down (▼) arrows, and by the **Auto** button (Fig. 21, Axis Control).

- Toggling option to switch between linear and logarithmic x-axis scaling (see **Log** checkbox Fig. 21).
- **Keep Scale** function to fix y-axis scaling when viewing a series of fast kinetics recorded at different y-axis scales (Fig. 21, y-axis scaling).

More options of the <Fast Kinetics> window are explained below.

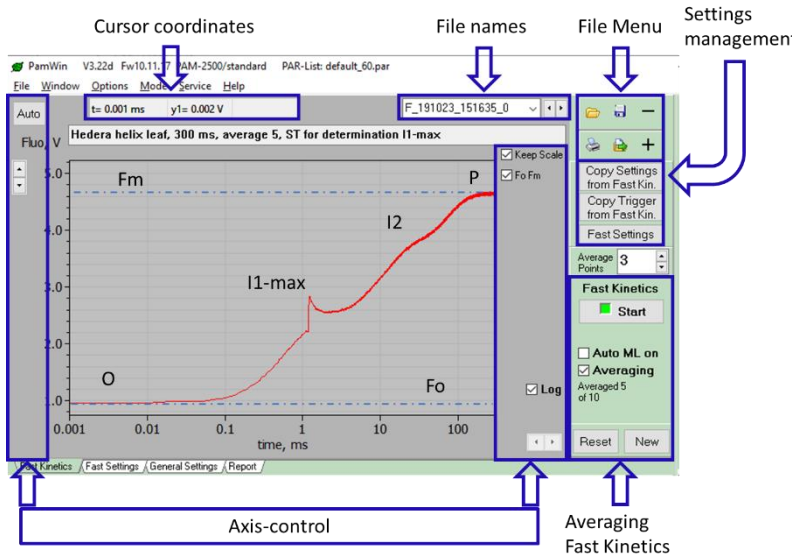


Fig. 21: Fast Kinetics Window. For the transient shown in the figure, 5 measurements (spaced 4-5 min apart) were averaged. A 50 µs ST was applied after 2 ms of illumination for the determination of the I1-max level.

4.5.2.1 Cursor Coordinates

Ordinate and linear abscissa data of the current cursor position are numerically displayed for evaluation of the fluorescence trace.

4.5.2.2 File Names

Each <Fast Kinetics> is saved using the file name format: **F_data_time_0**. The complete list of <Fast Kinetics> recordings is available by a left-click on the single downward-directing arrow: click on the file name to view the kinetics trace. To view kinetics sequentially, select a file name in the <Fast Kinetics> list, use the Up/Down arrow pair to the right of the file name display, or the up/down arrows on your keyboard, or the mouse wheel.

4.5.2.3 File Menu

<Fast Kinetics> which are not saved will be lost at closure of PamWin-3. Kinetics can be saved as **P**am **F**ast **K**inetics files (*.pfk) using the floppy disk icon in the <File Menu> (see Fig. 21). Also, data can be exported (icon below floppy disk icon) as *.csv files in ASCII or Unicode formats which are importable by most spreadsheet and graphing programs.

4.5.2.4 Fast Kinetics

A number of elements to manage fast kinetics are pooled in the <Fast Kinetics> panel (see Fig. 21).

Copy Settings from Fast Kin. – Each fast kinetics is saved with its trigger pattern and instrument settings. These particular trigger patterns and settings can be loaded and installed by the "Copy Settings from Fast Kin." command.

Copy Trigger from Fast Kin. – This command works similarly to the previous one, except that only the trigger pattern is copied. Clicking this icon overwrites the current trigger pattern with the trigger pattern associated with the currently displayed kinetics. The feature aids to reproduce kinetics.

Fast Settings – This command induces a switch from the <Fast Kinetics> to the <Fast Settings> window.

Average Points – The noise on the measured signal can be graphically reduced by increasing the <Average Points> number which is applied to the displayed figure. The "graphical averaging" does not affect the original data but at a certain point will start to dampen the graphically observed features.

Start – The command starts immediate execution of the current, loaded, trigger pattern.

Auto ML On – If checked, the Measuring Light is switched on at the start of a <Fast Kinetics> measurement.

Averaging – When <Averaging> is active, the mean of consecutively recorded <Fast Kinetics> will be calculated. Clicking on **Start** triggers execution of the loaded script file and leads to a single measurement. The user controls the time interval between measurements. The number specified for <Target Average> in the <Fast Settings> window determines the maximum number of <Fast Kinetics> transients to be averaged. With <Averaging> switched on, the <Clock> triggers only the number of <Fast Kinetics> specified as <Target Average> so that the entire averaging process can carried out automatically.

4.5.2.5 Polyphasic Fluorescence Rise

Here, the default trigger pattern of PamWin-3 (<Poly300ms.FTM>) is used to demonstrate aspects of trigger design for <Fast Kinetics>. The <Poly300ms.FTM> trigger pattern is shown in Fig. 19, and a fluorescence curve elicited by this trigger pattern is shown in Fig. 21.

Details of the <Poly300ms.FTM> trigger pattern are summarized in Table 7. In short, the triggering results in a <Fast Kinetics> measurement, which establishes a baseline in the absence of Measuring Light at negative time values. Shortly (1 ms) before time "zero", high frequency Measuring Light is switched on to establish the Fo level fluorescence. At time zero, a polyphasic fluorescence kinetics measurement is initiated by a strong multi turn-over pulse and, simultaneously, the time resolution of measurement is further increased by switching to the maximum frequency of Measuring Light. Fig. 21 also depicts Fo

Table 7: Default Trigger Pattern < Poly300ms.FTM>

	SETTINGS	COMMENT
S T A R T C O N D I T I O N		
Auto ML **ML**	OFF OFF	switch on Measuring Light by <F ML> trigger command
MF-H	ON	always use high frequency Measuring Light
MF-max	200 kHz	use highest frequency for Measuring Light for best time resolution
Zero Time Shift	10 000 µs	start Record 10 ms before fluorescence rise
T R I G G E R P A T T E R N		
F ML (trig. high)	-1000 to 300 000 µs	start Measuring Light 1 ms before <MT> to establish Fo, switch off at end of <MT>
MT (trig. high)	0 to 300 000 µs	expose sample to a 300 ms <MT>
MF-max (trig. high)	0 to 300 000 µs	use maximum Measuring Light frequency while <MT> on
S&H off (trig. high)	-10 to 10 µs 299 990 to 300 010 µs	switch off sample & hold amplifier at start and end of <MT>

and Fm level fluorescence which were established by an <Fo, Fm> determination prior to recording a <Fast Kinetics>.

Generally, to become experienced with PamWin-3 trigger management, it is recommended to start with one of the trigger files provided by the software and then modify the default settings. In Section 4.8 the creation of trigger files is discussed a second time using a slightly different approach.

4.6 View Mode

The <View Mode> of PamWin-3 is dedicated to analyses of fluorescence measurements. Three windows are available in <View Mode>: the <Report>, <Slow Kinetics> and <Light Curve> windows. The operation of the latter two windows is as described previously (Sections 4.4.1 and 4.4.2).

The <Report> window in the <View Mode> provides new tools for data management and navigation; the <Icon Bar>, the <Sidebar>, and a panel for data selection (

Fig. **22**).

4.6.1 Icon Bar

The <Icon Bar> provides commands for opening Records, viewing comments and exporting data either as CSV data (comma separated values) or to the clipboard (compare

Fig. **22**). The functions of the <Icon Bar> are also available in the <File> menu.

4.6.2 Sidebar

The foremost function of the <Sidebar> (

Fig. **22**) is navigation within a <Report>. The various navigation functions for moving around a <Report> are outlined in

Fig. **22**.

4.6.3 Pick Data

The <Pick Data> panel gives access to all Saturation Pulse data and fluorescence ratio parameters except the C/Fo data. The selection of a parameter by ticking a checkbox results in the display of the respective data in the <Record> data window: only the selected parameters are exported as CSV data or to the clipboard.

4.6.4 Pick Record Window

Information on the selected <Record> are provided in the main window of the Report Tab. If the **Comment** and **Line** buttons of the Field Screen were used, the results can be seen here (Fig. 23). The Field Screen Comment text is found on the comment line at the top of the Record Window. The first time the **Line** button is clicked and a text is added, this text is found just below the SP data it comments on.

Clicking the **Line** button a second and third time, the second and third text is place each time at the top of the Record Window. Based on the time of creation indicated in the time column, the comment can be associated with a particular saturation pulse.

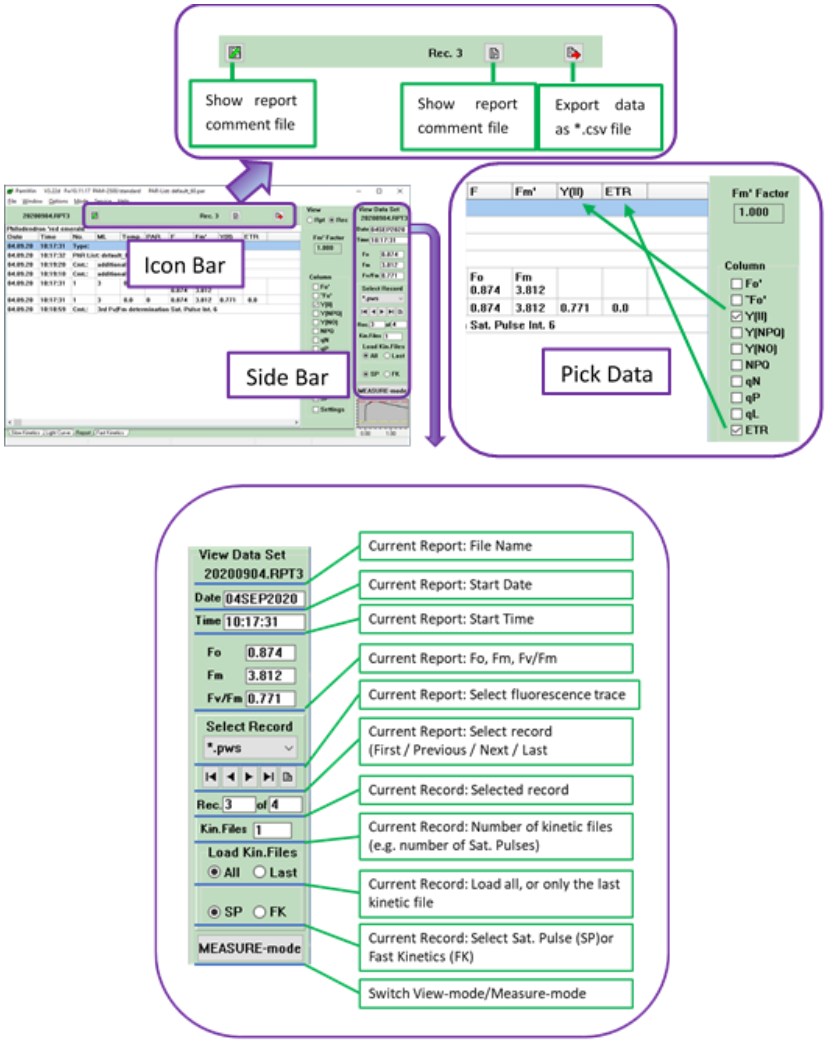


Fig. 22: View Mode Window.

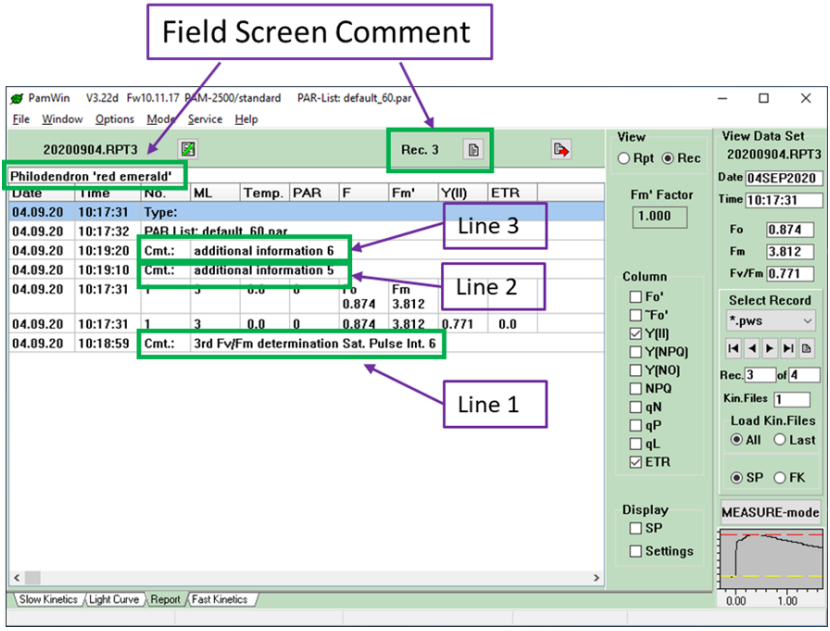


Fig. 23: View Mode Report Window: Field Screen Comment and Line function effects.

4.7 Script Files

Script files are used for automatic execution of experimental procedures of various complexities. Carrying out experiments by Script files can be advantages when the same type of analysis needs to be repeated frequently or when complicated protocols must be exactly reproduced.

Generally, all manual operations and adjustments in the PamWin-3 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 create <Script File>:

- Open <Script File> dialogue window by clicking on the **Load** icon of the Script panel (sidebar of <Field Screen> or bottom bar of all <Advanced> windows.

- Click **Open** without selection of an existing <Script File> and click **Cancel**. Enter a <Script File> name in the window which appeared in response to the **Cancel** command.
  Alternatively, open an existing <Script File> and click on the **New Script File** icon (Fig. 24: file icons).

- The <Command Box> of the <Script File> window (Fig. 24) contains the available commands divided into 8 sections. In addition, the <Program Control Commands> are displayed on top of the <Command Box>. The various functions of the latter commands are comprehensibly elucidated in the <Help> attached to the <Command Box>.

- All other command groups match the commands of manual instrument operation except the group <Condition Commands>, which permit the execution of instructions depending on the state of a parameter like the fluorescence level or temperature.

- A number of <Editing Tools> (Fig. 24) are provided to compose a Script file. Similar as for the <Command Box>, the <Help> associated with the <Editing Tools> comprehensibly explains their function.

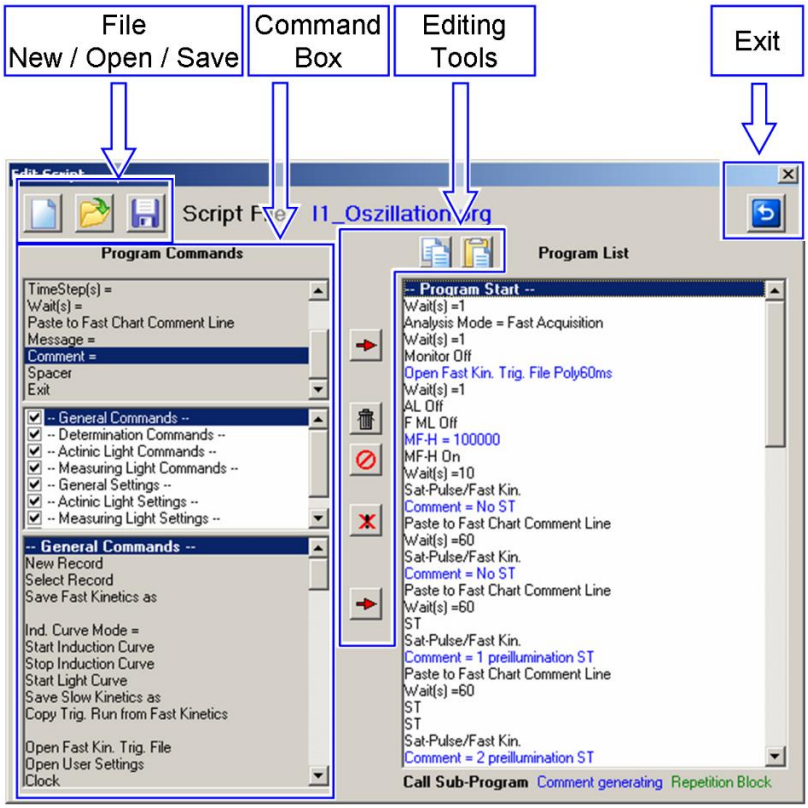


Fig. 24: Script Window.

4.7.1 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_3\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 "filename.TXT" which is associated with the script file "filename.PRG".

4.7.2 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 on the 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 on 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. 24)

- Select with the mouse cursor in the script file window (right text window of Fig. 24) 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 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.

4.7.3 Command Box (List of Script File Commands)

The command box of the script file window (see Fig. 24) 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 panel hides the corresponding group of commands in the bottom panel.

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

See Section 4.9 for some comments on writing script files.

PARAMETER	COMMAND, COMMENT	INPUT
Section 1: -- Program Control Commands --		

PARAMETER	COMMAND, COMMENT	INPUT
Call	Executes another PamWin-3 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. 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 interval in seconds

PARAMETER	COMMAND, COMMENT	INPUT
	time scale along which actions can be defined.	
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.	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”.	None

PARAMETER	COMMAND, COMMENT	INPUT
	Execution of the command requires that a Fast Kinetics measurement has been started.	
Message =	Halts script execution and displays a message. Clicking OK on the message window or hitting the Enter key continues 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 (will write the comment to one of two graph windows).	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

PARAMETER	COMMAND, COMMENT	INPUT
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 the execution of a light curve; equivalent to the stop button of Light Curve window.	None
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_3\Fast Kin Trigger”	File name

PARAMETER	COMMAND, COMMENT	INPUT
Open User Settings	Activates stored instrument settings. File name format: filename.DEF. Default settings: Walz2500.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 Fm' value is calculated on the basis of the O-I1 amplitude	Checked is on/unchecked is off

PARAMETER	COMMAND, COMMENT	INPUT
Stirrer	Switches stirrer on/off. Stirring is only brought in standby mode by the stirrer switch on the front panel of the control unit.	Check on/off
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 Fo and Fm fluorescence level and calculates Fv/Fm.	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 Fo fluorescence level.	None

PARAMETER	COMMAND, COMMENT	INPUT
Fo’	Determines Fo’ fluorescence level.	None
Fo’-mode	Saturation Pulse is followed by 5 s of far-red light to determine Fo’.	None
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
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. Command requires separate PS I lamp.	Check on/off

PARAMETER	COMMAND, COMMENT	INPUT
PS I Light =	Switches between far-red and blue PS I light. Command requires separate PS I lamp.	Check Far 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
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

PARAMETER	COMMAND, COMMENT	INPUT
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
Auto ML on	Turns on measuring light just before start fast kinetics measurements, and switches measuring light off immediately thereafter.	Checkbox
Keep Fo,Fm	Off: Determines Fo and Fm values at start of each induction or light curve. On: Uses the initial Fo and Fm values as reference for all subsequent saturation pulse analyses.	Check on/off

PARAMETER	COMMAND, COMMENT	INPUT
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
Section 6: -- Actinic Light Settings --		
	Sets intensity levels of:	
AL-Int.	Actinic light,	1 to 20
BL-Int.	Blue light,	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
	Sets illumination time interval for	
AL Width	Actinic light	0 - 900 s
BL Width	Blue light	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

PARAMETER	COMMAND, COMMENT	INPUT
	Sets time interval of	Drop down list
ST Pulse Width =	Single turnover flash.	5 µs - 50µs
MT Pulse Width =	Multiple turnover pulse.	1 ms- 300 ms
TR us-Pulse Width =	Short (sub ms) 5 V trigger pulse.	10 µs- 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),	Number of increments 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).	

PARAMETER	COMMAND, COMMENT	INPUT
ST sequence number	Defines the number of single turnover flashes in a flash sequence, or increases or decreases the flash number of a flash sequence.	Number of flashes, number of decrease (- #) or increase (+ #) of flashes. Valid entries: 1 to 20
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 --		
MF-max	Sets the maximum frequency for the measuring light	1000-200000 (Drop down list)
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).	Number of increment to decrease (- #) or to increase (+ #)

PARAMETER	COMMAND, COMMENT	INPUT
MF-L Step	Stepwise increases/decreases the low measuring light frequency (variable increments depending on present frequency).	Number of increment 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 measurement.	Check on/off

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

PARAMETER	COMMAND, COMMENT	INPUT
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

4.8 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. 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.

4.9 Writing scripts

In Section 4.7 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

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 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

However, what is in this case the time interval between these two single turnover flashes? It is not defined in the script. To find out, we have to go to the pulse definition window (click with the right mouse button on either the MT, ST or TR Pulse buttons to open this window). In my case the ST flash width was 100 ms, but any value between 10 and 500 ms can be chosen in the pulse definition window.

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

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 Section 4.5.2).

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. 24.

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 image though, that most users will never make use of these conditional commands.

5 Definitions and Equations

5.1 Relative Fluorescence Yields

Typically, five different types of fluorescence levels are acquired by Saturation Pulse analyses. Two of these levels need to be established with the dark-acclimated sample. The three remaining levels are repeatedly measured during subsequent sample treatments (e.g., exposure to Actinic Light; see Fig. 25).

5.1.1 Measurements with dark-acclimated samples

Fo Minimum fluorescence level excited by very low intensity of Measuring Light to keep PS II reaction centers open.

Fm Maximum fluorescence level elicited by a strong light pulse which closes all PS II reaction centers.

5.1.2 Measurements with light-exposed (treated) samples

Fo′ Minimum fluorescence level during a (light)treatment. When the measuring routine for Fo′ is active, the Fo′ 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 reaction centers and, thus, to quickly remove intersystem electrons and open PS II reaction centers (see Fig. 25, time point 75 s).

Alternatively, the Fo′ can be estimated according to Oxborough and Baker (1997):

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

Fm′ Maximum fluorescence level during a treatment; is induced by a saturation light pulse which temporarily closes all PS II reactions centers. Fm′ is decreased with respect to Fm by non-photochemical quenching.

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

5.2 Fluorescence Ratio Parameters

To quantify photochemical use and non-photochemical losses of absorbed light energy, fluorescence ratio expressions have been derived which use, as data input, the relative fluorescence yield measurements introduced above. Table 8 compiles the fluorescence quotients available in the PamWin-3 software. Subsequently, these fluorescence quotients will be briefly explained.

Fv/Fm and Y(II) Maximum and effective photochemical quantum yield of PS II

Both fluorescence quotients estimate the fraction of absorbed quanta used for PS II photochemistry, (i.e., for stable charge separation in the PS II reaction center). For measurements of Fv/Fm, 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. Requirements for dark acclimation can differ between plants: in extreme shade leaves, substantial closure of PS II centers can occur already at PAR values of 0.1 μmol photons/(m^2·s) but many sun leaves exhibit mostly open PS II centers even at 10-40 μmol μmol photons/(m^2·s).

The Y(II) value estimates the photochemical use of excitation energy in the light. To derive from Y(II) information on the overall state of photosynthesis, control of light conditions is required because a leaf may have a severely damaged Calvin cycle and still show a high value

of Y(II) in weak light. Therefore, photosynthetic performance should be assessed during steady state illumination at a photon flux density that is somewhat below saturation in a control sample.

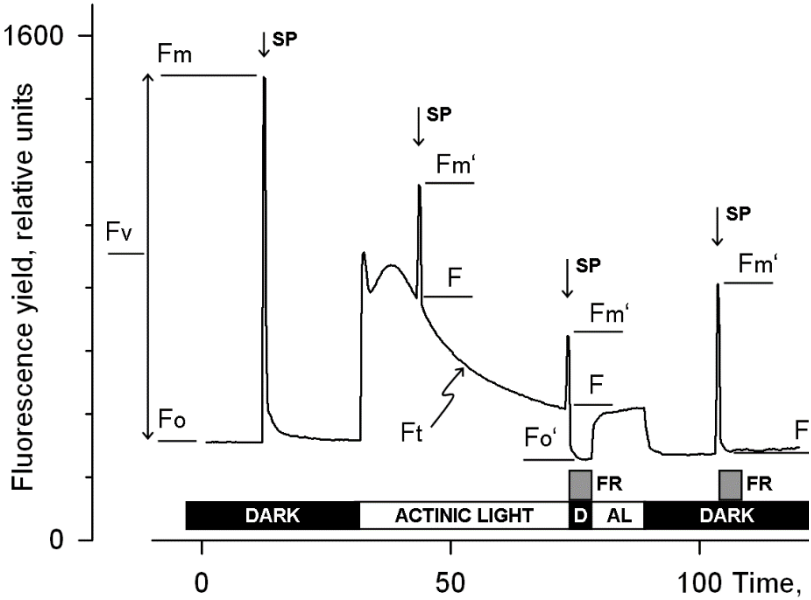


Fig. 25: Measurements for Saturation Pulse Analysis. AL, Actinic Light; D, dark; SP, Saturation Pulse; FR, far-red illumination.

Table 8: Fluorescence Ratio Parameters.

Source	Equation
Maximum photochemical quantum yield of PS II (Kitajima and Butler, 1975)	$\frac{Fv}{Fm} = \frac{Fm - Fo}{Fm}$
Effective photochemical quantum yield of PS II (Genty *et al.*, 1989)	$Y(II) = \frac{Fm' - F}{Fm'}$
Coefficient of photochemical fluorescence quenching (Schreiber *et al.* 1986 as formulated by van Kooten and Snel, 1990)	$q_P = \frac{Fm' - F}{Fm' - Fo'}$
Coefficient of photochemical fluorescence quenching assuming interconnected PS II antennae (Kramer *et al.* 2004)	$q_L = q_P \cdot \frac{Fo'}{F}$
Coefficient of non-photochemical fluorescence quenching (Schreiber *et al.* 1986 as formulated by van Kooten and Snel, 1990)	$q_N = 1 - \frac{Fm' - Fo'}{Fm - Fo}$
Stern-Volmer type non-photochemical fluorescence quenching (Bilger and Björkman, 1990)	$NPQ = \frac{Fm}{Fm'} - 1$
Quantum yield of non-regulated heat dissipation and fluorescence emission: this quenching type does not require the presence of a transthylakoid ΔpH and zeaxanthin (Genty *et al.* 1996)*	$Y(NO) = \frac{F}{Fm}$
Quantum yield of light-induced non-photochemical fluorescence quenching (Genty *et al.* 1996)*	$Y(NPQ) = \frac{F}{Fm'} - \frac{F}{Fm}$

* Kramer *et al.* (2004) have derived more complex equations for Y(NO) and Y(NPQ). Klughammer and Schreiber (2008) have demonstrated that the equations by Kramer *et al.* (2004) can be transformed into the simple equations of (Genty *et al*. 1996) which are used by the PamWin-3 software.

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 concept of separated PS II antennae (puddle model) whereas the q_L assumes interconnected PS II antennae (lake model) which appears to be the more realistic situation in leaves (*cf.* Kramer *et al.*, 2004). However, it should be kept in mind that in the presence of DCMU there is one single charge separation between Fo and Fm, whereas in uninhibited leaves Q_A becomes reduced and again oxidized 20-30 times between Fo and Fm, which may affect the impact of connectivity on the fluorescence kinetics. Determinations of q_P an q_L do not require fluorescence measurements with dark-acclimated samples unless Fo′ is not measured but calculated according to Oxborough and Baker (1997) (see Section 5.3).

q_N and NPQ Parameters of non-photochemical quenching

Both parameters are associated with non-photochemical quenching of excitation energy by thylakoid lumen pH- and zeaxanthin-dependent processes. In contrast to Y(II), qP and qL, calculations of the qN and the NPQ parameters always require fluorescence measurements with the sample in the dark-acclimated and in the light-exposed state (see Table 5.1). Calculation of NPQ (or SV_N; Gilmore and Yamamoto, 1991) is based on the Stern-Volmer equation for fluorescence quenching which predicts proportionality between fluorescence quenching (NPQ) and the concentration of fluorescence-quenching centers in the photosynthetic antennae (e.g. zeaxanthin).

Y(NO) and Y(NPQ) Yields of non-photochemical quenching

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, expressed in terms of the complementary quantum yields of

Y(NO) sum of non-regulated heat dissipation and fluorescence emission,

Y(NPQ) regulated thermal energy dissipation involving ΔpH- and zeaxanthin-dependent photoprotective mechanisms, and

Y(II) photochemical conversion

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 to analyze the partitioning of absorbed light energy in plants. For instance, under high light-conditions, a much higher Y(NPQ) than Y(NO) indicates that excess excitation energy is safely dissipated, that is, photosynthetic energy fluxes are well-regulated. In contrast, high values of Y(NO) would signify that excess excitation energy is mostly channeled off *via* basal quenching mechanisms and, hence, that energy fluxes are inadequately controlled.

5.3 Constant Fraction of Fo Fluorescence (C/Fo)

A number of data suggest that PS I contributes significantly to room temperature fluorescence from leaves. Typical fractions of PS I contribution to total Fo fluorescence are 30 and 50% for C_3 and NADP-ME C_4 plants (e.g., maize), respectively (Genty *et al.* 1990, Pfündel 1998, Agati *et al.* 2000, Peterson *et al.* 2001, Franck *et al.* 2002).

Here, the assessment of <C/Fo> is based on the comparison of the Fo′ calculated according to Oxborough and Baker (1997) (see Section 5.1.2) with the Fo' determined during a post-Saturation Pulse illumination with far-red light. Considering that PS I fluorescence contributes a constant quantity, F^{PSI}, to each fluorescence level, the Oxborough-Baker equation can be reformulated:

$$Fo' = F^{PSI} + \frac{1}{\frac{1}{Fo^{PSII} - F^{PSI}} - \frac{1}{Fm - F^{PSI}} + \frac{1}{Fm' - F^{PSI}}}$$

In the latter equation, the F^{PSI} is subtracted from the measured fluorescence levels Fo, Fm, and Fm′ so that the total fraction in the equation above describes Fo′ originating in PS II. Clearly, the latter fraction plus the F^{PSI} make up the total Fo′ signal. Compared to the original equation, the introduction of F^{PSI} increases the calculated Fo′ because the addition of F^{PSI} overcompensates the effect of F^{PSI} subtraction. Consequently, when the F^{PSI} is disregarded, the theory underestimates Fo′, and this is consistent with the observation that the calculated Fo′ is often lower than measured Fo′. Consequently, to assess the C/Fo, the PamWin-3 program iteratively varies the level of F^{PSI} until the calculated Fo′ matches the measured Fo′.

In practice, determinations of C/Fo require:

- Fully dark-acclimated material for accurate determination of Fo and Fm.
- Following Fo, Fm determinations, illumination with a light intensity that induces substantial non-photochemical quenching but does not cause photoinhibition to measure Fo′ and Fm′.

5.4 Relative Electron Transfer Rate (ETR)

Relative electron transfer rates are calculated according to:

$ETR = PAR \cdot ETR\text{-}Factor \cdot P_{PS2}/P_{PPS} \cdot Y(II)$.

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

PAR Photosynthetically active radiation (400-700 nm)

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

ETR-Factor Absorptance of photons by photosynthetic pigments

The ETR-Factor corresponds to the fraction of incident photons absorbed by photosynthetic pigments. The default value for the ETR-Factor is 0.84, which reasonably matches the average absorptance in the visible range (400-700 nm) of many green leaves. Anthocyanins, however, can reduce availability of blue photons for photosynthesis (Pfündel *et al.* 2007) and, hence, lower the ETR-Factor.

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

The default value for P_{PS2}/P_{PPS} is 0.5. This value is reasonable if one assumes only linear electron transport, 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 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 e. g. may arise from photorespiratory electron flow, nitrite reduction or electron cycling at PS II
- fluorescence information primarily originates from the topmost chloroplast layers, while gas exchange is integrated over all layers; 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.

PAR can be measured at the same spot of the leaf where fluorescence is measured, when the mini-quantum-sensor is moved into the beam. This is possible without substantial loss in signal amplitude. The properties of the mini-quantum-sensor are such that its response to spectral composition and incidence angle of the impinging light approximates that of the leaf

The combined information of ETR, PAR and Temperature provides profound 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 (see next section).

5.5 Rapid Light Curves

The <Light Curve> feature of PamWin-3 exposes the sample to increasing intensities of Actinic illumination. To allow complete equilibration at each light intensity of several minutes are required. Often, much shorter time intervals for each light intensity are chosen to reduce the length of these measurements. In that case, full equilibration of photosynthetic reactions will not be reached. Some ecologist hope to determine the actual state of the sample at the start of the Light Curve, by making the time interval so short that little acclimation is

expected (hoped for) during the set of light intensities measured. So-called “Rapid Light Curves” (RLC) may want to characterize a state, or provide information on the ability, or lack thereof, of the photosynthetic apparatus to respond to rapid changes in the light intensity and are not to be confused with classical photosynthetic light response curves in which photosynthetic rates under steady state conditions are plotted against light intensities. By plotting ETR versus PAR (see previous section), Rapid Light Curves provide three key parameters:

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

The function used by the PamWin-3 software has been derived by Eilers and Peeters (1988) using a mechanistic model which considers the processes of photosynthesis and photoinhibition. The final model function is:

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

To describe the data points of a Rapid Light Curve, the three free parameters (a, b, and c) of the final model function are varied until the best fit between function and data is achieved.

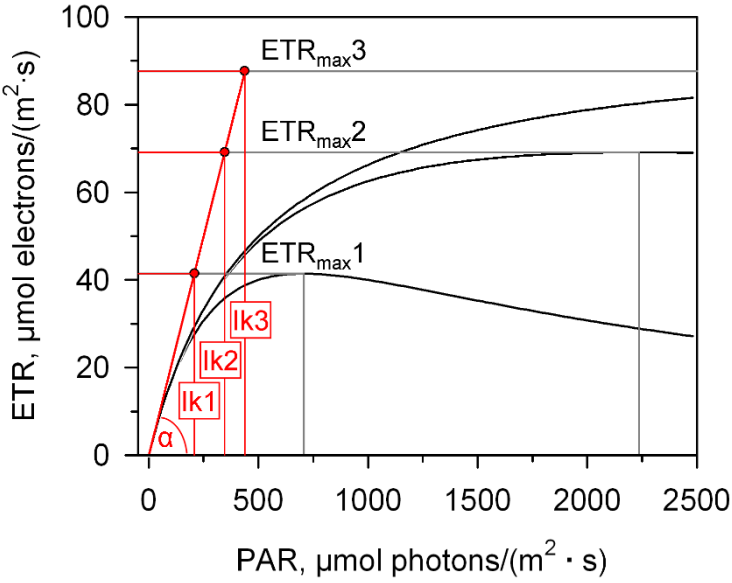
With estimates for the free parameters, the cardinal points of the Rapid Light curves are calculated according to the following equations:

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

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

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

Fig. 26 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.



Curve	Parameter					
	a	b	c	α	ETR_{max}	Ik
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. 26: Theoretical Examples of Eilers and Peeters (1988) Light Curves.

5.5.1 Some Papers related to Rapid Light Curves

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6 Some Reviews on Chlorophyll Fluorescence

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7 Specifications

7.1 Basic System

7.1.1 General Design

Signal detection: PIN-photodiode protected by long-pass filter (T(50%)=715 nm). Selective window amplifier

Sockets: Connector for Special Fiberoptics 2010-F. USB socket. Sockets for Leaf Clip Holder 2030-B or Mini-Quantum/Temperature-Sensor 2060-M, Battery Charger MINI-PAM/L or external 12 V battery *via* MINI-PAM/AK cable, and external lamp/trigger output

Communication: USB. Bluetooth Version 2.0 + EDR Class 2

User interface: Windows (tablet) computer with PamWin-3 software

Power supply: Rechargeable, built-in and sealed, lead-acid battery 12 V/2 Ah; Battery Charger MINI-PAM/L (100 to 240 V AC)

Power consumption: Basic operation 1.6 W, 8 W with all internal light sources operated at maximum output (measuring light, red and blue actinic light, and far red light). Saturation Pulse at maximum intensity, 30 W

Recharging time: approximately 6 hours (with the PAM-2500 turned off) *via* Battery Charger MINI-PAM/L

Operating temperature: -5 to +40 °C

Operating humidity range: 20 to 95% RH (to avoid condensation)

Dimensions: 23 cm x 10.5 cm x 10.5 cm (L x W x H) aluminum housing

Weight: 2.5 kg (including battery)

7.1.2 Light sources

Measuring light: Red LEDs, maximum emission at 630 nm, FWHM (full width at half maximum) 20 nm. 1 µs pulses at modulation frequencies 10 to 5000 Hz for Fo determinations (200 Hz default), and 1 to 100 kHz during actinic illumination, fast kinetics with 100 or 200 kHz, 20 intensity levels, frequency dependent effective PAR ranging from 0.001 to 100 µmol photons/(m^2·s)

Blue actinic light: LEDs, maximum emission at 455 nm, FWHM 20 nm, PAR up to 800 µmol photons/(m^2·s), 20 intensity levels

Far-red light: LED, maximum emission at 750 nm, FWHM 25 nm, 20 intensity levels

Red actinic light: LEDs, maximum emission at 630 nm, FWHM 15 nm, PAR up to 4000 µmol photons/(m^2·s), 20 intensity levels

Saturation pulses: Red LEDs (see red actinic light), PAR up to 25 000 µmol photons/(m^2·s), adjustable between 0.1 and 0.8 s, 20 intensity levels

Multiple turn-over flashes: Red LEDs (see red actinic light), PAR up to 25 000 µmol photons/(m^2·s), adjustable between 1 and 300 ms, 20 intensity levels

Single turn-over flashes: Red LEDs (see red actinic light), PAR up to 125 000 µmol photons/(m^2·s), adjustable between 5 and 50 µs

7.1.3 Special Fiberoptics 2010-F

Design: Flexible, steel-spiral, plastic-covered bundle with three-pin optical connector

Joint end (measuring site): Active diameter 6 mm, outer diameter 8 mm

Length: 100 cm

Weight: 300 g

7.1.4 Distance Clip 60° 2010-A

Design: Metal clip with fiber holder and 11 mm sample hole: 5.5 cm x 1.4 cm (L x W)

Fiber holder: 1.2 cm length, mounted 0.7 cm above base, with lateral screw to fix fiber optics. Angle between fiber optics axis and sample plane: 60°. Two spacer rings to vary the distance between fiber end and leaf surface

7.1.5 Leaf-Clip Holder 2030-B

Mini quantum sensor: Magnetically attached, swivel-mounted sensor, selective PAR measurement, 0 to 20000 µmol photons m^{-2} s^{-1} PAR

Thermocouple: Ni-CrNi, diameter 0.1 mm, -20 to +60 °C

Output: PAR, high sensitivity range: 0 to 1000 µmol m-2 s-1 (output 0 to 2.5 V for each range). Leaf temperature, -20 to +60 °C (0 to 0.8 V). Remote trigger button, signal line connected to ground.

Power supply: PAM-2500 leaf clip socket (5 V/4 mA)

Cable length: 100 cm

Dimensions: 17 cm x 5.7 cm (max.) x 8 cm (max.) (L x W x H)

Weight: 310 g

7.1.6 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

7.1.7 External Voltage Supply Cable MINI-PAM/AK

Design: 90 cm cable with fuse for over-current protection. The cable bypasses the internal battery of the PAM-2500 fluorometer and supplies voltage from an external 12 V battery directly to the electronics

7.1.8 System Control and Data Acquisition

Software: PamWin-3 System Control and Data Acquisition Program

Fluorescence parameters:

Measured: Ft, Fo, Fm, F, Fo′ (also calculated), Fm′. Fast polyphasic rise and decay kinetics (time resolution up to 10 µs). PAR and °C, using the "Leaf-Clip Holder 2030-B" or the "Mini-Quantum/Temp.-Sensor 2060-M)"

Calculated: Fo′ (also measured), Fv/Fm and Y(II) (maximum and effective photochemical yield of PS II, respectively), qL, qP, qN, NPQ, Y(NPQ), Y(NO) and ETR (electron transport rate), C/Fo (constant fraction of Fo that is assumed to originate from PS I)

7.1.9 Carrier Bag

Carrier: Robust field carrier bag with shoulder and hip belt

7.1.10 Transport Box 2040-T

Design: Aluminum box with custom foam packing for PAM-2500 and accessories

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

Weight: 5 kg

7.1.11 Minimum Computer Requirements

Processor: 0.8 GHz; RAM: 512 MB; Hard disc space: 50 MB; Screen resolution: 1024 x 600 pixels; Interface: USB 1.1 or USB 2.0; Optional interface: Bluetooth Version 2.0 + EDR, Widcomm Stack; Operating system: Microsoft Windows 7/8/10.

7.2 Accessories

7.2.1 Touchscreen tablet computer for Field Research

7.2.1.1 Computer box

Design: Aluminum compartment, 23.5 cm x 15.0 cm x 4.5 cm (L x W x H), on top of PAM-2500 chassis to keep an touchscreen tablet computer

7.2.1.2 Touchscreen tablet computer

The touchscreen tablet computers sold with the instrument change every 2-3 years due to developments in the computer industry. Our choice is determined by ruggedness and long battery life.

7.2.2 External battery 000160101314

Battery: 12 V battery, 2.1 Ah, 0.84 kg

Dimension: 3.4 cm x 17.8 cm x 6.0 cm. Charge cycle, 2 hours

7.2.3 Automatic charger 000190101099

Description: Charger for external battery 000160101314, input 100-230 V, 50-60 Hz AC, 0.4 kg

7.2.4 Leaf-Clip Holder 2030-B

Mini quantum sensor: Magnetically attached, swivel-mounted sensor, selective PAR measurement, 0 to 20 000 µmol photons/(m^2 s) PAR

Thermocouple: Ni-CrNi, diameter 0.1 mm, -20 to +60 °C

Output: PAR, high sensitivity range: 0 to 1000 µmol photons/(m^2·s) PAR; normal sensitivity range: 0 to 20 000 µmol photons/(m^2·s) PAR (output 0 to 2.5 V for each range). Leaf temperature, -20 to +60 °C (0 to 0.8 V). Remote trigger button, signal line connected to ground

Power supply: *Via* PAM-2500: leaf clip socket (5 V/4 mA)

Length of power cable: 100 cm

Dimensions: 17 cm x 5.7 cm (max.) x 8 cm (max.) (L x W x H)

Weight: 310 g

7.2.5 Arabidopsis Leaf Clip 2060-B

Design: Aluminum clip with 3.2 mm diameter viewing area designed to position small leaves below the fiberoptics of the PAM-2500, prepared to accommodate PAR and temperature sensors of the Mini Quantum/Temperature-sensor 2060-M

Dimensions: 7.6 cm x 3.0 cm (max.) x 5.2 cm (max.) (L x W x H)

Weight: 55 g

7.2.6 Dark Leaf Clip DLC-8

Design: Clip made of aluminum with felt contact areas and sliding shutter (to close the clip)

Dimensions: 6.5 cm x 2 cm (max.) x 1.5 cm (max.) (L x W x H)

Weight: 3.6 g

7.2.7 Mini Quantum/Temperature-Sensor 2060-M

Mini quantum sensor: Selective PAR measurement, 0 to 20 000 µmol photons/(m^2·s) PAR

Thermocouple: Ni-CrNi, 0.1 mm diameter, -20 to +60 °C

Output: PAR, high sensitivity range: 0 to 1000 µmol photons/(m^2·s) PAR; normal sensitivity range: 0 to 20 000 µmol photons/(m^2·s) PAR (output 0 to 2.5 V for each range). Leaf temperature: -20 to +60 °C (0 to 0.8 V)

Power supply: *Via* PAM-2500: leaf clip socket (5 V/4 mA)

Cable length: 100 cm

Length of sensor cables: 30 cm

Dimensions: 16 cm x 3 cm x 1.7 cm (L x W x H)

Weight: 220 g

7.2.8 Suspension Cuvette KS-2500

Design: Round stainless steel cuvette (7.5 mm wide, 9.0 mm deep) with top window adapter for connecting the Fiberoptics; embedded in POM (polyoxymethylene) body with injection port for Hamilton syringes and hose nozzles for connecting an external flow-through water bath (not included). Including three 6.0 x 1.5 mm magnetic stir bars

7.2.9 Magnetic Stirrer MKS-2500

Design: Magnetic stirrer to drive the magnetic stir bar in the KS-2500 Suspension Cuvette; with PVC ring for centering the cuvette and miniature stand to fix the Fiberoptics on top of the cuvette

7.2.10 Compact Tripod ST-2101A

Adjustable height: In steps between 24 cm and 87 cm

Weight: 400 g

*Subject to change without prior notice

8 Trouble Shooting

- **Erroneous graphical appearance**, e.g. some elements are missing.

  Close PamWin-3.

  Switch off PAM-2500.

  Delete (or better rename) <PAM_WIN.INI> which is in the C:\PamWin_3\Data_2500 directory. Start instrument and software.

- **Unable to establish communication** between the computer and the PAM-2500.

  Close PamWin-3 AND switch off the PAM-2500 fluorometer.

  After several seconds, switch PAM-2500 on again, make sure that the green signal LED flashes and start software.

- **Cannot open report data.** Occurs after changing the name of the folder in which the Report is located.

  Reason is that NAME of data folder (in C:\PamWin_3\Data_2500\Report) must be identical to be identical to NAME of RPT3 file.

- **After system crash: PAM-2500 not found.**

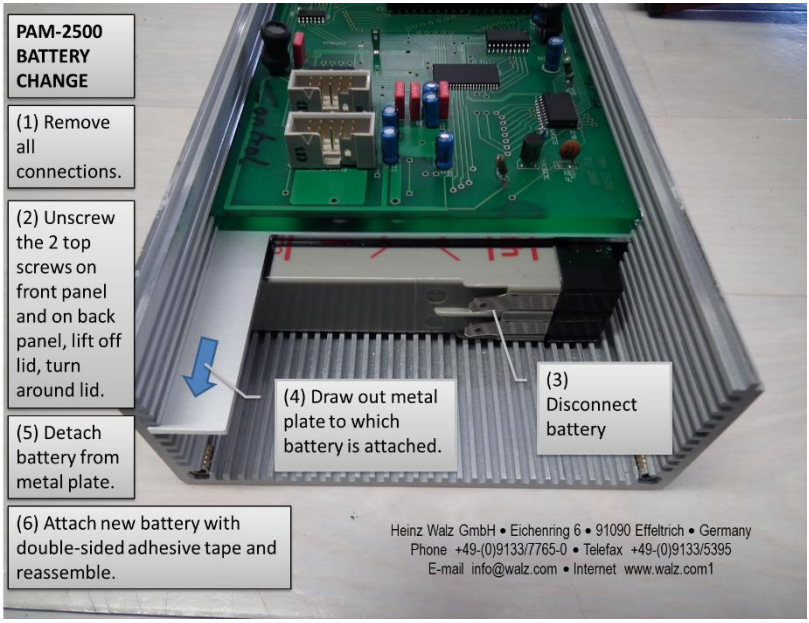
  Switch off fluorometer, unplug USB cable, reconnect USB cable, switch fluorometer on.

- **Connection cannot be established, or saturation pulse crashes the system.**

  If the battery is older than 3-5 years or if the instrument has not been used for many months and there is a risk of deep discharge, the above-mentioned symptoms may point to an

exhausted or dysfunctional battery, requiring a battery replacement.

8.1 Battery replacement



Ordering information for the new battery is provided on the Walz website.

https://www.walz.com/support/support.html, open “Battery Table”

9 Guarantee

9.1 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 the 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.

9.2 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, 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 underwater version of 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.

9.3 Instructions

To obtain warranty service, please follow the instructions below:

- The Walz Service Information Form available at http://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.

9.4 Applicable law

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

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