

DUAL-KLAS-NIR

**Measuring System for P700, Plastocyanin,
Ferredoxin & Chlorophyll Fluorescence**

Manual

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1 Safety Instructions

1.1 General Safety Instructions

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

1.2 Special Safety Instructions

- The DUAL-KLAS-NIR 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 DUAL-KLAS-NIR can emit very strong light! To avoid harm to your eyes, never look directly into the light port of the DUAL-KLAS-NIR or its Perspex optics.
- Keep all ports and plugs clean.
- Do not force a plug into the wrong socket. Orientate 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 bushing of the plug.
- Do not open housing of the DUAL-KLAS-NIR. There are no serviceable parts inside the DUAL-KLAS-NIR.

2 Preliminary manual for the DUAL-KLAS-NIR

The text below I would define as a preliminary manual. In it, many details on the operation of the DUAL-KLAS-NIR can be found but not everything. How to write scripts has for example not been treated. The DUAL-KLAS-NIR software has many similarities with the DUAL-PAM-100 software. If the user wants to know something that he/she cannot find in the present text, it may be an idea to have a look at the manual of the DUAL-PAM-100. The determination of the maximum signal amplitudes of P700, PC and Fd and the P700, PC and Fd Model Spectra (DMP's) is treated twice. First by Gert Schansker (4.2-4.12) and subsequently, by Ulrich Schreiber (4.13) each with their own approach to treating these topics. Hopefully, this leads to synergy for the reader/user.

3 **Extent of Delivery**

Power-and-Control-Unit	DKN-C
Emitter Unit with NIR ML and Green Fluorescence Excitation	DKN-E
Detector Unit with Blue Sources of Illumination	DKN-D
Linear Positioning System	3010- DUAL/B
Power Supply	MINI-PAM/L
Transport Case	DKN-T
Standard USB-A to USB-A cable	

4 Starting up the DUAL-KLAS-NIR

4.1 Linear Positioning System 3010-DUAL/B

Just like a microscope, where the object table is moved towards the lens using a coarse/fine drive, the Linear Positioning System does the same thing, moving either the emitter or detector head relative to the other. The fine drive is mounted on a metal baseplate to which a 13 cm metal rod is attached, which in turn can be connected to another standing metal rod, standing on a wooden plate (Fig. 1).

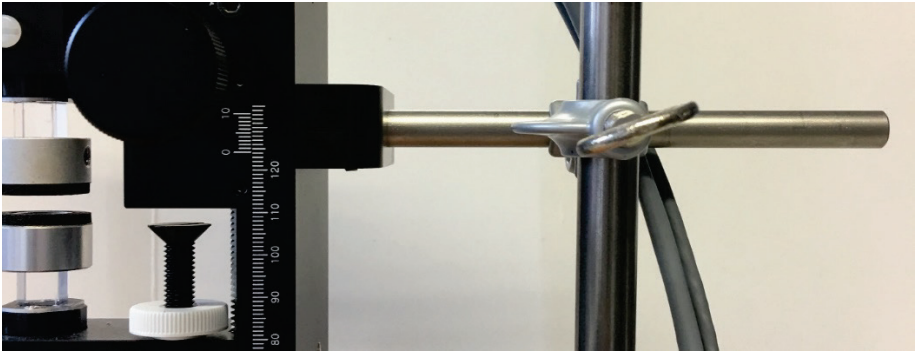


Fig. 1: Connection between Linear Positioning System and standing metal rod.

There is another parallel with the microscope, where it is in principle possible to screw the lens into the sample, destroying it. Also, in the case of the Linear Positioning System considerable power can be generated that potentially can cause damage to the emitter and detector heads, if the two light guide rods are screwed against each other.

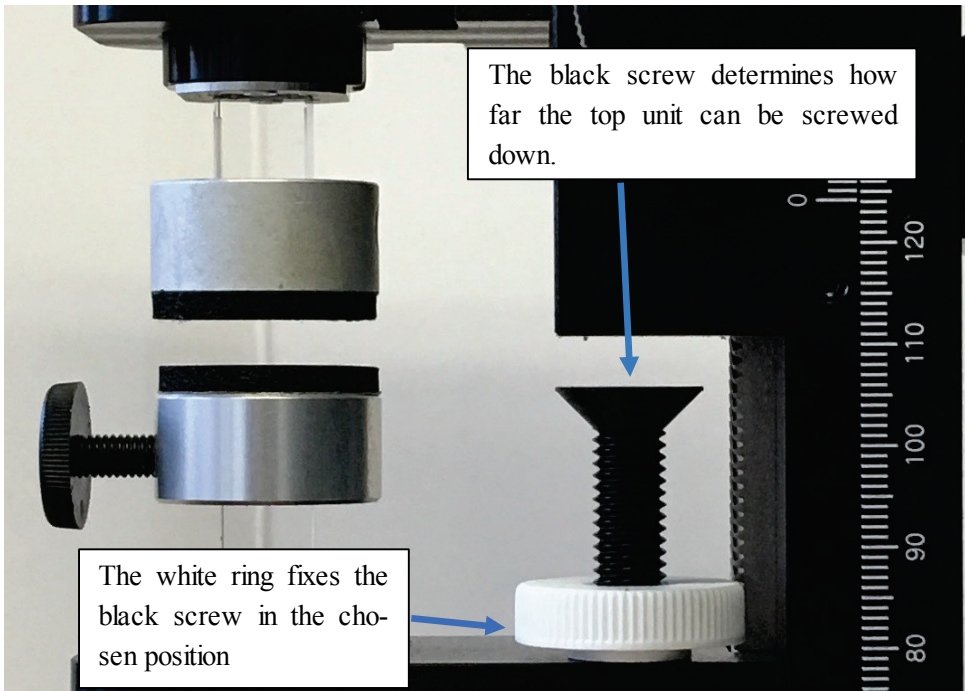


Fig. 2: Black screw and white ring.

The Linear Positioning System has a build-in system with which the risk of damage can be minimized, but it has to be adjusted in order to make it effective. This safety system consists of a black screw and a white ring (Fig. 2). The black screw can block the Linear Positioning System. This means that the screw has to be positioned in such a way that the rings that are placed at the end of the light guides can touch each other but no more. With the white ring the position of the black screw can then be fixed.

4.2.1 How to position the black screw?

The instrument can be built in the following way: position the emitter unit on the bottom side, move the Perspex light guide through the hole and fix the unit in position with the white plastic

screw. Then position the silver colored ring with black soft material on top of the Perspex light guide (see Fig. 2). Lower the Linear Positioning System, bringing the emitter head closer to the wooden base plate. In case the screw was not tightened enough, and the Emitter would drop out, it will not drop far. Add the detector unit at the top side by moving the Perspex light guide through ring of the Linear Positioning System. Screw the white plastic screw tight and place the other silver ring with black soft material on top of the Perspex light guide of the Detector. Then use the coarse/fine drive to place the silver caps against each other. Subsequently, screw the black screw against the coarse/fine drive and fix it in place with the white ring. Now the light guides of Emitter and Detector cannot be screwed into each other anymore unless a very thick object is placed between them.

4.2 First settings

The DUAL-KLAS-NIR needs some time to warm up. This is important for a stable baseline.

Once the DUAL-KLAS-NIR has warmed up for about hour, it is time to open the KLAS-100 software.

The first place to go to may be the 'Settings/Mode' window.

Here it is possible to choose between <Standard SP> and <Fast Acquisition> (Fig. 3). <Standard SP> is for applications like the quenching analysis and light curves. In this mode all kinds of parameters are automatically calculated and can be made visible in the 'SP Analysis' window. In the <Fast Acquisition> mode no automatic calculation of parameters occurs.

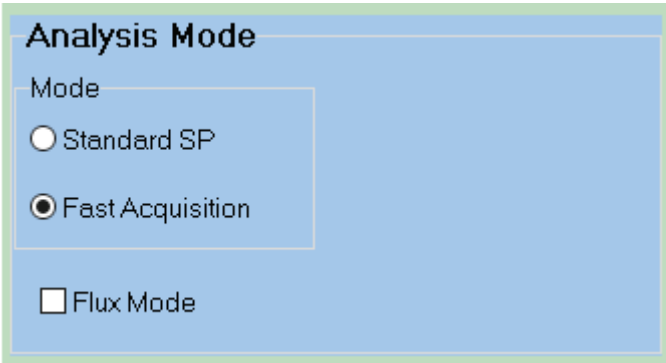


Fig. 3: Analysis Mode panel.

Another choice that can be made in this window is between single, dual and six channel mode.

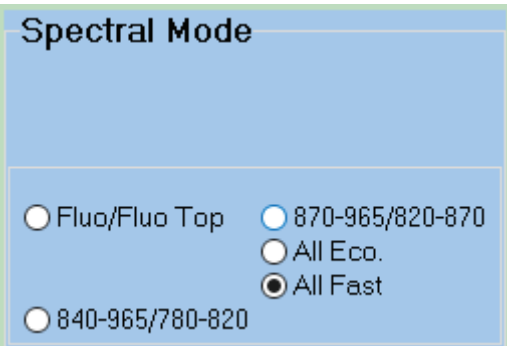


Fig. 4: Spectral Mode panel.

In the ‘Spectral Mode’ sub-window, a first choice can be made (Fig. 4). <All Fast> is the 6-channel mode the user would choose if the instrument is connected to the mains. <All Eco.> is the 6-channel mode the user should choose if the instrument gets its energy from the battery.

This choice gives two upper right panels (Fig. 5). Via the <Fluo panel>/<NIR panel> buttons the user can toggle between these two windows.

Fluo panel Full Screen

PC	P700	Fd
Maximum	Maximum	Maximum
PC 3.050	P700 7.400	Fd 0.350
Oxidation	Oxidation	Reduction
PC 0.000	P700 0.000	Fd 0.000
Rel. PC	Yield	Rel. Fd
Ox. 0.000	Y(I) 0.000	Red. 0.000
SP 0.000	Y(ND) 0.000	SP 0.000
Ratio	Y(NA) 0.000	Ratio
PC/P700		Fd/P700
0.000		0.000
870-965	820-870	
0.000 Offset	0.000 Offset	
+0.00 V	+0.00 V	
840-965	780-820	
0.000 Offset	0.000 Offset	
+0.00 V	+0.00 V	
PAR	Temp	U Batt.
0 uE	—	13.9 V

NIR panel Full Screen

Fluo	Fluo Top
Fo 0.000	Fo 0.000
F(I) 0.000	F(I) 0.000
Fv/Fm 0.000	Fv/Fm 0.000
Fm 0.000	Fm 0.000
Y(II) 0.000	Y(II) 0.000
F 0.000	F 0.000
Fm' 0.000	Fm' 0.000
Fo' 0.000	Fo' 0.000
qP 0.000	qP 0.000
qL 0.000	qL 0.000
qN 0.000	qN 0.000
NPQ 0.000	NPQ 0.000
Fo, Fm	Fo, Fm
Fluo	Fluo Top
0.000 Offset	0.000 Offset
+0.00 V	+0.00 V
PAR	Temp
0 uE	—
	U Batt.
	13.9 V

Fig. 5: Spectral mode sub-window.

Measure Mode

Single Channel Dual Channel

☐ Fluo
☐ Fluo Top
☐ Ext. 1
☐ Ext. 2

☒ Fluo+Fluo Top
☐ Fluo Top+Ext. 1
☐ Fluo+Ext. 2
☐ Ext. 1 + Ext. 2

Spectral Mode

☒ Fluo/Fluo Top
☐ 840-965/780-820

Fig. 6: Measure Mode sub-window.
Selection Fluo/Fluo Top

Clicking the <Fluo/Fluo Top> button gives in the ‘Measure Mode’ sub-window several dual-channel options as well as the <Fluo> and <Fluo Top> single channel options (Fig. 6). It is important to be aware of the fact that following installation of the software the <Fluo Top> measuring light is set to 0 and that makes this channel invisible. To register the fluorescence of this channel the measuring light intensity has to be set to a value larger than 0. The <Ext.> channels are for the moment a little bit theoretical.

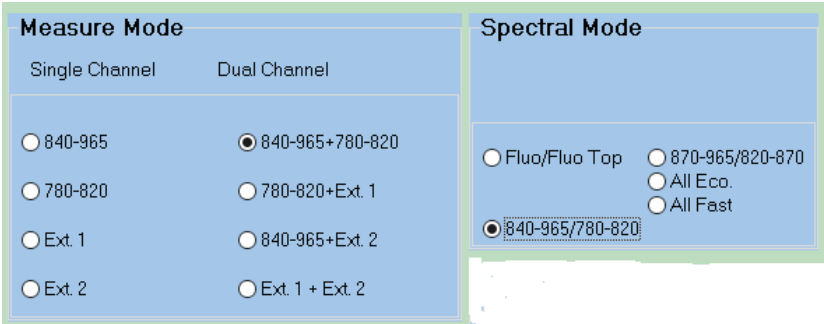


Fig. 7: **Measure Mode sub-window.**
 Selection 840-965/780-820.

Choosing the two wavelength pairs: <840-965/780-820> or <870-965/820-870> also induces changes in the left upper panel of the ‘Settings/Mode’ window (Fig. 7).

Fd		PC		P700	
Maximum		Maximum		Maximum	
Fd	0.350	PC	3.050	P700	7.400
dI/I	0.000	dI/I	0.000	dI/I	0.000
V	0.000	V	0.000	V	0.000
Max.		Max.		Max.	
Reduction		Oxidation		Oxidation	
Fd	0.000	PC	0.000	P700	0.000
Rel. Fd		Rel. PC		Yield	
Red.	0.000	Ox.	0.000	Y(I)	0.000
SP	0.000	SP	0.000	Y(ND)	0.000
Ratio		Ratio		Y(NA)	
Fd/P700	0.000	PC/P700	0.000		
840-965	780-820	870-965	820-870		
0.000 Offset	0.000 Offset	0.000 Offset	0.000 Offset		
+0.00 V	+0.00 V	+0.00 V	+0.00 V		
PAR	Temp	PAR	Temp	U Batt.	
0 uE	—	0 uE	—	13.9 V	

Fig. 8: Spectral mode sub-window.

The two panels of Fig. 8 show the two right side panel options for the <840-965/780-820> pairs and for the <870-965/820-870> pairs, respectively. The first choice focuses on Fd and the second choice focuses on P700 and PC.

Analysis Mode		Ch 1	Ch 2
Mode		Gain	Gain
<input checked="" type="radio"/> Standard SP <input type="radio"/> Fast Acquisition		<input type="radio"/> 1 (Low) <input checked="" type="radio"/> 5 (High)	<input type="radio"/> 1 (Low) <input checked="" type="radio"/> 5 (High)
SP		Damping	Damping
<input type="radio"/> Sat-Pulse 1 <input type="radio"/> Sat-Pulse 2 <input type="radio"/> Sat-Pulse 3 <input type="radio"/> Fluo SP <input checked="" type="radio"/> P.+F. SP		<input type="radio"/> 10 μ s (Low) <input checked="" type="radio"/> 1 ms (High)	<input type="radio"/> 10 μ s (Low) <input checked="" type="radio"/> 1 ms (High)
		Zero Offset	Zero Offset

Fig. 9: Analysis Mode panel.

Choosing in the 'Analysis Mode' sub-window the option <Standard SP> sets the Gains of channel 1 and 2 to High and the (signal) Damping to High as well (Fig. 9). In addition, this option allows the user to choose from several types of saturation pulses: <Sat-Pulse 1>, <Sat-Pulse 2>, <Sat-Pulse 3> (the properties of these three pulse types can be studied in the 'Settings/SP Trigger' window), <Fluo SP> and <P.+F. SP>, where <Fluo SP> is a standard saturation pulse used for quenching analysis experiments and <P.+F. SP> is the standard pulse used to determine not only F_m or F_m' but also the maximum NIR-absorption changes ($\sim P_m$).

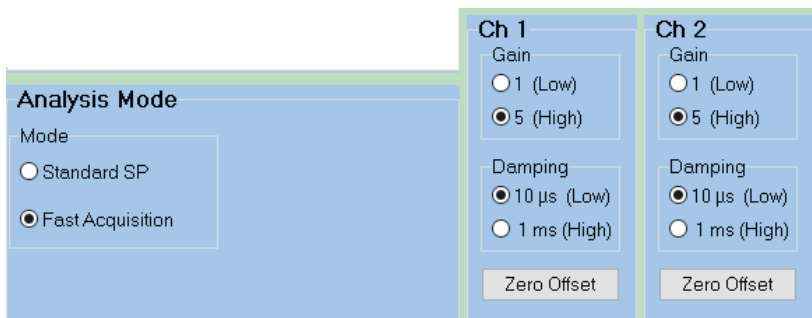


Fig. 10: Analysis Mode panel.

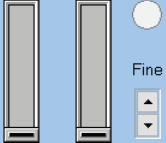
Choosing in the 'Analysis Mode' sub-window the option <Fast Acquisition> (Fig. 10) sets the Gains of channel 1 and 2 to High and the (signal) Damping to Low. In the <Fast Acquisition> mode, experiments are mainly based on trigger patterns and script files, and no special saturation pulse options are offered.

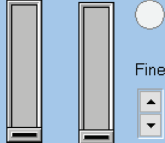
4.3 NIR Meas. light window

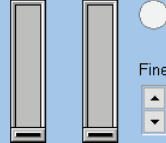
The next window relevant for making the DUAL-KLAS-NIR ready for measurements is the 'NIR Meas. light' window.

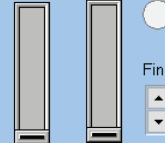
Clicking on the <NIR Bal.> button will change something, see the before and after situation below (cf. Fig. 11 and Fig. 12):


Mode NIR Meas. Light Fluo Meas. Light Actinic Light Slow Kin. Trig. Run Sat. Pulse SP Trigger

'P700' Meas. Light
 820 nm 870 nm


'PC' Meas. Light
 870 nm 965 nm


'Fd' Meas. Light
 780 nm 820 nm


'All' Meas. Light
 840 nm 965 nm


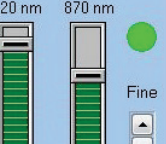
Balance

 Target mV
 0
 Fine Adjust Step
 1
 ML Intensity
 9
 Set to default values

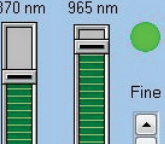
Deconvolution
 Please choose model spectra for online deconvolution
 DKN Model Spectra

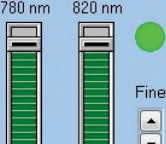
Comp.	Model spectrum	100%
P700	P700_ivy_50ms_Febr_2_#8.K	3.730
PC	PC_ivy_50ms_Febr_2_#8.KMS	1.230
Fd	Fd_ivy_50ms_Febr_2_#8.KMS	0.400

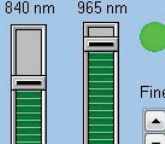
☐ No PC ☐ No Fd


Fig. 11: NIR measuring light window.
Before command "Balance"

'P700' Meas. Light
 820 nm 870 nm


'PC' Meas. Light
 870 nm 965 nm


'Fd' Meas. Light
 780 nm 820 nm


'All' Meas. Light
 840 nm 965 nm


Balance

 Target mV
 0
 Fine Adjust Step
 1
 ML Intensity
 9
 Set to default values

Deconvolution
 Please choose model spectra for online deconvolution
 DKN Model Spectra

Comp.	Model spectrum	100%
P700	P700_Orchid_#X.KMS	7.070
PC	PC_Orchid_#X.KMS	3.170
Fd	Fd_Orchid_#X.KMS	0.690

☐ No PC ☐ No Fd

Fig. 12: NIR measuring light window.
After command “Balance”

So far, I have worked with a NIR measuring light intensity of 9, which worked well for me. In the case of fluorescence measuring light the user always has to consider the actinic effect of this light; in the case of NIR measuring light, this does not play much of a role. The NIR measuring light has a slight FR-effect (between FR1 and FR2) despite the fact that these wavelengths are far beyond 700 nm. After long dark adaptation the NIR measuring light may cause some PC oxidation.

This window also contains a ‘Deconvolution’ sub-window, which is crucial for the deconvolution of the NIR-signals. It is here, where the differential model plots (DMPs) are imported. Double-click on the cell next to P700. This opens the KLAS-100\Model Spectra\DualKlasNIR\DLN Model Spectra directory (Fig. 13). Here, a P700_*.kms file can be chosen, relevant for the sample to be measured.

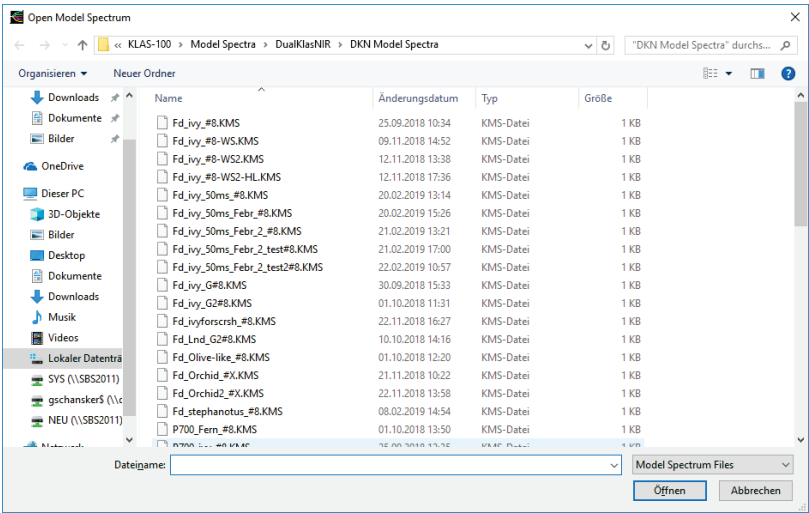


Fig. 13: Folder DKN Model Spectra.

Then double click on the cell next to PC and import the accompanying PC_*.kms file and finally double click on the cell next to Fd and import the accompanying Fd_*.kms file.

Fine tuning of the calibration is possible in this window, but it can be done as well in the 'Slow Kinetics' window, see below.

4.4 Slow Kinetics window

The next window that is important for making the instrument ready for measurements is the 'Slow Kinetics' window. At this point it is important that a leaf/sample has been placed in the instrument.

With a dark-adapted leaf in the instrument, the first step is to click on the <NIR Bal.> button. When a sample has been replaced by another the following unbalanced situation may arise (Fig. 14):

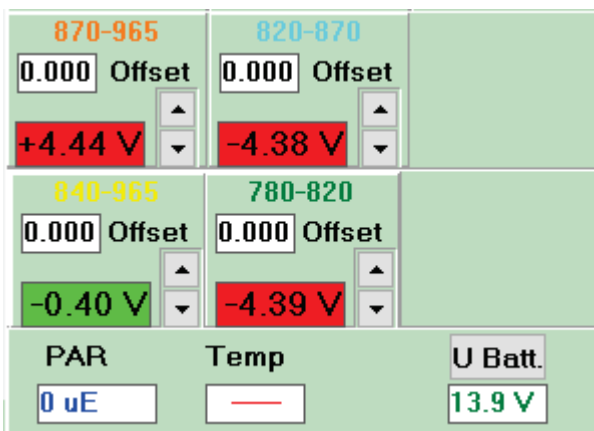


Fig. 14: Signal levels.
Not balanced.

Three of the four fields indicate that the situation is unbalanced, and one field is a little bit off. After clicking the <NIR Bal.> button and a bit of fine tuning the following situation is observed (Fig. 15):

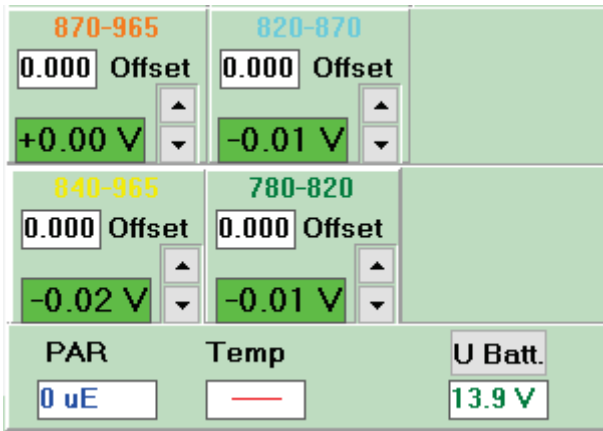


Fig. 15: **Signal levels.**
Balanced.

All four fields are close to zero and we have a balanced situation. With the little arrows next to the fields fine tuning is possible (up arrows increases the value and down arrows decrease the values).

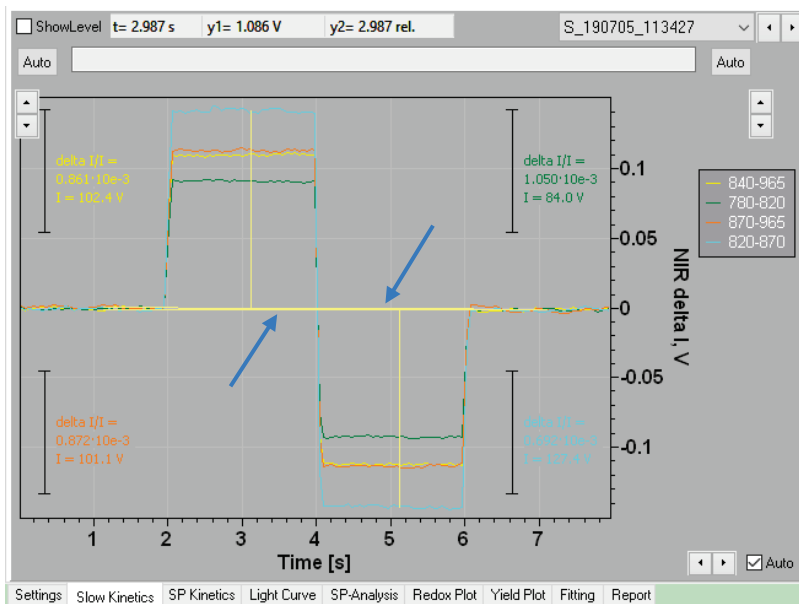


Fig. 16: Slow Kinetics window.

Signal calibration.

The next step is to click the <Calib.> button. This initiates the execution of a short script (Fig. 16).

The two yellow lines in the middle of Fig. 16 are essentially flat and parallel. This means that there is a flat base line and the sample is ready for measurements. Starting version 65 of the software, signal amplitudes for the four wavelength pairs are indicated in the calibration graph.

To characterize the leaf and the NIR-signals a bit more, a final protocol is run.

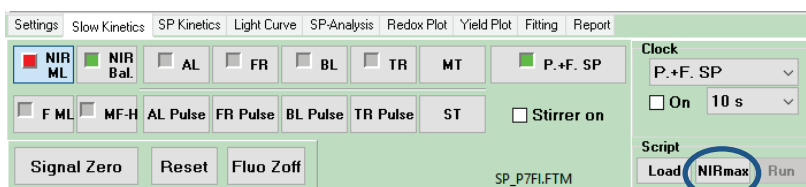
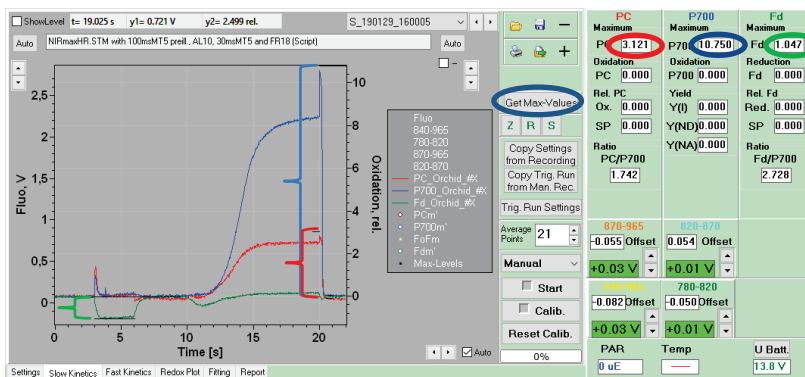


Fig. 17: Control panel.

Command <NIR max> marked.

On the left bottom panel there is the <NIRmax> button (Fig. 17). Click on this button to start a script to determine the maximum Fd, P700 and PC signals (Fig. 18).

**Fig. 18: Slow Kinetics window.**

Determination of max signals.

The standard NIRmax script consists of two elements: The first element is a 3 s pulse of light on top of which a 30 ms MT after 800 ms is given. With this pulse of light, it is possible to reduce all Fd, which in the subsequent 4 s of darkness re-oxidizes again. The second element consists of a 10 s pulse of far-red light with at the end an MT pulse. The FR-light oxidizes P700 and PC to a large extent. The MT pulse at the end, oxidizes the remaining reduced PC and P700 and then, subsequently, reduces them again.

Once the script is finished, click the <Get Max[-Values]> button, this extracts the maximum P700, PC and Fd amplitudes from the measurement and transfers them to the Maximum fields at the top of the right panel (Fig. 18). With respect to the <Get Max[-Values]> button several points can be made: 1. The default value

for the Max-Values is one. This means that all measurement values larger than 1 are cut off and the result is a truncated signal; 2. The obtained Max-Values are sensitive to the DMPs used. It is, therefore, important to determine the Max-Values with the correct DMPs imported. If a new set of DMPs is going to be determined the Max-Values have to be determined again after importing the new DMPs; 3. Sometimes the line segments indicating the minimum and maximum values are positioned incorrectly. This may happen in the case of pine needles where the Fd-signal immediately starts to go up again after reaching its lowest value. In that case the mouse can be used to determine the correct minimum and maximum values (Fig. 19). Moving the mouse over the graph area of the Slow Kinetics window the t-value relating to the time base, the y1-value relating to the fluorescence-axis and the y2-value relating to the 'NIR delta I/I'-axis change. By pointing with the mouse at the minimum Fd-value, the corresponding y2-value can be found. In Fig. 19, $t = 3.242$ s yields $y2 = -0.643 \cdot 10^{-3}$. The maximum Fd-value around 7 s gives $y2 = 0.082 \cdot 10^{-3}$, which then gives an amplitude of $0.725 \cdot 10^{-3}$. Then go to the NIR Meas. Light window and click in the Deconvolution sub-window twice on the Fd-Max cell. This activates the cursor in this cell (Fig. 20) and the value 0.725 can be inserted manually.

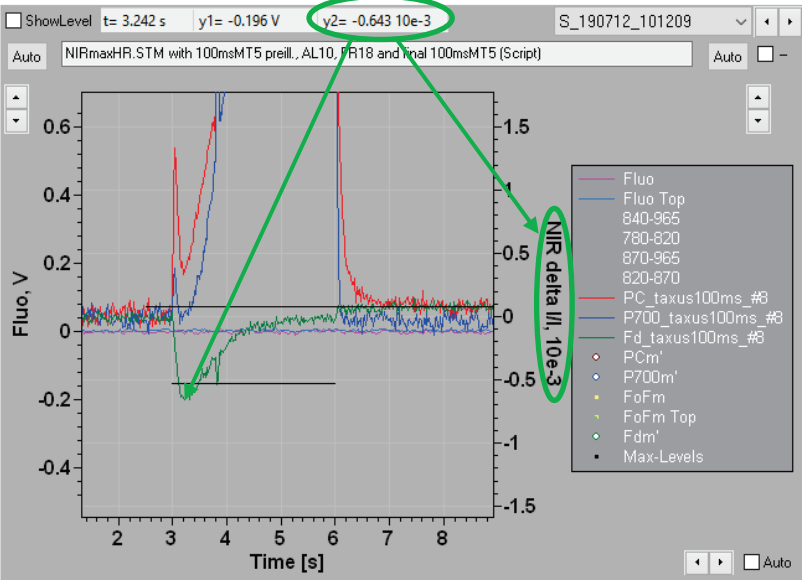


Fig. 19: Getting the correct Fd-min value in the Slow Kinetics window.

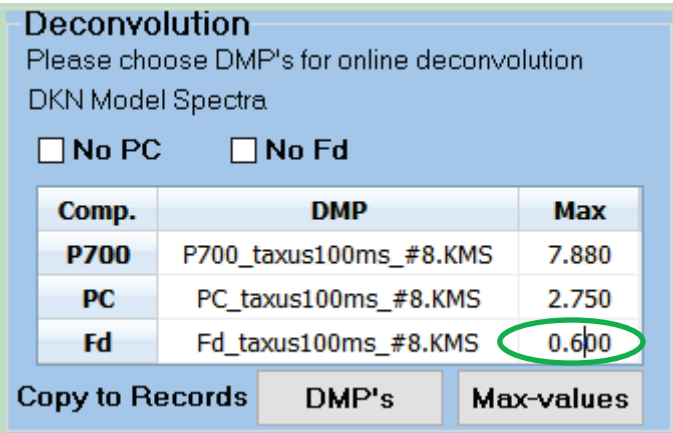


Fig. 20: Manually changing the Fd-Max value in the NIR Meas. Light window.

Table 1 NIRmax measurement made on a sun-exposed ivy leaf (Ivy HL) deconvoluted with 4 sets of DMPs.

	P700 (*10 ⁻³)	PC (*10 ⁻³)	Fd (*10 ⁻³)	PC/P700	Fd/P700
Ivy HL	6.09	3.08	1.01	3.03	4.64
Ivy LL	5.75	3.27	1.83	3.42	8.91
Cherrylaurel	6.49	2.59	1.00	2.39	4.31
Bergenia	6.11	3.17	1.16	3.11	5.32

In Table 1 a single NIRmax measurement of a sun-exposed leaf of ivy was taken. In the top row of the table the signals were deconvoluted with the DMPs determined for this leaf (Ivy HL). Then the same measurement was deconvoluted with DMPs of respectively an ivy leaf grown under shade conditions (Ivy LL), a cherrylaurel leaf exposed to similar light conditions as the sun-exposed ivy leaf and a bergenia leaf. Using DMPs of other types of leaves yields quite different amplitudes and ratios and this becomes especially clear when comparing the calculated ratios, which vary for Fd/P700 between 4.31 and 8.91 (a factor 2.07) and for PC/P700 between 2.39 and 3.42 (a factor of 1.43). This table very clearly indicates that for a correct deconvolution of the signals it is important to determine for each species and growth light condition (sun-exposed vs shade) specific DMPs.

Provided that good DMP files are available for this plant species/sample the sample is now ready for measurements.

4.5 Changing a sample/leaf

Every time a new sample is placed in the instrument, the same sequence of four steps has to be followed:

- a. Click on the <NIR Bal.> button and if necessary fine tune
- b. In the Slow Kinetics window click on the <Calib.> button
- c. Run the <NIRmax> script
- d. Click on the <Get Max[-Values]> button

Without points c. and d., it is also possible to make measurements, but then they cannot be related to the maximum signal amplitude and there is a risk the signals will be truncated (see above).

4.6 Determining differential model plots (DMPs)

The NIR spectra Fd, P700 and PC do not have much structure and isosbestic points are missing. In order to be able to deconvolute the NIR-signals an alternative strategy was developed. It is possible to design experimental protocols that contain time windows during which only one of the three components shows redox changes. These time windows are defined and then used for the individual DMPs. There are separate scripts for the P700, PC and Fd DMPs.

For the P700 and PC DMPs the electron transport chain has to be activated (best, activated PS I acceptor side and activated Calvin-Benson cycle). This state can always be induced. For the Fd DMP on the other hand it is important that the acceptor side of PS I remains in the inactive state during the ten repetitions of the standard protocol and for the <NIRmax> protocol it is important that the acceptor side of PS I is in the inactive state at the beginning of the script.

For higher plants (most angiosperms) this requirement can easily be met since the activation of the PS I acceptor side is, in general, a slow process. In gymnosperms, green algae, phytoplankton etc. a single saturating pulse is enough to activate the PS I acceptor side.

This means that the standard Fd-DMP script that was developed for angiosperm leaves will not work for organisms other than angiosperms.

Therefore, another strategy was chosen that can be applied to gymnosperm needles.

In the original script a 600 ms AL pulse ($\sim 650 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) was used. In angiosperm leaves at the end of such a pulse of light Fd, P700 and PC are all in the reduced state. In the subsequent 3.4 seconds of darkness P700 and PC remain reduced whereas Fd is re-oxidized. If activation of the PS I acceptor side occurs during the pulse, re-oxidation of P700, PC and Fd and fast re-reduction of P700^+ and PC^+ by electrons from the PQ-pool take place in parallel to equally fast re-oxidation of Fd by electron donation to NADP^+ , a clean separation of the Fd-signal is prevented.

The effect of the activation of the PS I acceptor side on the Fd re-oxidation kinetics was demonstrated by Schreiber (measured on *Hedera helix* leaves; Fig. 21).

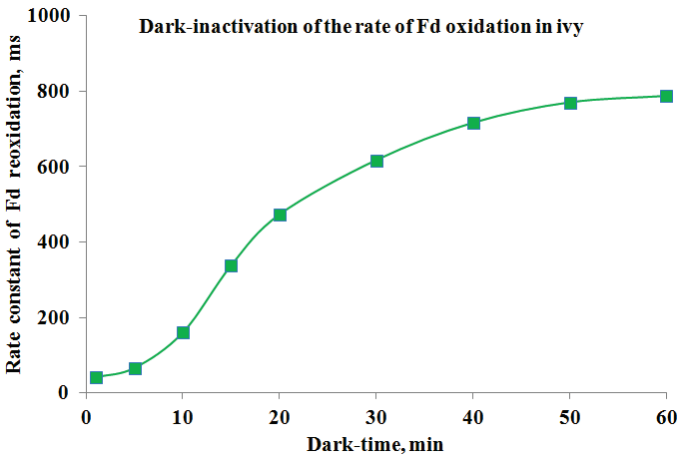


Fig. 21: Fd re-oxidation in the dark.

For needles (from gymnosperm trees) it was possible to change the 600 ms AL pulse into a 50 ms MT pulse which was short enough to avoid activation of the PS I acceptor side even in the course of ten repetitions. It was subsequently found that a 100 ms MT pulse is still short enough to avoid PS I acceptor side activation in needles of gymnosperms.

The idea behind this modified script is the following: during the 50 ms pulse Fd will become to a large extent reduced. Reduction of Fd means initially oxidation of PC and P700. At the same time the PQ-pool starts to become reduced. Then, after about 20 ms of illumination electrons generated by PS II start to re-reduce PC and P700. After 50 ms of illumination, Fd will be largely reduced and P700 and PC will be partially reduced. On lights off, PC and P700 that are still in the oxidized state will become reduced within 100 ms by electrons from the PQ-pool. At longer times (longer than 100 ms darkness) it will be the re-oxidation of Fd that determines the change in the NIR signals. If, for the organism the user works with, 50 ms is still too long, the user can also try MT pulses of 40 or 30 ms.

It was found that this approach does not work for angiosperm leaves. Re-oxidation of Fd during the fluorescence rise in angiosperm leaves is much faster than at Fm. Possibly a relatively fast transfer of electrons from the PS I acceptor side to the PQ-pool is possible as long as the PQ-pool is not yet fully reduced. It has then to be assumed that such a fast transfer does not occur in needles.


Further, the <NIRmax> script, which is used to determine the maximum Fd, PC and P700 amplitudes will probably activate the PS I acceptor side as well. It is, therefore, important to run the <Fd-DMP> script before the <NIRmax> script. A modified <NIRmax> script, more suitable for gymnosperms, algae, etc., is available.


For the DMP-determination the measurements should be made in the following order:

- a. Run the modified <Fd-DMP> script
- b. Run the modified <NIRmax> script
- c. Run the <P700-DMP> script
- d. Run the <PC-DMP> script (3. and 4. can also be done in the inverse order if a separate ~5 min AL 8-10 pre-illumination is given beforehand, to activate photosynthesis)

4.7 Determination of the Fd-DMP

Click on the <Load> button. This will open the KLAS-100\script files\NIR_Script subdirectory. If not, navigate there. If the wrong

script was opened it is possible via the <Open Directory>  icon to navigate to the correct script. Select Fd_NIR_Ref.prg for angiosperm leaves or Fd_NIR_Ref_NeedleAlgae.prg for other

organisms. This imports this script into the script window. Then close the script window clicking on the <U-turn arrow>  icon or the <cross>, both in the upper right corner of the script window. Then click <Run>, to start the selected script. Starting version 65 of the software, the standard DMP determination scripts are placed in a separate folder: KLAS-100\Script Files\NIR_Script\NIR_Ref.

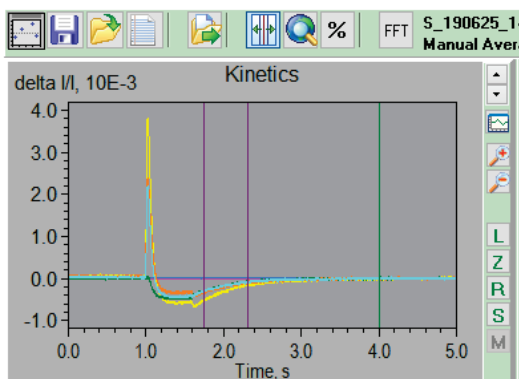
After execution of the script the software will automatically go to the 'fitting window'.

In the 'fitting window' it is important to make all the steps in the right order:

In the left-hand top side window, the raw measurement data (transients of the four wavelength pairs) are presented (Fig. 22) and among other things two purple vertical lines. With these lines the segment of the curve where only Fd-redox changes are expected to occur should be limited. With the left purple line, the pair can be moved from left to right or right to left. With the right purple line, the distance between the two purple lines can be modified. In addition, there are two green lines that define the reference value for the measurement. In newer versions of the software the green lines for the Fd-DMP determination are set on the right-hand side of the window, where NIR-measuring-light-induced PC oxidation does not play a role.

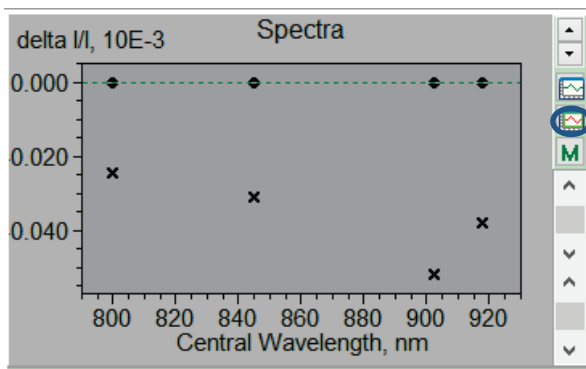
On the right-hand side of the 'Fit' window there are many small icons. In practice, the user will probably only use the <Vertical Zoom> buttons to allow an easier choice of the time window defined by the purple lines. In that case, position the purple lines around the segment that should be amplified. For the Fd-DMP, the selective part of the transient is the segment where the signals return from negative values to 0. If the sample is not well dark adapted or if the PS I acceptor side became activated dur-

ing the 10 repetitions of the measurement, the kinetics of this increase will be very fast, and it will be difficult to define an appropriate time window with the purple lines.



- ← Vertical zoom
- ← Autoscaling
- ← Horizontal zoom
- ← Show Legend
- ← Zero first cut
- ← Zero Reset
- ← Slope correction
- ← Maximum determination

Fig. 22: Kinetics fitting window.



- ← Vertical zoom
- ← Get model spectrum

Fig. 23: Spectra fitting window.

The 'Spectra' window (Fig. 23) has on its right-hand side one very important icon called <Get Model Spectrum>. Once the purple lines have been positioned, the user should click on this icon. This will open a pop-up window (Fig. 25).

In more recent versions of the DUAL-KLAS-NIR software the <Get Model Spectrum> icon has been moved closer to the ‘Kinetics’ sub-window (Fig. 24).

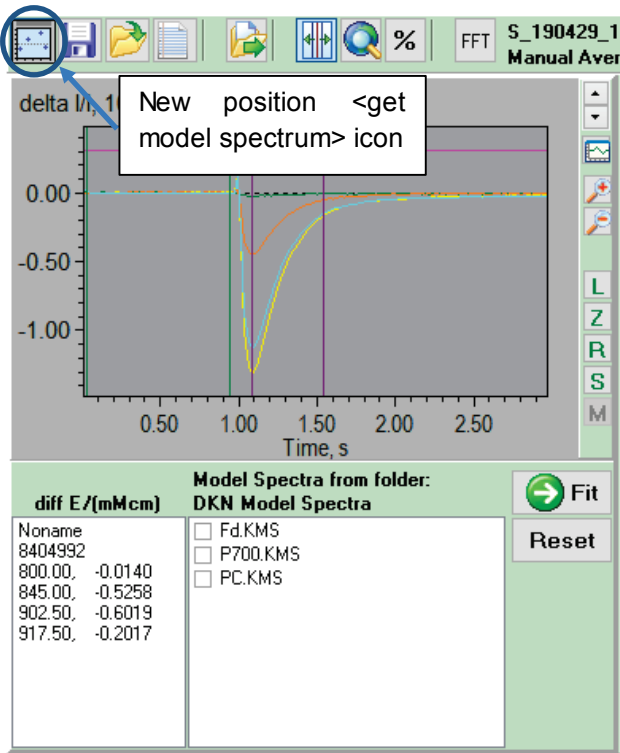


Fig. 24: New position of the <Get Model Spectrum> icon.

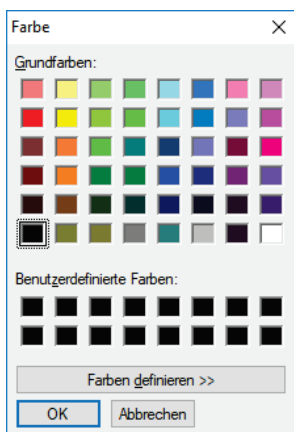


Fig. 25: Color table.

The user has to click on a color in the pop-up window and then click <OK>. The user is of course free to choose his or her color coding, but so far, we have used a variation of green for Fd, a variation of blue for P700 and a variation of red for PC. On clicking <OK> the software will extract non-normalized DMP information from the transients.

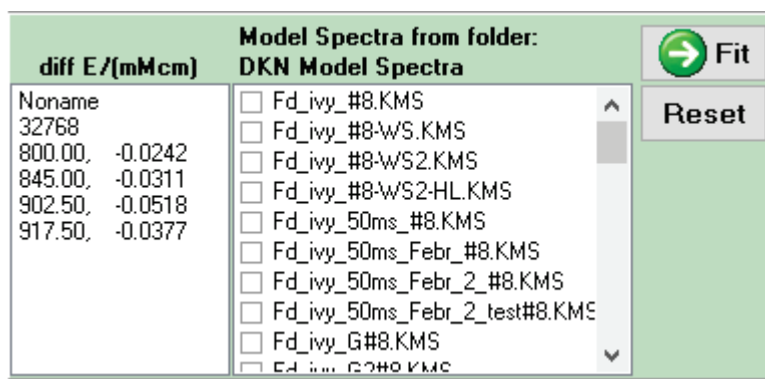


Fig. 26: Non-normalized model spectra.

These data can be found in another sub-window (Fig. 26).

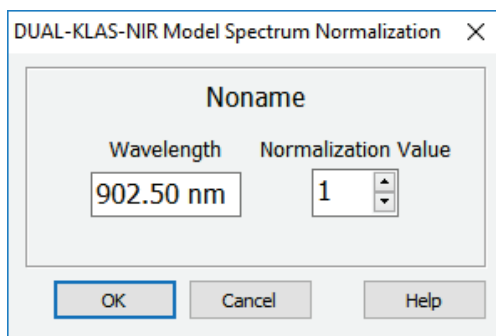


Fig. 27: Conditions for normalization.

The values in the left column give the middle-values of the four wavelength pairs that are measured by the DUAL-KLAS-NIR. The values in the right column are the non-normalized data extracted from the time window between the purple lines, where in this case only Fd-redox changes occurred. The third value is always the most negative. Double click on this value and a pop-up window will open (Fig. 27).

Click <OK> to accept that this value will be normalized to 1 (Fig. 28).

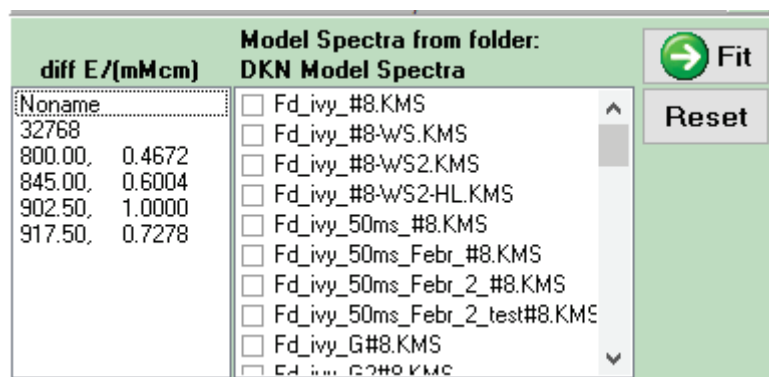



Fig. 28: Normalized model spectrum.

The result is a set of four normalized numbers that define the Fd-DMP. This should be saved using the  button. This will open the KLAS-100\Model Spectra\DualKlasNIR\DKN Model Spectra directory, where the Fd_*.KMS file can be saved. It may be a good idea to start the file name of the Fd-DMP with 'Fd_'. On saving the file, the DMP-data will be shown in the 'Differential Model Spectra' window (Fig. 29).

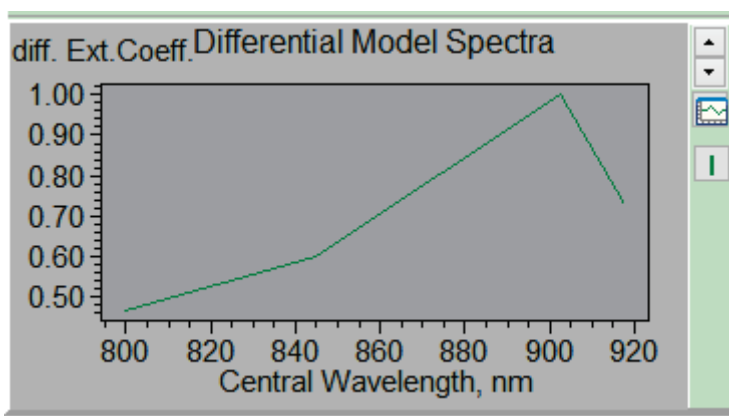


Fig. 29: 'Differential Model Spectra' window.

The DMPs of P700 and PC are very reproducible. Only with respect to Fd there is some variation between leaves (Fig. 30).

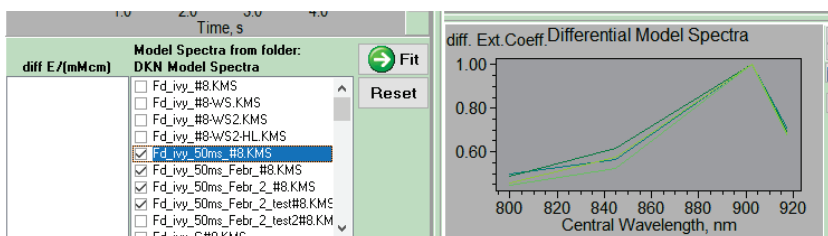


Fig. 30: Comparison of Fd model spectra.

The reason for this may be that the Fd-signal is by far the smallest signal, and it may, therefore, be more sensitive to contamination of the signal by P700 or PC, or there is really variability in

the properties of the signal. Contamination by PC or P700 may occur if the purple line was positioned too far to the left where redox changes of PC and P700 still occurred. It may, therefore, be an idea to try, initially, several positions for the left purple line to get a feeling how far it has to be moved to the right (accepting a smaller signal amplitude) to avoid contamination with P700 and PC redox changes.

4.8 'NIRmax' script

Once the Fd-DMP is determined, the next step is to run the 'NIRmax' script (Fig. 31). Also, for this protocol it is important that the acceptor side of PS I is initially in the inactive state. In non-angiosperm samples it will become activated by the 3 s pulse of light. In the original script an MT pulse is given after 800 ms to maximally reduce Fd, however, in the case of gymnosperms, green algae/phytoplankton, etc. this is too late. In the modified protocol this MT is already given after 200 ms.

The <NIRmax> button is associated with a particular script (e.g. NIRmaxHR.prg). By clicking with the right button of the mouse on the <NIRmax> button the KLAS-100\Script Files directory will be opened. The relevant scripts can be found in the KLAS-100\Script Files\NIR_script directory. Navigate there and open NIRmax_NeedleAlgae.prg. Now the <NIRmax> button is associated with this file. Click with the left mouse button on the <NIRmax> button, which will initiate execution of the chosen script file. After the measurement is made, click on the <Get Max[-Values]> button.

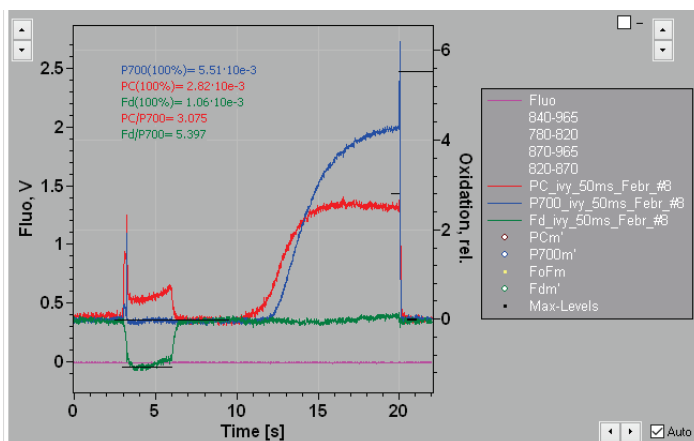


Fig. 31: Redox kinetics induced by the <NIRmax> script.

The flat black lines in Fig. 31 represent the determined minimum and maximum values for each component.

These data are then transferred to the Maximum P700, PC, Fd cells (Fig. 32).

PC	P700	Fd
Maximum	Maximum	Maximum
PC <input type="text" value="2.824"/>	P700 <input type="text" value="5.509"/>	Fd <input type="text" value="1.062"/>
Oxidation	Oxidation	Reduction
PC <input type="text" value="0.000"/>	P700 <input type="text" value="0.000"/>	Fd <input type="text" value="0.000"/>
Rel. PC	Yield	Rel. Fd
Ox. <input type="text" value="0.000"/>	Y(I) <input type="text" value="0.000"/>	Red. <input type="text" value="0.000"/>
SP <input type="text" value="0.000"/>	Y(ND) <input type="text" value="0.000"/>	SP <input type="text" value="0.000"/>
Ratio	Y(NA) <input type="text" value="0.000"/>	Ratio
PC/P700 <input type="text" value="3.075"/>		Fd/P700 <input type="text" value="5.397"/>

Fig. 32: Table with Max values.

The next step is to determine the P700 and PC DMPs. The P700 DMP script has to be run first, because it contains a 60 s illumination event to activate the Calvin-Benson cycle. Apart from this activation element in the P700 DMP script, the order is not important, and these scripts also do not have to be modified in the case of gymnosperms or phytoplankton. However, it was reported to us that high (chloro)respiratory activity may cause complications.

4.9 Determination of the P700-DMP

If we start with the P700-DMP:

It is always good to click on the <NIR Bal.> button if the values in the green fields are not close to 0 anymore (e.g. Fig. 33):

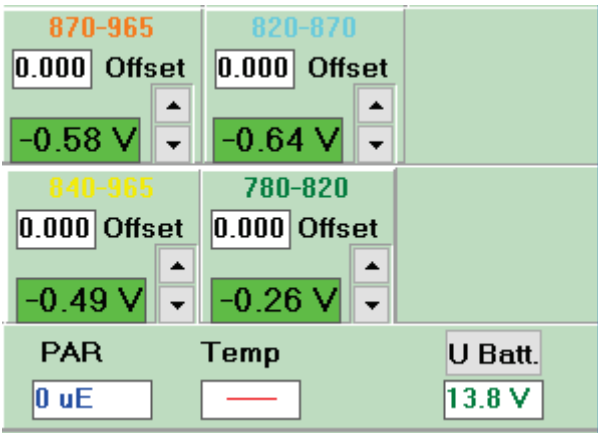



Fig. 33: Signal levels.
Balancing recommended.

The values shown here, really require a click on the <NIR Bal.> button. The values can be fine-tuned using the little arrows next to the green fields (up: more positive; down: more negative).

Now, click on the <Load> button, then navigate to the KLAS-100\Script Files\NIR_script directory and open P700_NIR_Ref.prg. This file is then imported into the script win-

dow. Close the script window by clicking on the  button or on the <cross>, both found in the upper right corner of the script window.

Click on <Run> to start the measurement. The progress of the measurement can be followed in the script window that pops-up when clicking on <Run> (Fig. 34).

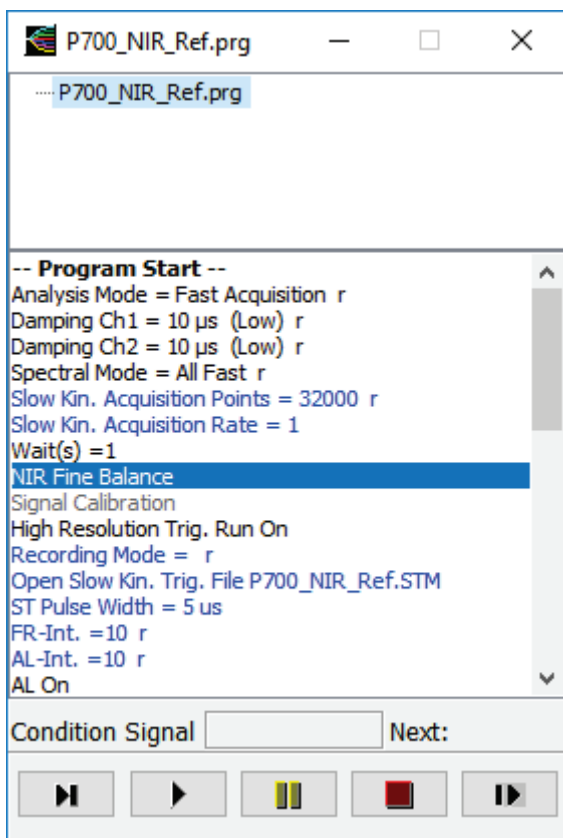


Fig. 34: Script file window.

Highlighted is the currently executed command.

When the measurement is finished, the software will automatically go to the 'Fit' window. Now the same procedure has to be followed as described for the Fd-DMP. For this particular script it is a good idea to have a look at the slow kinetics window (Fig. 35).

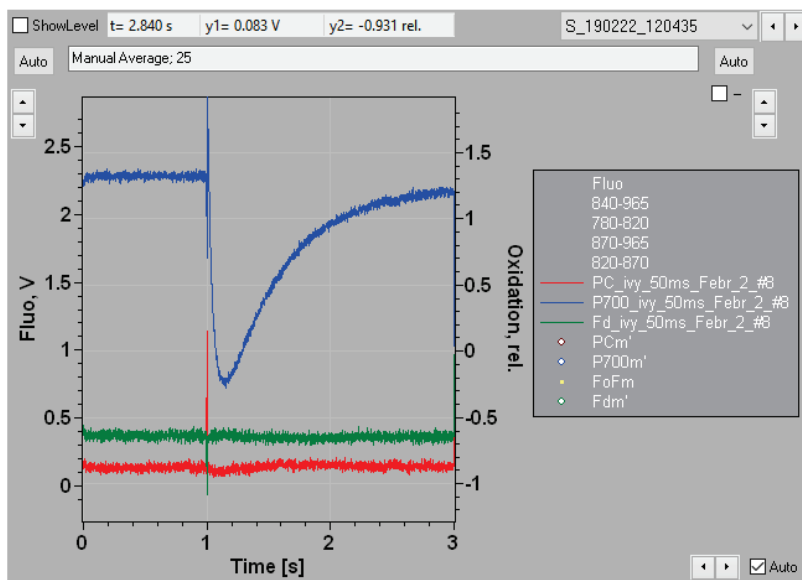


Fig. 35: Slow kinetics window.

In the slow kinetics window, the deconvoluted signals (based on the imported DMP files) of the P700-DMP measurement are shown. What is special is that the protocol has nearly no effect on the PC and Fd signals and that, therefore, choosing a time window with the purple lines is very easy (see Fig. 36). This nearly perfect separation is achieved by giving a 1 ms AL pulse on a background of relatively strong far-red light. The 1 ms AL pulse induces initially a transient oxidation of P700 which is followed by a broader transient reduction of P700 due to electrons generated in the PS II reaction centers by the 1 ms AL pulse.

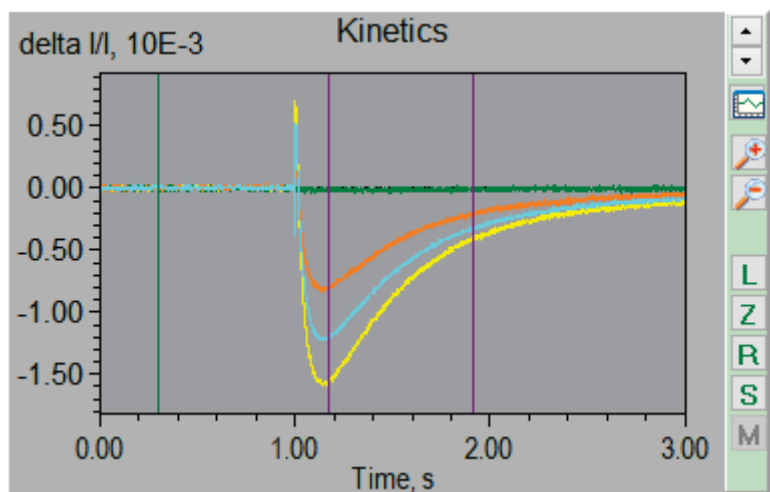


Fig. 36: Slow kinetics window.

Once the purple lines are positioned, click on the <Get Model Spectrum> icon above the 'Kinetics' window (see Fig. 24).

Then choose a blue color in the popped-up window and click <OK>.

Now four sets of numbers show up on the left-hand side. Double click on the third pair (the biggest number) and then click <OK> in the popped-up window to accept that this value is normalized to one and the other three are divided by the same number.

Then save the normalized numbers as for Fd as P700_*.KMS.

Making a comparison between different P700-DMP determinations (Fig. 37) demonstrates that these DMP-values are extremely reproducible:



Fig. 37: Comparison of P700 model spectra.

4.10 Determination of the PC-DMP

We repeat the same procedure for the PC-DMP:

Again, first go to the 'Slow Kinetics' window.

Click on the <NIR Bal.> button to bring the green values back to approx. 0 if necessary.

Then click on <Load> and find the PC_NIR_Ref file in the KLAS-100\Script Files\NIR_script directory. Open it, which imports it into the 'Script' window and then close the 'Script' window followed by clicking on the <Run> button.

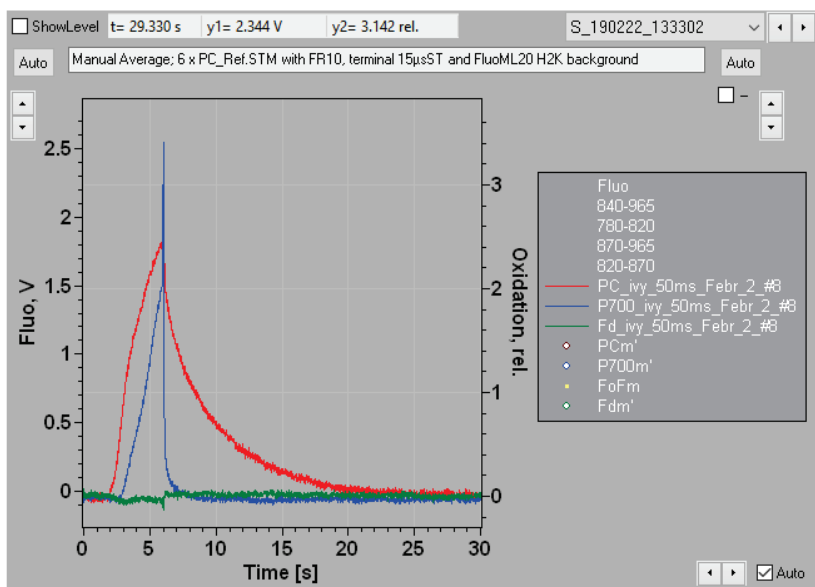


Fig. 38: Slow kinetics window.

The deconvoluted signal of the PC-DMP script demonstrates the principle behind this measurement (Fig. 38). With strong far-red light and an ST-flash P700, PC and Fd all are brought in the oxidized state. During the subsequent period of darkness Fd remains oxidized and P700 becomes re-reduced (by stromal electrons via PC) much faster than PC. Around 10 s into the script, the P700 transient is more or less flat and only changes in the PC redox state still occur.

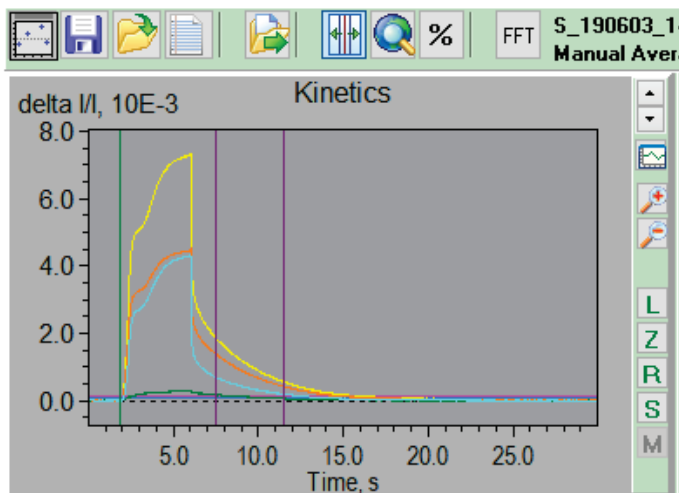


Fig. 39: Slow kinetics window.

This translates into the following position of the purple lines (Fig. 39). Then, again, the same sequence:

Click on the <Get Model Spectrum> icon above the 'Kinetics' sub-window.

Choose a red color.

Double click on the third pair of numbers.

Accept to normalize the third value to one.

Save the PC-DMP as PC_*.KMS.

Again, we make a comparison of different PC-DMP determinations:

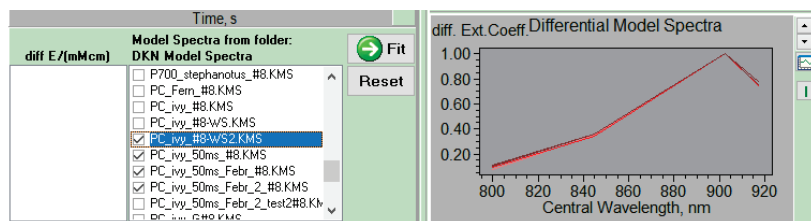


Fig. 40: Comparison of PC model spectra.

The PC-DMPs do not overlay on top of each other as perfectly as the P700-DMPs (Fig. 40), but they are still quite reproducible.

4.11 The fit-button of the Fitting window

Below the 'Kinetics' sub-window of the 'Fitting' window, there are two buttons: <Fit> and <Reset>. With the <Fit> button it can be tested how well the lines were positioned and how well the de-convolution works. In Fig. 41 and Fig. 42 this function is illustrated.

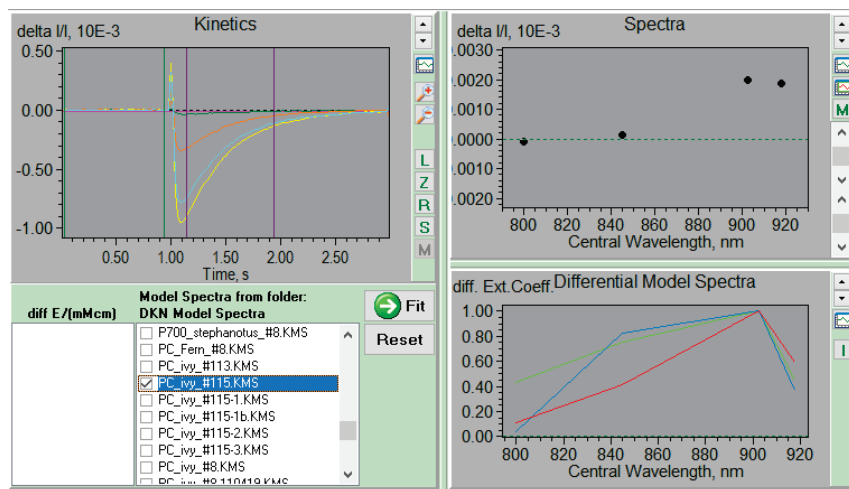


Fig. 41: Before clicking <Fit>, but after choosing DMPs.

As a first step Model Spectra (DMPs) for Fd, P700 and PC have to be chosen from the list (checked). Then click on the <Fit> button. This deconvolutes the P700-DMP signals (see Fig. 41).

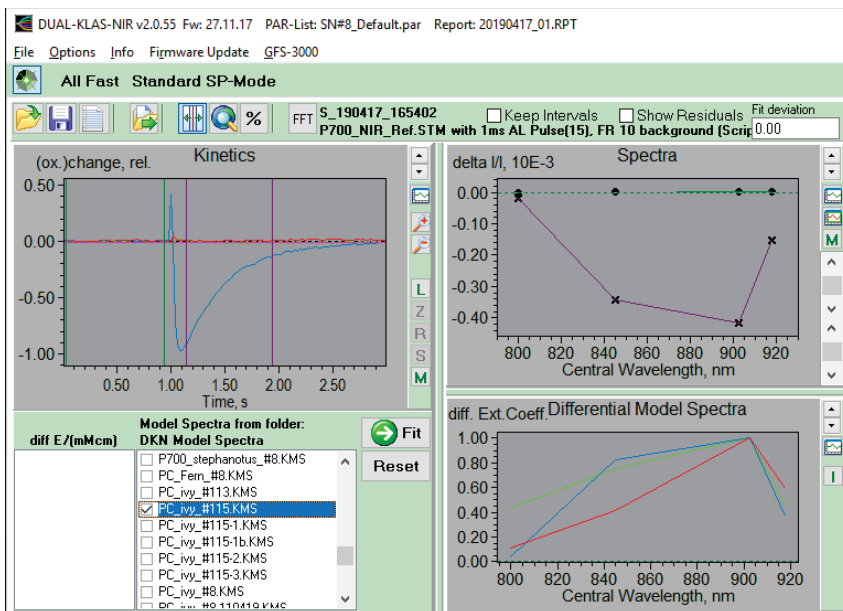


Fig. 42: The kinetics window following clicking on 'Fit'.

The deconvolution works well in this case. Between the purple lines only P700 changes occur. The P700 DMP script is expected to have mainly an effect on the P700 signal and the kinetics window shows that this is the case (Fig. 42). Clicking on <Reset> returns the window to the original un-deconvoluted state.

4.12 Importing a set of DMP files

To finalize the whole procedure, go to the 'Settings' window clicking on <Settings> underneath the graph window and then on <NIR Meas. light> above the graph window.

Then look at the ‘Deconvolution’ section, double-click on the field next to P700 and import the newly determined P700-DMP. Then double-click on the field next to PC and import the newly determined PC-DMP and finally double-click on the field next to Fd and import the newly determined Fd-DMP. With this new set of DMPs the Max-values have to be determined again for the ‘NIRmax’ measurement. To allow the user to associate a set of Max-values with selected records a new function was introduced in the ‘Deconvolution’ sub-window (Fig. 43 and Fig. 44).

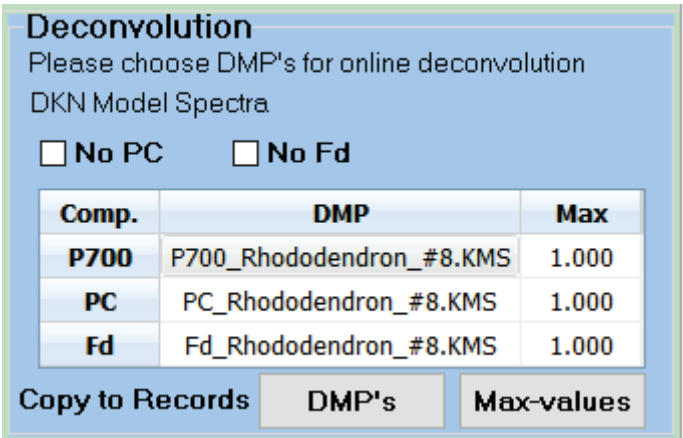


Fig. 43: The <DMP's> and <Max-values> buttons.

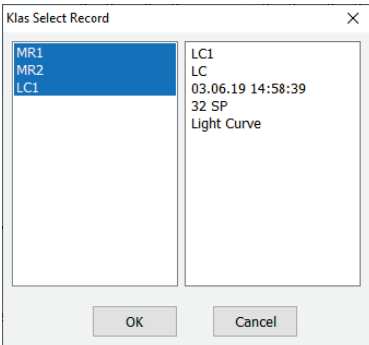
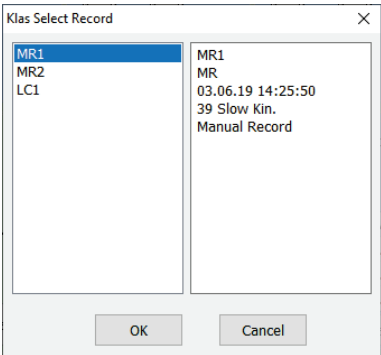


Fig. 44: Pop-up window showing up when either the <DMP's> or <Max-values> button is clicked. With the mouse the 'Records' to be associated can be selected.

After determining the DMPs, go to <Report> and select the first record of the active Report in which normally the NIRmax measurement can be found. Select the NIRmax measurement in the 'Slow Kinetics' window (either by clicking on the downward arrow next to the file name to activate a dropdown menu with the file names or on the left and right arrows next to the downward arrow with which it is possible to click through the list of files, see Fig. 45), then go via 'Settings' to the 'NIR meas. Light' window to import the new DMPs in the 'Deconvolution' sub-window as described above. Go back to the 'Slow Kinetics' window and click on the <Get Max-Values> button to get the Max-Values for P700, PC and Fd. I normally make a screenshot (alt Print) of the 'Slow Kinetics' window and paste it in 'Paint' to have a record of the Max-Values. Then go back to the 'NIR Meas. Light' window to associate the DMPs and Max-Values with the relevant records as shown in Fig. 43 and Fig. 44.

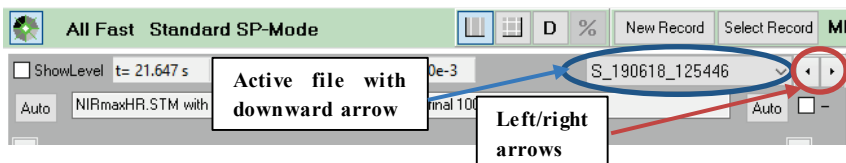


Fig. 45: Downward and left/right arrows with which a file from the active record can be selected.

The user is now ready to do measurements with his/her sample specific DMPs.

4.13 An alternative 'getting started' text

Automated routine for determination of the maximum transmittance changes caused by P700, PC and Fd in a giv-

en sample upon transition from the fully reduced to the fully oxidized state and vice versa.

Sample preparation: Dark-acclimated for at least one hour. In the case of light green leaves, signal quality can be significantly improved by putting two layers of leaf on top of each other, with the upper sides outside. However, in general adequate signals can be obtained with single layer lighter green leaves.

Note: Calibration (click the <Calib.> button in the 'Slow Kinetics' window) is required before running the NIRmax Script (i.e. one calibration per sample/measured area). Optimal deconvolution of the obtained data can be expected, if the Model Spectra (P700-DMP, PC-DMP and Fd-DMP) are measured with the same type of sample in the same optical geometry (i.e. single or double layer), ideally starting with Fd_NIR_Ref.prg right after the NIRmax measurement, i.e. while the sample is still dark-acclimated. [It should be noted that for quite a few plant species the NIRmax script will undo the dark-acclimation. In that case it is better to let the Fd-DMP determination precede the NIRmax measurement.]

Details of the Script:

After clicking the <NIRmax> button, the recording of the kinetics and all illumination steps are fully automated:

a) The transmittance changes are measured with the High-Resolution Slow Trigger file NIRmaxHR.STM. Approximately 30 s before this recording is started, a 100 ms MT is applied to ensure full reduction of PC, which at high settings of NIR-ML intensity may be partially oxidized in the "dark".

b) At the beginning of the Slow Kinetics the sample is illuminated for 3 s at AL10, on top of which a 30 ms MT is applied, leading to full reduction of Fd, because the reactions downstream of Fd are dark-inactivated. 10 s after the start of the recording FR18 is switched on for 10 s and simultaneously with FR-off a 100 ms

MT is applied, which induces a transient full oxidation of both P700 and PC.

c) After the recording, the deconvoluted P700, PC and Fd signals are autoscaled.

For the final step of the determination of the maximum transmittance changes the <Get Max[-Values]> button has to be pressed.

Then the 100% oxidation and reduction levels are automatically determined and the amplitudes of 100% redox changes calculated. These values are entered into the DKN Model Spectra table under 'Settings/Deconvolution' and thereafter used for the % oxidation display of the deconvoluted kinetics.

Note: The 100% values depend on the correctness of the Model Spectra applied for deconvolution.

Upon clicking the <Get Max[-Values]> button, based on the obtained Max-values and assumptions on the relative molar extinction coefficients of P700, PC and Fd in the 780-965 nm range, the molar ratios of PC/P700 and Fd/P700 are calculated and displayed.

It is also possible to determine separately Maximum Fd and Maximum P700 plus Maximum PC making use of the <Max.> buttons (NIR panel). This alternative approach may be advantageous, as the optimal conditions for Fd on one hand and for P700 plus PC on the other differ from each other. While Maximum Fd determination requires thorough dark acclimation (dark inactivation of the reactions downstream of PS I), Maximum P700 plus PC determination benefits from activated reactions beyond the PS I acceptor side, as encountered after pre-illumination.

Automated routine for determination of the Differential Model Plot (DMP, 'Model Spectrum') of ferredoxin, Fd.

Sample: Either dark-green leaf (like ivy) or two leaf layers with lower chlorophyll content (adaxial sides outside, so that both upper leaf sides are directed towards the perspex light guides). [It should be noted here that also for lighter leaves decent signal intensities and qualities can be obtained working with a single leaf layer.]

Sample pretreatment: Dark-acclimated for at least one hour; signals must be calibrated (click on the <Calib.> button in the 'Slow Kinetics' window [at the beginning of the measurement series]). The Fd-DMP normally is measured before the P700-DMP and PC-DMP, as the latter are determined after pre-illumination of the sample.

Rationale: Dark-acclimation causes inactivation of the reactions downstream of Fd. Hence, Fd, PC and P700 can be almost completely reduced by a short (600 ms) pulse of light, after which the re-oxidation of Fd is slow. Since P700 and PC remain reduced in darkness, their redox states do not change following the pulse of light. If some activation of the reactions at the acceptor side of PS I occurs and there is some oxidized PC at the end of the pulse of light, electrons from the (partially) reduced PQ-pool will re-reduce these PC molecules within 100-200 ms of darkness. Transmittance changes observed at times longer than 200 ms after the light pulse may be considered selective for Fd.

Possible problem: The main problem that may affect the Fd-DMP determination is a fast activation of the PS I acceptor side leading to fast re-reduction kinetics of Fd, making it difficult to define a Fd-specific window. Another problem has to do with the FR-effect of the NIR measuring light. During longer term dark-acclimation the rate of dark reduction of the intersystem electron

transport chain via NDH declines. As a consequence, it may no longer compensate the FR-effect of the NIR measuring light and this may lead to a partial oxidation of PC. It can be reversed by a pre-illuminating MT. In addition, the Zero-interval for the Fd-DMP is defined at the end of the recording, where PC may be assumed to be fully reduced.

Details of the Script:

- a) First a 10 ms MT is applied, by which that part of PC is reduced that had been slowly oxidized by the pulse-modulated NIR measuring light during the long preceding dark period.
- b) 30 s later the first High Resolution recording of the Triggered Run Fd_NIR_Ref.STM is started, which features a 600 ms AL pulse (at intensity setting 13) and the recording of the subsequent redox changes over a 3.4 s period.
- c) This measurement is repeated ten times and then the 10 recordings are averaged.
- d) The averaged response is transferred to the 'Fitting' window in order to derive the Fd Model Spectrum. Interval 1, over which the averaged signal points are automatically zeroed, is defined at the end of the recording (4.4 to 4.9 s). Interval 2, in which the signals are assumed to display Fd-selective differences with respect to Interval 1, is defined at 1.8 to 2.4 s [i.e. 200 to 600 ms after the end of the pulse of light (see Fig. 21)]. However, the interval that can be used for the Fd-DMP depends strongly on the Fd-reduction rate and may be considerably shorter than 400 ms.

All further steps have to be carried out manually:

Once the two Fitting Intervals are defined, calculation of the Fd Model Spectrum can be carried out manually by clicking the <Get Model Spectrum> icon [at the left-hand side of the <Save> icon above the <Kinetics> display (see Fig. 24)]. After selecting a

color for display, a list of the calculated differential extinction coefficients appears at the left side (under <Kinetics> display, see Fig. 26). For normalization, the largest value (normally central wavelength 902.50 nm) is double clicked and a preset normalization value of 1 is confirmed (see Fig. 27). Thereafter, the new Fd Model Spectrum is displayed. For further use, it has to be saved in a .KMS file. Together with this file a text file is saved, which contains details on data origin and Interval-definition. The text may be edited and extended by the user.

Automated routine for determination of the Differential Model Plot (DMP, 'Model Spectrum') of P700.

Sample: Either dark-green leaf (e.g. ivy) or two layers of leaf with lower chlorophyll content (adaxial sides outside, so that upper leaf sides are directed towards perspex light guides). [Again, also single leaf layers of less dark green leaves give adequate signals.]

Sample pretreatment: Preferentially light acclimated sample (however, the Script also provides some light activation: 60 s AL 10); signals must be calibrated (click the <Calib.> button in the 'Slow Kinetics' window at the beginning of the measurement series). The Script normally is started after running the Script for Fd-DMP measurement and a 5-10 min pre-illumination at moderate AL (setting 8-10).

Rationale: Far-red illumination causes almost complete oxidation of PC, strong oxidation of P700 and minor reduction of Fd, because re-oxidation of Fd occurs during the FR-light pulse. When in this state a 1 ms pulse of strong AL is applied, PS II pumps electrons with high quantum efficiency to PS I, whereas quantum efficiency of PS I is low (most P700 is oxidized). While some of the P700 that was not oxidized by the FR will be oxi-

dized by the AL-pulse, this will be rapidly (with a delay of about 20 ms) re-reduced by PS II action. Simultaneously with an AL-pulse induced P700 oxidation some Fd will be reduced which, however, may be expected to be rapidly re-oxidized via the light-activated reactions downstream of Fd. Hence, the AL-pulse induced transmittance changes are strongly dominated by rapid PS II driven reduction of P700, followed by slower P700 re-oxidation driven by the FR background light. The transmittance changes at times longer than 100 ms after the AL-pulse may be considered to be exclusively due to P700 reduction.

Details of the Script:

- a) First the sample is pre-illuminated for 1 min with AL (intensity setting 10).
- b) 15 s thereafter FR background illumination (intensity setting 10) is started
- c) 20s thereafter the first High Resolution recording of the Triggered Run P700_NIR_Ref.STM is started, which features a 1 ms AL-pulse (at intensity setting 15) and the recording of the following relaxation kinetics over a 2 s period.
- d) This measurement is repeated 25 times and then the 25 recordings are averaged.
- e) The averaged responses are transferred to the 'Fitting' window in order to derive the P700 Model Spectrum. Interval 1 is defined for the baseline preceding the AL-pulse (started at 1 s) and then automatically zeroed. Interval 2, in which the signals are assumed to display P700-selective differences with respect to Interval 1, is defined at 1.1 (close to the signal peak) to 1.4 s.

All further steps have to be carried out manually:

Once the two Fitting Intervals are defined, calculation of the P700 Model Spectrum can be carried out manually by clicking

the <Get Model Spectrum> icon [at the left-hand side of the <Save> icon above the <Kinetics> display (see Fig. 24)]. After selecting a color for display, a list of the calculated differential extinction coefficients appears at the left side (under the <Kinetics> display, see Fig. 26). For normalization, the largest value (normally central wavelength 902.50 nm) is double clicked and a preset normalization value of 1 is confirmed (see Fig. 27). Thereafter, the new P700 Model Spectrum is displayed. For further use it has to be saved in a .KMS file. Together with this file a text file is saved, which contains details on data origin and Interval-definition. The text may be edited and extended by the user.

Automated routine for determination of the Differential Model Plot (DMP, 'Model Spectrum') of plastocyanin, PC.

Sample: Either dark-green leaf (like ivy) or two layers of leaf with lower chlorophyll content (adaxial sides outside, so that upper leaf sides are directed towards perspex light guides). [Single leaf layer measurements will also give adequate signals for less dark green leaves if the FR-intensity is set to 20 (maximum value) and the number of measurements is increased to 10.]

Sample pretreatment: Light-activated sample; signals must be calibrated (click the <Calib.> button in the 'Slow Kinetics' window at the beginning of the measurement series). The script can be started directly after running the script for P700-DMP measurement.

Possible problem: At high intensity settings the FR effect of the NIR measuring light may lead to partial oxidation of PC via PS I action. The latter can be effectively counteracted by the application of 540 nm fluorescence measuring light (Fluo ML at maximum intensity), which keeps PC reduced during the "dark" period preceding FR application.

Rationale: When a light-activated sample is illuminated by strong FR, this leads to almost complete oxidation of PC, partial oxidation of P700 and minor Fd changes. The electrons set free in PS II by a 15 μ s ST end up almost exclusively in P700, which has a much higher oxidation potential than PC. Hence, when the 15 μ s ST is applied at the end of a 4 s FR illumination, P700 is rapidly re-reduced, whereas re-reduction of PC is much slower, due to the fact that reduced PC can only accumulate after reduction of P700, because several PC molecules per P700 have to be reduced before full reduction is reached and finally because this process depends on non-photochemical reduction of the PQ-pool. Any changes related to Fd should be reversed within a couple of seconds. Therefore, the light induced changes of transmittance observed 2-6 s after the FR pulse may be considered selective for PC.

Details of the Script:

a) First 540 nm Fluo ML (intensity setting 20, MF-High) is switched on, which serves to fully reduce PC, without changing the redox states of P700 and Fd. The weak green light counteracts the weak PS I action of the pulse-modulated NIR measuring light, which tends to slowly oxidize PC in the dark.

b) About 1 min later the first Slow Kinetics recording of the Triggered Run PC_NIR_Ref.STM is started, which features 4 s FR illumination (at intensity setting 10), a 15 μ s ST upon FR-off and recording of the relaxation kinetics over the following 24 s period.

c) This measurement is repeated six times and then the 6 recordings are averaged.

d) The averaged responses are transferred to the 'Fitting' window in order to derive the PC Model Spectrum. Interval 1 is defined for the baseline preceding the start of FR-illumination (at 2 s) and then is automatically zeroed. Interval 2, in which the sig-

nals are assumed to display PC-selective differences with respect to Interval 1, is set between 8 and 12 s, i.e. starting 2 s after FR-off.

All further steps have to be carried out manually:

Once the two Fitting Intervals are defined, calculation of the PC Model Spectrum can be carried out manually by clicking the <Get Model Spectrum> icon [at the left-hand side of the <Save> icon above the <Kinetics> display (see Fig. 24)]. After selecting a color for display, a list of the calculated differential extinction coefficients appears at the left side (under <Kinetics> display, see Fig. 26). For normalization, the largest value (normally central wavelength 902.50 nm) is double clicked and a preset normalization value of 1 is confirmed (see Fig. 27). Thereafter the new PC Model Spectrum is displayed. For further use it has to be saved in a .KMS file. Together with this file a text file is saved, which contains details on data origin and interval-definition. The text may be edited and extended by the user.

4.14 Exponential fits of selected sections of the measurement

Starting v65 of the software, several new functions are hidden underneath the <Get Max> button (formerly <Get Max-values> button). Clicking on the small arrow on the right-hand side of the <Get Max> button will make a short dropdown menu visible with the following options: <Get Max>, <Exp. Fit>, <Get Fd-RCF> and <Get Poolsize>. With the <Exp. Fit> option it is possible to fit a user-defined section of a measurement (either P700, PC or Fd signal). Fig. 46 gives an example of such a fit. The same <Exp. Fit> option can be found in the 'SP Kinetics' window in the dropdown menu of the <Recalibrate> button. See section 4.32

for a discussion of the <Get Fd-RCF>, <Get PC-RCF> and <Get Poolsize> functions.

In Fig. 47 the pop-up window that shows up following clicking on <Exp. Fit> is shown.

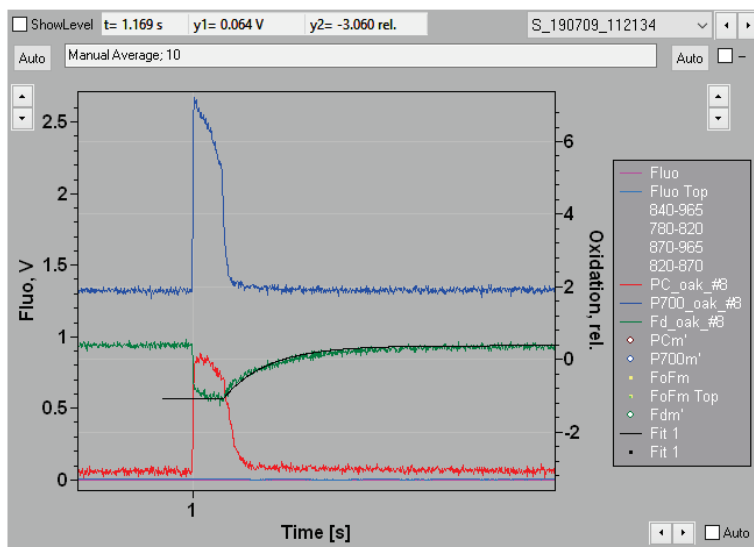


Fig. 46: Exponential fit of the re-reduction kinetics of Fd following a short MT pulse applied to a needle of a gymnosperm tree.

Fig. 47 gives the parameter menu on the basis of which the measurement is fit. In Fig. 47 under 'fit settings' the Fd-signal was selected for analysis. In the upper right-hand side, the number of exponentials with which the signal has to be fit has to be indicated. Here, one exponential was chosen. Very important is to select <Begin-Level manually>. There, the y2-value the users wants to use as a starting point for the fit can be indicated. Go with the mouse to this start point, note the y2 value above the Slow Kinetics graph window and use it as an input for the parameter list. The script applied consisted of 1 s without actinic light followed by a 50 ms MT.

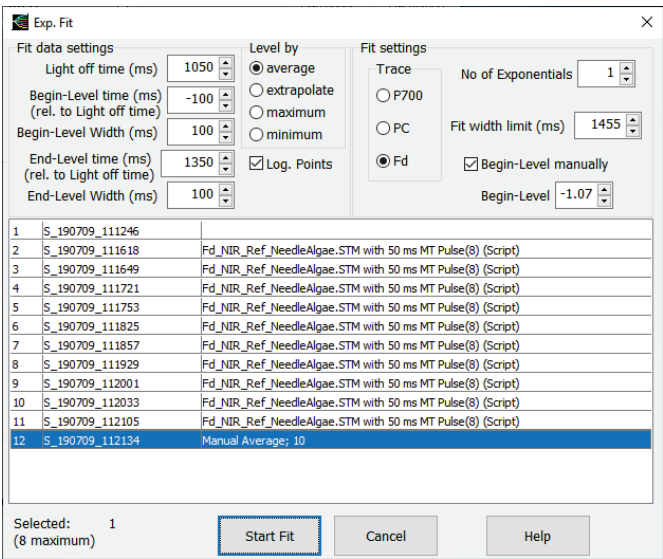



Fig. 47: Popup window showing up after <Exp. Fit> is clicked.

Therefore, lights off occurred at 1050 ms. The end point of the fit can again be determined by putting the mouse on the wished for end point and using this time point (minus 1050 ms) as input for the parameter sheet. For the width values each time the smallest value was chosen (either positive or negative). Then the measurement to be analyzed was selected (here, the average of 10 measurements for the determination of the Fd-DMP). Once values for all fitting parameters have been added to the sheet, the <Start Fit> button can be clicked, and the fitting procedure will start. The result is the addition of a fit curve to the figure (Fig. 46) and another pop-up window with the fit results (Fig. 48).

Exp. Fit

Nr.	Filename	Comment	Low I	High I	Trace	PAR	A(tot.)	A0	A1	A2	A3	Tau1	Tau2	Tau3	Error
		dim.	10e-3	10e-3		$\mu\text{E}/\text{m}^2\text{s}$	Volt	%	%	%	%	ms	ms	ms	rel.
1	S_190708153810	Manual Average: 10	-1.080	-0.522	Fd Raw	0	0.558	0.0	100.0			382.08			2.2

OK



creases in this wavelength interval for both plants by 15-20%. The PC signal is smaller than the P700 signal and the effective contamination of the P700 signal will be 10-15%. This would be the contamination without deconvolution.

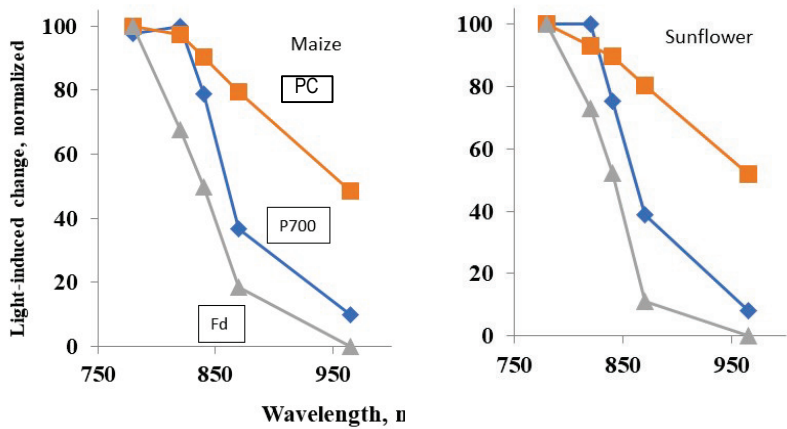


Fig. 49: Absorption spectra of PC, P700, and Fc.

For PC the wavelength pair 870-965 was chosen. PC (orange) is by far the most difficult component to resolve. Its absorption decreases gradually beyond 820 nm. Between 870 and 965 nm PC absorption decreases by about 35% for both plants. At the same time P700 decreases by ~30% in both plants and the Fd absorption decreases by 10-15%. Without deconvolution the PC-signal would be heavily contaminated by P700, but still, it is the best wavelength pair we have. The spectra of the two plants further show that there is spectral variability between plants. In addition, the spectra also make clear that there is little difference between 870-965 nm and 870-950 nm (both 965 and 950 nm LEDs have been used so far) with respect to a deconvolution strategy.

4.16 Measurement strategy and time resolution of DUAL-KLAS-NIR measurements

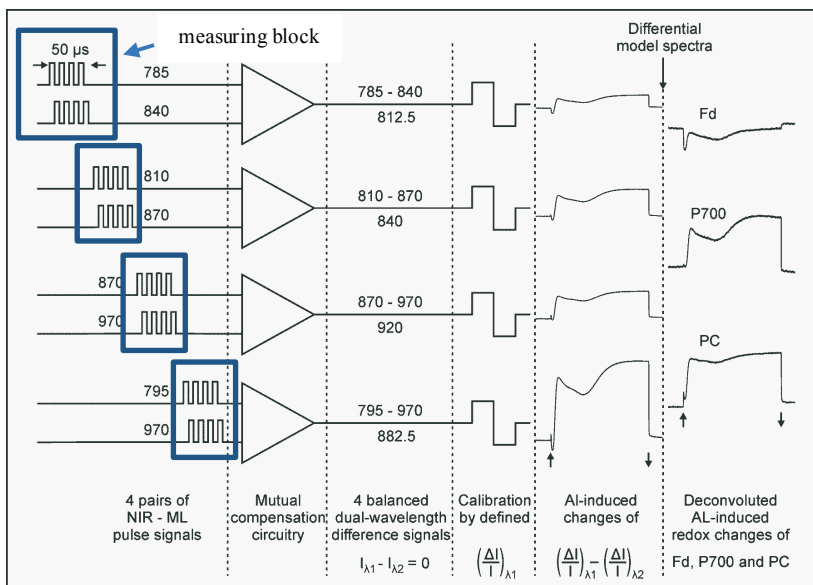


Fig. 50: Scheme outlining data acquisition and deconvolution.

The measuring strategy for the DUAL-KLAS-NIR is the same as for the DUAL-PAM-100. All channels used are measured sequentially. Each wavelength pair is measured for 50 μs. This is called a block (see Fig. 50). As more channels are measured, the time needed to go through a whole cycle increases and the time resolution of the measurement decreases. However, for single channel measurement the signal is measured continuously (no blocks) and a time resolution of ~35 μs is achieved. For a dual channel (block) measurement, the time resolution is ~150 μs and for a full six-channel measurement the time resolution goes down to ~1 ms, where it is assumed that the time resolution equates two cycles, with each cycle taking 500 μs.

4.17 Light Curves

The DUAL-KLAS-NIR software offers a special window for Light Curve measurements: the 'Light Curve' window. A light curve means that a sample is illuminated for a defined time at a particular light intensity after which the light intensity is either increased or decreased one step.

On the right-hand side of the graph window, the <Edit> button can be found. Clicking on this button opens a pop-up window in which the length of the illumination steps (6 = 60 s, 12 = 120 s) can be defined and the user can further indicate, which light intensities of the PAR lists should be included in the Light Curve measurement.

Once this is done, the pop-up window can be closed, maybe a last click on <NIR Bal.> and the experiment can be started by clicking on the <Start> button.

The progress of the measurement can be followed on the screen of the 'Light Curve' window where, after each light level, selected parameters, e.g. $Y(I)$, $ETR(I)$, $Y(II)$ and $ETR(II)$, are calculated and added to the plot.

Once the measurement is finished (Fig. 51), the light intensity dependencies of the parameters $ETR(I)$ and $ETR(II)$ can be fit with either the Eilers-Peeters model or by the model of Platt et al. (click on either the <EP> or <Platt et al.> button and then click on the <Fit> button).

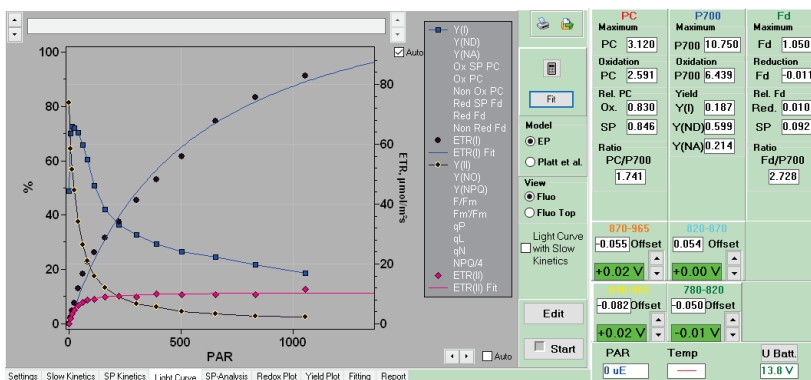



Fig. 51: Light Curve window.
ETR(I) (blue) and ETR(II) (pink) were fit by Eilers and Peeters.

Once the data are fit, the fit and parameter data can be exported clicking on the  button and providing a file name. The data are then normally saved to the KLAS-100\Export folder. The user can, however, select his or her own folder for this purpose.

It is also possible to measure the light curve up and down or down and up. Click on <Edit> and manually create the following list of light intensities: 1-2-3-4-5-6-7-8-9-10-9-8-7-6-5-4-3-2-1 or 2-4-6-8-10-12-14-16-18-20-18-16-14-12-10-8-6-4-2 if the high light intensities are important, or 10-9-8-7-6-5-4-3-2-1-2-3-4-5-6-7-8-9-10 if the user wants to study the reversibility of the light adaptation process (Fig. 52).

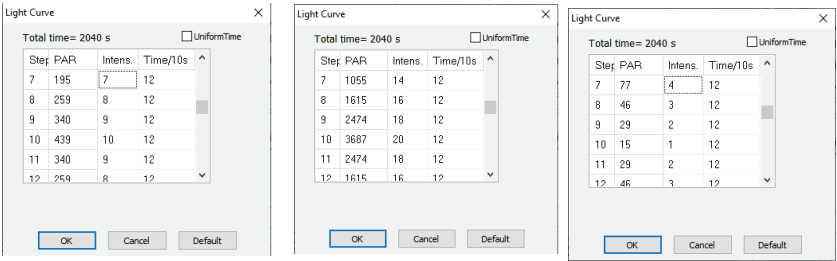


Fig. 52: Different types of protocols for Light Curve experiments

In the ‘SP-Analysis’ window many fluorescence and absorption parameters can be plotted to get a feeling for the relationships between different parameters and processes.

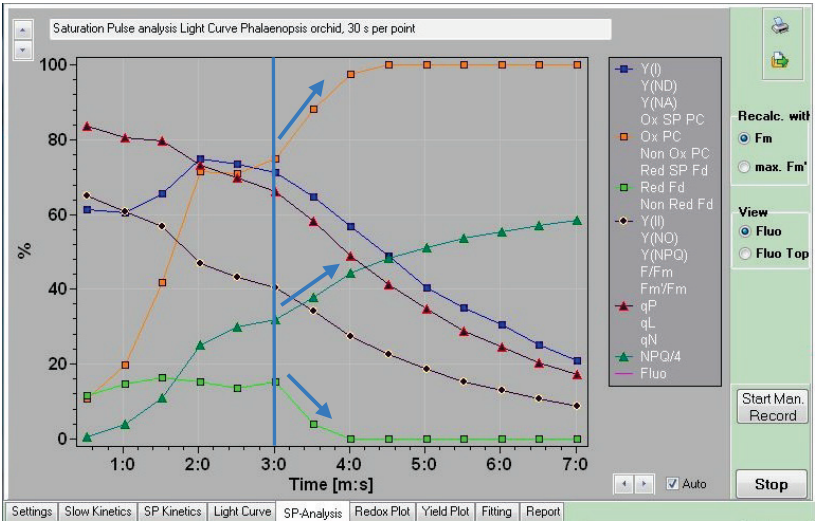


Fig. 53: SP-Analysis window.

Fig. 53 shows that after 3 min of illumination (after the 6th light intensity, $\sim 80 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) of the leaf measured a further increase of NPQ is paralleled by a further oxidation of PC and Fd

suggesting a decrease of the lumen pH and stronger photosynthetic control (lower re-oxidation rate of PQH₂ by the cyt b6/f complex).

With the DUAL-KLAS-NIR the actinic light intensity can be increased all the way up to $\sim 3500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, but to go through all 20 light steps may in most cases not be a good idea. It takes a lot of time, provides little additional information and in practice only leads to extensive photoinhibition of the sample. As a rule of thumb, for green algae, etc. a maximum light intensity of $\sim 500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ will in most cases be enough, for leaves acclimated to a moderate light intensity $\sim 1000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ will in most cases be enough and for sun leaves even higher light intensities will still be reasonable.

The other parameter to consider is the length of the light steps. Light Curve measurements were originally developed for studies of the steady state. Using 10 s steps will keep the sample far from steady state and the observed pattern will mainly say something about the rapidity with which plants can adapt to a new situation. If the user would choose 2 min light steps, he/she would probably come already quite close to steady state and more information can be extracted from the data. We would suggest the user to use a step length of at least 30 s, preferably longer. Especially for short step lengths it is important to pre-activate photosynthesis (e.g. give a 2 min $100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ pre-pulse) to avoid interference of photosynthetic induction with the Light Curve measurements.

4.18 Quenching Analysis

Also, the traditional quenching analysis can be carried out without too much trouble.

Go to the ‘Slow Kinetics’ window. To the right-hand side of the graph window, there is a button with a draw down menu that could read <Manual> (the default value). Click on this button and this will give a choice between <Manual>, <Ind. Curve>, <Ind. + Rec.> and <Trig. Run>.

The <Ind. Curve> option only records the kinetics of the induction of photosynthesis, with the goal to reach the steady state. The <Ind. Rec.> option does the same thing but continues measuring for a while in darkness after the actinic light is switched off. With the option <Trig Run> a previously recorded <Manual> run can be repeated in detail (see below).

Let’s choose the <Ind. Rec.> option.

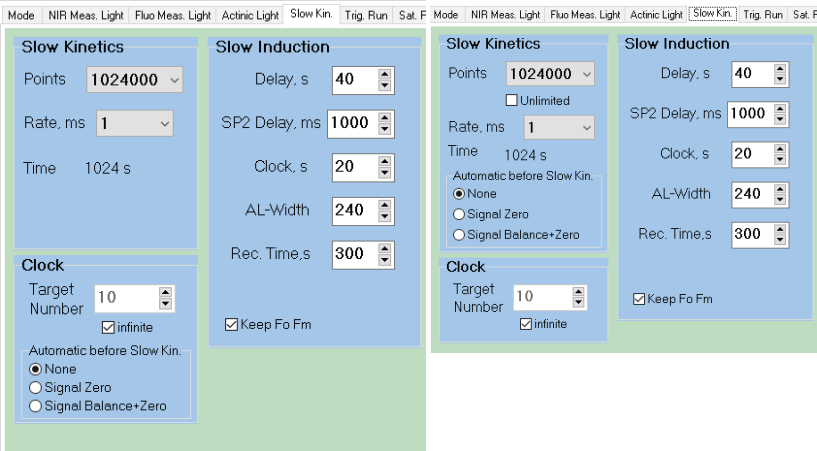


Fig. 54: Slow Kinetics Settings, with the layout starting v65 on the right-hand side.

To define the details of the quenching analysis protocol, click on the ‘Settings’ window at the bottom and then ‘Slow Kin.’ on top. In this window several choices have to be made (Fig. 54).

In the ‘Slow Induction’ section, the AL-Width may for example be 240 s and Rec. (= Recovery) time may be 300 s. These two

numbers together mean that the protocol will run for at least 540 s.

If we would then choose the maximum rate of 1 ms between points, we would need at least 540000 points and this requirement is only met if we choose in the <Points> drop down menu the maximum number: 1024000.

Instead of 1 ms, it is also possible to choose a rate of 2, 5 or 10 ms. As the rate is decreased, the number of points needed will also decrease: 128000 points at 5 ms (640 s) is also enough to run the protocol.

Starting v65 an <Unlimited> box is added underneath <Points>. Checking this box allows recording of an unlimited number of points.

In addition, the three options (<None>, <Signal Zero>, <Signal Balance + Zero>), to be carried out at the start of the measurement have been moved from the 'Clock' sub-window to the 'Slow kinetics' sub-window.

If fewer points are chosen than needed to run the <Ind. Rec.> or <Ind. Curve> protocol, the software will give a warning and will refuse to run.

In the manual of the DUAL-PAM-100 details are given for this protocol.

If we look at the slow induction section:

The <Delay, s> gives the time between the first 'P.+F. SP' and the turning on of the actinic light. The default value is 40 s. The range is 5-600 s.

The <SP2 Delay, ms> is the time between the moment the actinic light is turned on and the moment the second 'P.+F. SP' is triggered. The default value is 1000 ms and the range is 100-

2000 ms. The position of this pulse should more or less coincide with the position of P(eak).

<Clock, s> gives the time between subsequent saturation pulses. The default value is 20 s, which gives a high time resolution for NPQ and qP changes, but I could also imagine that the user takes a value of around 100 s.

<AL-Width> gives the time the actinic light is on plus the delay between the first saturation pulse and actinic light on. The default value is 240 s, which is 40 s delay + 200 s actinic light. The range is 10-600 s. The user should see to it that the math is right, because <AL-Width> is not automatically adapted to the new reality. It is also not possible to make use of the whole range: <AL-Width> = 10 s is numerically possible, but from a protocol point of view is not (theoretical minimum is 15 s).

The <Rec. Time, s> field defines the time in darkness during which the instrument keeps measuring after the actinic light was turned off. The default time is 300 s. The range is 10-600 s. During the illumination phase the time between pulses can be kept constant. During the recovery phase this issue is more complicated. A clock time of 20 s is maybe good enough for the relaxation of a large part of qE but may perturb further dark-relaxation. One option is to choose e.g. a clock time of 100 s between pulses, but in that case, there is zero time resolution with respect to the relaxation of qE. For the DUAL-PAM-100 and the DUAL-KLAS-NIR a different strategy is chosen. The interval between pulses is continuously increased. The first pulse is given 15 s after turning off the actinic light and then the subsequent pulses are given, respectively, 18 s, 22 s, 25 s, 31 s, 37 s, 44 s, 53 s, 64 s, 76 s, 92 s, 110 s, 132 s, 158 s after the previous saturation pulse. This yields a maximum relaxation time of about 15 min.

At the bottom of the 'Slow Induction' section there is <Keep Fo Fm>. When this field is checked, the software will use the Fo and Fm values of the first saturation pulse for the calculation of for example qN and NPQ of all subsequent saturation pulses.

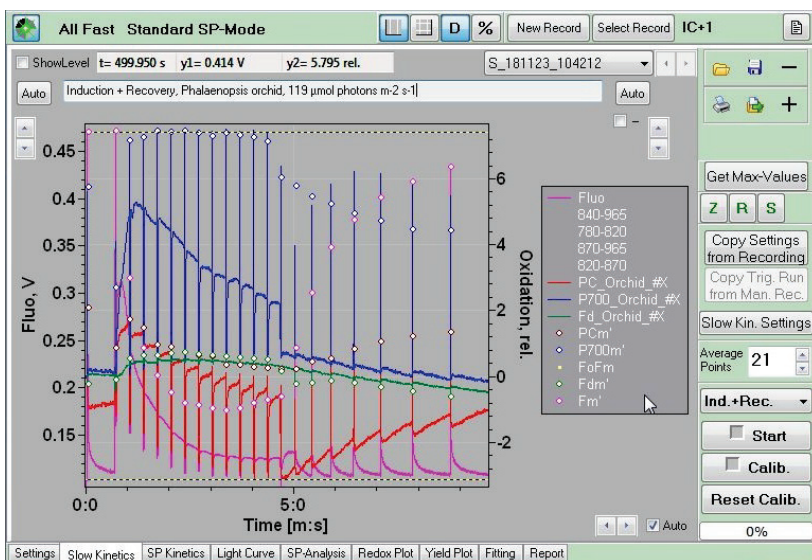


Fig. 55: Induction/Recovery Experiment.

In the 'Clock' section there are two options. It is possible to check <Infinite> in that case the <Target [P. + F. SP] Number> will adapt to the length indicated in the protocol. Unchecking <Infinite> allows the user to set a <Target Number>, but in that case the <AL-Width> time (10 x clock interval + e.g. 40 s) will have to be adapted to that number.

An example of such a default <Ind. Rec.> measurement is shown in Fig. 55.

4.19 Manual Recording

With the <Manual> recording option, pushing on <Start> means that the instrument starts recording and that the user then manually can turn on the measuring light, actinic light, can give pulses etc., just as the user feels is convenient. The problem with a manual recording may be the reproducibility. Repeating a Manual run may give differences in the timing. The DUAL-KLAS-NIR has a solution for this problem. After the Manual recording has been ended by clicking on the <Stop> button, the user then has to click on <Copy Trig Run from Man. Rec.> and save the file with all the necessary information on the Trigger pattern, etc. These data can then later be used to repeat the Manual recording in detail (as a <Trig. Run>).

4.20 The Pulse Width Window

One of the better hidden windows in PAM software in general and the DUAL-KLAS-NIR as well is the Pulse Width window. It can be made visible by a right mouse click on the <AL Pulse>, <FR Pulse>, <BL pulse>, <TR Pulse> or <ST> buttons. However, in the case of the DUAL-KLAS-NIR, the 'Pulse Width' window can also be accessed via the Actinic Light Window where a <Pulse Widths> button can be found. The settings in this window are only active in the <SP Analysis> mode and do not affect measurements in the <Fast Acquisition> mode. A click with the left mouse button will trigger a pulse with a duration as defined in the 'Pulse Width' window. Given that the default values are rather short it is important to be aware of the settings in this window. The default <AL> and <FR/BL Pulse Widths> are 1 ms (range 1-1000 ms), the <TR Pulse Width> is 10 μ s (range 10-250 μ s) when the <Sub ms> field is checked and 1 ms (range 1-1000

ms) if that is not the case. For the <TR Pulse Width>, it is also possible to check a <S+H off> field. The default <MT Pulse Width> is 50 ms (range 1-300 ms). For the <ST Pulse Width>, it is 5 μ s (range 5-50 μ s), with the <S+H off> field checked and a 5 μ s extended <S+H off> time (range 5-100 μ s).

An ST (single turnover flash) has the potential to strongly perturb a measurement. To minimize/eliminate this perturbation the instrument does not measure during the flash. This is called S+H (Sample & Hold) off. The time interval during which the instrument stops measuring can be defined by the Extended S+H off time.

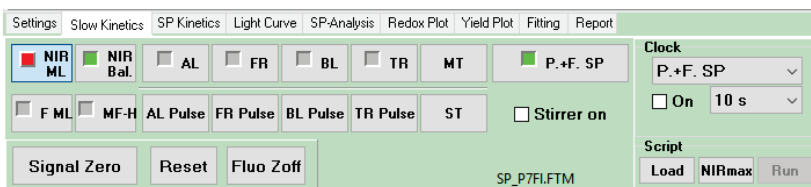


Fig. 56: Bottom panel.

The Bottom panel (Fig. 56) contains a set of buttons for the triggering of different light sources.

<NIR ML> – Clicking on this button turns the near-infrared (NIR) measuring light on. Clicking on the neighboring <NIR Bal.> button also turns the NIR ML on. Only when the NIR ML is on, absorption changes can be measured.

<NIR Bal.> – The function of this button has been treated above. Clicking on this button balances the NIR ML, which in practical terms means that the four ‘green’ (sometimes ‘red’) fields in the right-hand panel go to values close to zero.

<AL> – Actinic light, refers to the red-light source in the emitter head of the DUAL-KLAS-NIR; clicking on this button will turn the actinic light on. The intensity of this light is defined in the ‘Set-

tings/Actinic Light' window. If in that window the Width is <man.> (manual) it means that the actinic light remains on until it is manually turned off, or until a command in a script turns it off. If the hole in the <AL> button is red, the AL light is on.

<FR> – Far-red light; clicking on this button will turn the far-red light on. The intensity of the FR light is defined in the 'Settings/Actinic Light' window. If, in that window, the Width is <man.> (manual) it means that the far-red light remains on until it is manually turned off, or until a command in a script turns it off. The <man.> setting is particularly important if the user wants to make use of background FR light. If the hole in the <FR> button is red, the FR light is on.

<BL> – Blue light, refers to the blue light source in the Detector head of the DUAL-KLAS-NIR (range 0~400 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$); clicking on this button will turn the blue actinic light on. The intensity of the blue light is defined in the 'Settings/Actinic Light' window. If in that window the Width is <man.> (manual) it means that the blue light remains on until it is manually turned off, or until a command in a script turns it off. If the hole in the <BL> button is red, the BL light is on.

<TR> – <Trigger> button; in the absence of external devices the TR function is 'on' in combination with the fluorescence measuring light (<F ML>) and the <MF-H> button which increases the measuring pulse frequency of F ML when the actinic light is triggered.

MT – Multiple Turnover pulse; MT is like a saturation pulse, but its purpose is not to reach F_m ; it may for example be 50 ms, instead of 200-300 ms, the minimum length for an SP pulse. Its intensity is defined in the Settings/Sat. Pulse window (range ~1000~25000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) and its length in the Pulse Width window.

<P.+F. SP> – Saturation Pulse whose purpose is not only to determine F_m , F_m' , but as well the maximum absorption change (between minimum and maximum) of P700 (and PC). It consists of a 10 s FR-illumination followed by an SP. The FR-illumination oxidizes most of P700 and PC and the subsequent SP first oxidizes the remaining P700 and PC and then reduces all P700 and PC again. In the DUAL-PAM-100 this button is called <Pm>.

<F ML> – fluorescence measuring light; its effective intensity is defined by intensity and by frequency. In the DUAL-KLAS-NIR the frequency is the block frequency, which is set in the 'Fluo Meas. Light' window. For <MF-low> the block frequency ranges between 1 Hz and 1000 Hz and for <MF-high> is 2000 Hz. Given that in 6-channel mode a complete block takes ~0.5 ms to complete, 2000 Hz is the maximum block frequency available. Switching to 2-channel mode, <MF-High> becomes available (range 100-10000 Hz). If Auto <MF-High> is checked, the block frequency will switch from <MF-low> to <MF-high> when the actinic light is triggered.

<MF-high> – High block measuring frequency, normally active when the actinic light is on. It is linked to the <TR> button; when <MF-high> is active, the hole in the <MF-H> button is red.

<AL Pulse> – Actinic Light pulse; when clicked the actinic light is turned on for a duration defined in the 'Pulse Width' window and an intensity defined in the 'Settings/Actinic Light' window.

<FR Pulse> – Far-red Light pulse; when clicked the far-red light is turned on for a duration defined in the 'Pulse Width' window and an intensity defined in the 'Settings/Actinic Light' window.

<BL Pulse> – Actinic Blue Light pulse; when clicked the blue actinic light is turned on for a duration defined in the 'Pulse Width' window and an intensity defined in the 'Settings/Actinic Light' window.

<TR Pulse> – Applies a 5 V pulse to the TRIGGER OUT socket.

<ST> – single turnover flash; when clicked a single turnover flash is triggered with a length defined in the ‘Pulse Width’ window.

<Stirrer on> – Switching on the stirrer in the control unit only puts the stirrer in standby mode; Checking the <Stirrer on> field is needed to activate the stirrer. This function is only relevant for aquatic samples.

The third row gives three further buttons:

<Signal Zero> – Setting the ‘green fields’ to zero using the <Signal Zero> button gives at the same time an offset value above the ‘green fields’. This, the <NIR Bal.> button does not do.

<Reset> – Subsequently clicking on the <Reset> button, sets all offset values to zero again, adding the off-set values to the signals in the green fields.

<Fluo Zoff> – Clicking on this button will set the presently measured fluorescence value to zero.

4.21 Clock Sub-Window

Using the clock-function it is possible to trigger, with a pre-defined frequency, a certain event. The trigger frequency range is 1 s – 60 min and the events that can be triggered are: a <P.+F. SP>, <ST>, <MT>, <AL pulse>, <FR pulse>, <BL pulse>, <TR pulse>, <Act+Yield>, <FR+Yield>, <AL on>, <FR on>, <BL on>, <Start Script>. Especially the <Start Script> option opens a whole range of possibilities. The clock-function is triggered by checking the box next to <on>.

4.22 Script Sub-Window

The three buttons of the 'Script' sub window: <Load> (opens the KLAS-100/Script Files window), <NIRmax> and <Run> (runs the open script) have been discussed above, discussing the determination of DMPs and the function of the <NIRmax> script.

4.23 Fluo Meas. Light window



Fig. 57: Fluo Meas. Light Window.

The DUAL-KLAS-NIR has two sources of fluorescence measuring light (Fig. 57). The Emitter head contains an LED emitting green measuring light. Green light penetrates deeper in the leaf, probing also deeper cell layers. The expectation is that green measuring light matches the NIR measuring light quite well. Green light is less efficiently absorbed, and the available measuring light intensity range is smaller ($3\text{--}37 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$)

than in the case of the blue measuring light LED. As a consequence, the signal is less strong compared with the signal induced by the blue measuring light. The green measuring light is referred to as 'Fluo'.

The blue measuring light LED is located in the detector head and is referred to as 'Fluo Top'. Both fluorescence channels are defined separately, measured separately and treated separately. In Fig. 57 the 'Fluo Top Meas. Light' is set to 0 and this makes the 'Fluo Top' channel in the recordings invisible!

In the third window of Fig. 57, the <Block Frequency> is defined. In the 6-channel mode (<All Fast>), the maximum Block Frequency is 2000 Hz and in that case <MF-high> is not available, or at least its value cannot be modified and the value is fixed at 2000 Hz.

4.24 Actinic Light Window

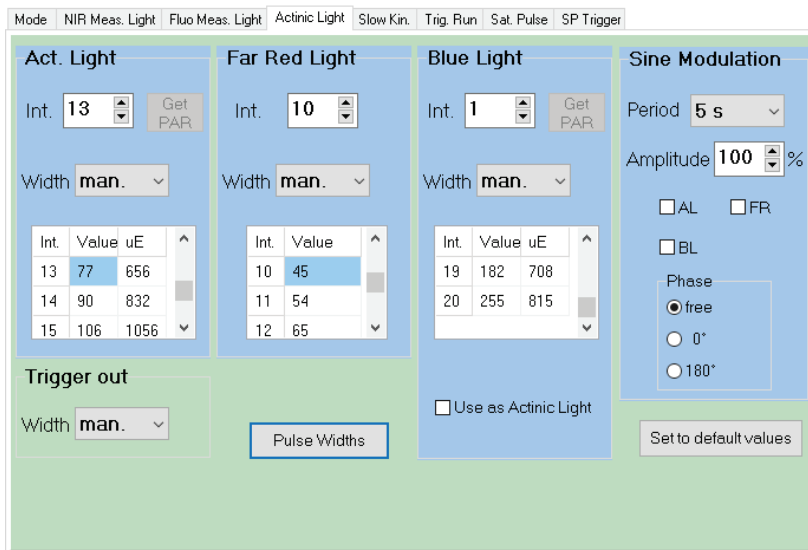


Fig. 58: Actinic Light Window.

Several aspects of the 'Actinic Light' window (Fig. 58) have been discussed above. An important feature of this window is the <Width> button associated with <Act. Light>, <Far Red Light>, <Blue Light> and <Trigger Out>. If these buttons are set to <man.> it means that turning off the <Act. Light>, <Far Red Light>, <Blue Light> and <Trigger Out> has to be done either manually by clicking on the accompanying button on the bottom panel or by a command in a script.

For <Act. Light>, <Far Red Light> and <Blue Light> intensities 20 levels are available.

At the bottom of the 'Blue Light' sub-window it is possible to check <Use as Actinic Light>. In that case the software will consider the blue actinic light instead of the red actinic light of the sub-window on the left side as the 'true' actinic light source.

If a light sensor is connected to the instrument the <Get PAR> buttons next to the 'Act. Light int.' and the 'Blue Light int.' menus can be clicked to determine the PAR lists for respectively the 'Act. Light' (red) and the 'Blue Light'.

The right-hand side 'Modulation' sub-window can modify the <Act. Light> (red), <FR Light> and <Blue Light> in a special way making it sinusoidal.

The dropdown menu of <Period> defines the time needed to go from peak value to peak value (range 1-10 s, 10-60 s, 1-10 min, 10-60 min, 1-24 h). With a 24 h sinusoid it would be possible to mimic a day/night rhythm, but in that case the leaf/sample would have to stay for more than 24 h in the DUAL-KLAS-NIR, which may be somewhat impractical.

With <Amplitude> it is possible to change the light intensity in steps of 10%, but it is of course also possible to change the intensity setting of the light source itself (20 levels available).

The light sources to be sine-modulated (<AL>, <FR>, <BL>) can be selected by checking the boxes next to respectively <AL>, <FR> and <BL>.

With respect to the <phase> the user can choose between free, 0° and 180°. In the case of 0° the sinusoid starts its rise the moment the light source is turned on. In the case of the 180° phase the intensity starts at the highest point and then goes down. Choosing free, the intensity of the light source will start randomly somewhere along the sinusoid and continue from there.

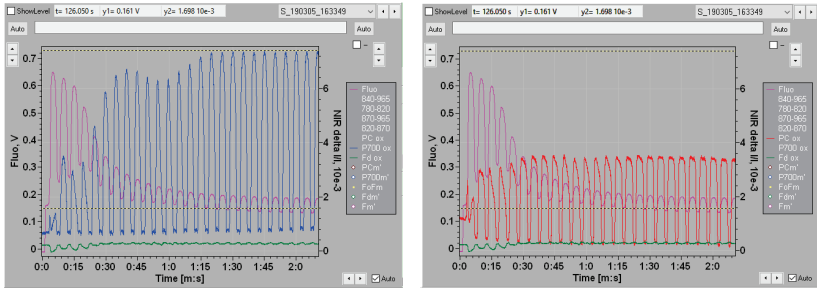


Fig. 59: Sinusoidal illumination.

The two panels of Fig. 59 give an example of the effects of sine-modulation (180°) on photosynthetic induction. The figure demonstrates that the P700 signal gives better oscillations than the fluorescence, PC or Fd signals.

A different type of experiment (Fig. 60) also demonstrates that the P700 signal is by far the most sensitive to periodical changes in the light intensity.

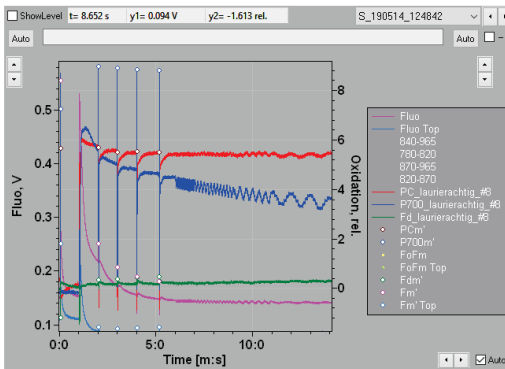


Fig. 60: Sinusoidal illumination of a leaf following a short induction period. The length of the periods of the oscillations in the light intensity was gradually increased.

4.25 Flux mode

Another special measurement mode is called 'Flux mode' and is only available in dual or single channel mode for the 4 absorption wavelength pairs. In that case not the absolute changes in the absorption levels are analyzed, but the changes (up and down) induced by the measuring pulses.

4.26 The Trig. Run window (for <Standard SP> and <Fast Acquisition> mode)

To create a trigger pattern with the DUAL-KLAS-NIR is really simple (see Fig. 61). As a first step choose the light sources/events that have to be included in the trigger pattern by checking the boxes next to, respectively, <AL> (actinic red light), <FR> (far red light), <BL> (actinic blue light), <MT> (multiple turnover pulse), <TR> (trigger pulse), <MF-max> (maximum measuring light frequency), <S&H off> (sample and hold off), <ST> (single turnover flash). For each selected light source/event a line with a different color shows up in the trigger pattern graph. At the same time, for each selected light source/event a column is created in the Table underneath the graph window.

Clicking with the right mouse button on the row number of one of the rows of the Table opens up a menu with the following options: <New Line>, <Delete Line>, <Clear Line>, <Copy Line> and <Copy Width (ms)>.

Clicking on <New Line> adds a line to the Table above the row nr on which was clicked.

Clicking twice on a <time> cell (a cell in the <second (time,ms)> column) makes that cell editable. A time can be typed in or

reached by clicking on the little up/down arrows that become visible after clicking twice on the cell.

Clicking twice on an <action> cell (a cell in the third (Action) column) makes a drop down menu visible from which an action can be chosen, e.g. a <Pm determination> or an <MT>. This action will be carried out at the indicated time. An important action option is <Trig. out>. Choosing this action will add 0's to the cells of the columns at the end (the <AL>, <FR>, <BL>, <MT>, etc, depending on the boxes that were checked on the right-hand side of the graph window). It is possible to toggle between 0 and 1 (off and on).

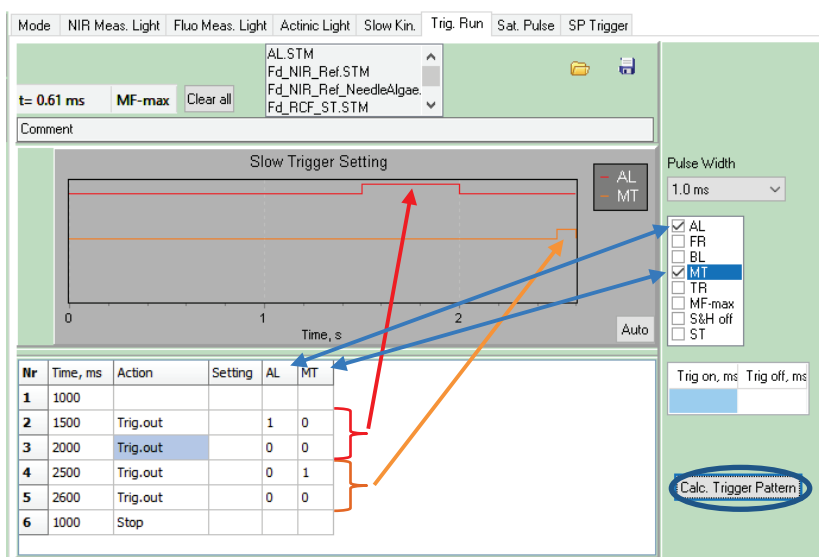




Fig. 61: Triggered Run Window.

With the <Calc. Trigger Pattern> button, the actions in the Table are translated into effects on the Trigger Pattern in the graph window.

With the <Clear all> button both the Slow Trigger Setting graph and the Information in the Table are cleared, reducing the Table

to a single line reading: 1000 (ms) – Stop. The ‘Slow Trigger Setting’ graph only shows flat lines of the selected events.

The menu in the middle above the graph window allows the selection of a Trigger Pattern that had been created and saved earlier in the KLAS-100\Slow Kin Trigger directory. If the user wants a trigger pattern that has been saved elsewhere he/she should

click on  and navigate to the directory where the searched for trigger pattern has been saved. Clicking on  allows the user to save a trigger pattern that has been created.

4.27 Sat. Pulse/Fast Acqui. Windows

The ‘Sat. Pulse’ (<Standard SP> mode) and the ‘Fast Acqui’ (<Fast Acquisition> mode) windows share features (see three panels of Fig. 62). Both have the same ‘Fast Kinetics’ sub-window. However, in the ‘Fast Acqui.’ window the variable <Points> can be varied between 500 and 128000 and in the Sat. Pulse window it is fixed to 16000 points. The second variable in this sub-window, <Rate, μ s>, is in both cases sensitive to the number of channels measured. In the 6-channel mode, it is fixed to 100 μ s and in the 2-channel (or single channel) mode it can be varied between 2.5 and 1000 μ s. The length of, respectively, the <SP> and <MT> pulses is <Rate, μ s> x <Points>. This number is shown at the bottom of the Fast Kinetics sub-window.

The name and format of the sub-window on the left side depends on the measuring mode. In the <Standard SP> mode, measuring only fluorescence it is called ‘Fluo. SP’. The sub-window allows the choice between 20 levels of MT/SP intensity and the Width of the pulse, which can be varied between 1 and 800 ms. If also, or only, absorption is measured the sub-window is called ‘P.+F.

SP', but further the choices that can be made are the same. However, in this case there is an extra 'SP-evaluation' sub-window below the 'P.+F. SP' sub-window, where the default values are 5 s[?] and 30 ms[?]. In the case of the 'Fast Acqui.' window, this sub-window is called 'MT'. In this case toggling between <6-channel> and <2-channel Fluo> mode does not affect the 'MT' sub-window. Here, the intensity can be chosen, but the Width is <Fast Trigger>, which means that it is determined by a Trigger Pattern or Script. In the 'Fast Acqui.' window there is an additional 'Fast Kin. Average' sub-window.

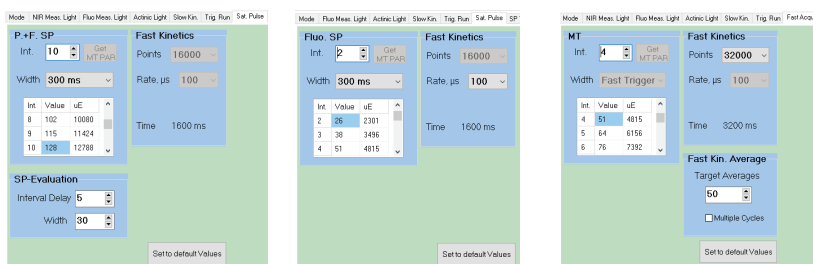


Fig. 62: Sat. Pulse/Fast Acqui. windows.

4.28 SP Trigger Window

The last Settings window is the 'SP Trigger' window. This has the same format as the Trigger window of the MULTI-COLOR-PAM (see the renewed MC-PAM manual how to create a trigger pattern in this window).

4.29 Slow Kinetics/SP Kinetics/Fast Kinetics Windows

When the user has selected either the 'Slow Kinetics' or 'SP Kinetics' window in <Standard SP> mode or either the 'Slow Kinetics' or 'Fast Kinetics' window in the <Fast Acquisition> mode a

set of options is shown next to the upper right corner of the graph window (Fig. 63).

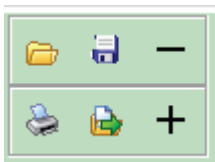


Fig. 63: File/Data Management Panel.

The first icon represents the well-known <open directory> icon. Selecting the <Standard SP> mode and then going to the 'Slow Kinetics' window it opens the KLAS-100\Slow Kin Data directory and in the 'SP Kinetics' window it opens the KLAS-100\Fast Kin Data directory. Selecting the <Fast Acquisition> mode and then going to the 'Slow Kinetics' window it opens the KLAS-100\Slow Kin Data window and in the 'Fast Kinetics' window it opens the KLAS-100\Fast Kin Data directory.

The second icon is the well-known <save> icon, which allows the user to save his/her data in the same directories as mentioned above for the different windows.

The first icon of the bottom row is the well-known <print> icon, which allows the user to print out the graph window of the active window.

The second icon of the bottom row is the <export file> icon. This saves the data as a *.csv file (a comma separated value file). These *.csv files can for example be imported into Excel via the import text option. However, in that case semicolons have to be chosen as separators to distribute the values over different columns. In more recent Excel versions these files can simply be opened by double clicking on them. It is necessary to save these files subsequently as *.xlsx files when figures are made. I

learned by experience that all figures are lost when a file is saved as *.csv file.

This leaves the <-> symbol in the top row and the <+> symbol in the bottom row. The <-> icon allows the user to subtract two measured transients from each other, for example to improve the base line or to make a feature visible that is hidden in one of the two transients.

On clicking on the <-> symbol the user is offered a series of measurements he/she can choose from. If no measurement has been opened yet in the window, the chosen transient will be subtracted from itself and the result is a set of flat lines that will be shown in the graph window.

If in the dialogue window the <Save Result in Report> box is checked the difference transient will be saved at the end of the same report file.

On clicking on the <+> symbol the same dialogue window is opened, in which the user can check the same <Save Result in Report> box. This time the user can select several (more than 1) measurements from the report file that have to be averaged. Having selected the measurements to be averaged, click on <OK> and the average is saved at the end of the Report file and at the same time shown in the graph window.

4.30 Top Panel

On top of the graph window (Fig. 64) more information is available, and several additional functions can be found.

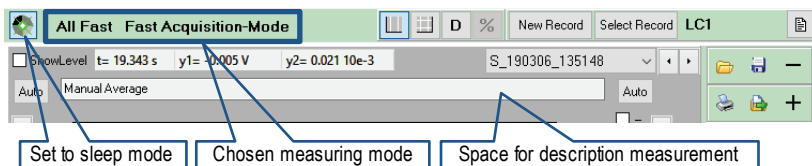


Fig. 64: File/Data Management Panel.

Clicking on the **<D>** button means that in the graph window only deconvoluted signals will be shown. In practice the button toggles between showing the deconvoluted P700, PC and Fd data and the raw data measured for the four wavelength pairs.

The percentage **<%>** button requires that the **<NIRmax>** script has been run and that **<Get Max[-Values]>** button has been clicked on to obtain the maximum amplitudes of the absorption changes. In that case the **<%>** button will turn absolute values into relative values.

Clicking on the **<New Record>** button starts a new record. Every time a measurement (or a set of related measurements) is finished it is a good idea to start a new record. That way the user can avoid that he/she gets a long spreadsheet with unrelated measurements.

With the **<Select Record>** button another Record in the active Report that has been created earlier that day can be opened.

Clicking on the downwards arrow next to the **S_*** field will open a dropdown menu with all the measuring files contained in the open Record. It is also possible to click through these measurements with the small arrows (left is up along the list and right is down along the list) to the right of the **S_*** field.

Clicking on the **<page>** icon above the **<->** icon opens the 'record comments file'. In it notes can be made on the experiment(s) associated with this record file, or the comments that were made earlier can be studied.

4.31 Redox Plot and Yield Plot

Two windows that have not yet been discussed are the Redox Plot and the Yield Plot. With the Redox Plot (Fig. 65) the redox state of PC and the redox state of P700 can be plotted against each other.

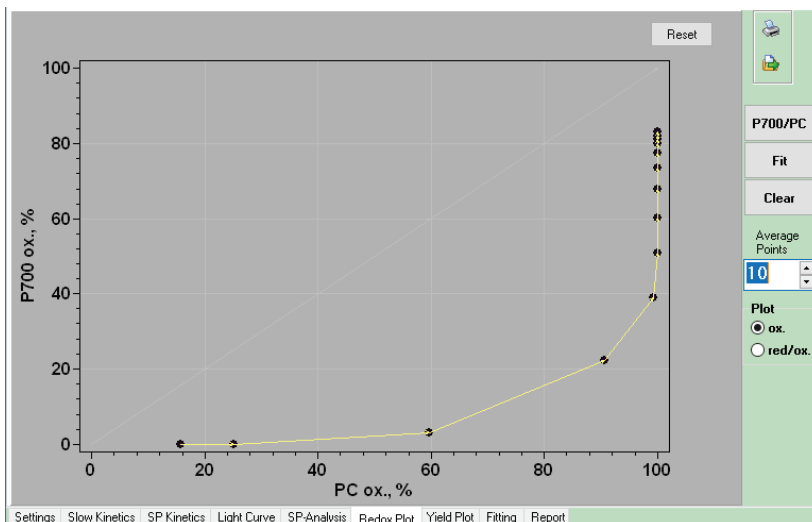


Fig. 65: Redox Plot.

The data set in Fig. 65 is derived from a light curve experiment with 2 min light intensity steps and light intensities up to $\sim 1000 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$. Already at low light intensities PC becomes completely oxidized. At that point approximately 40% of P700 is in the oxidized state.

The yield plot (Fig. 66) determines the relationship between the PS I and PS II quantum yields.

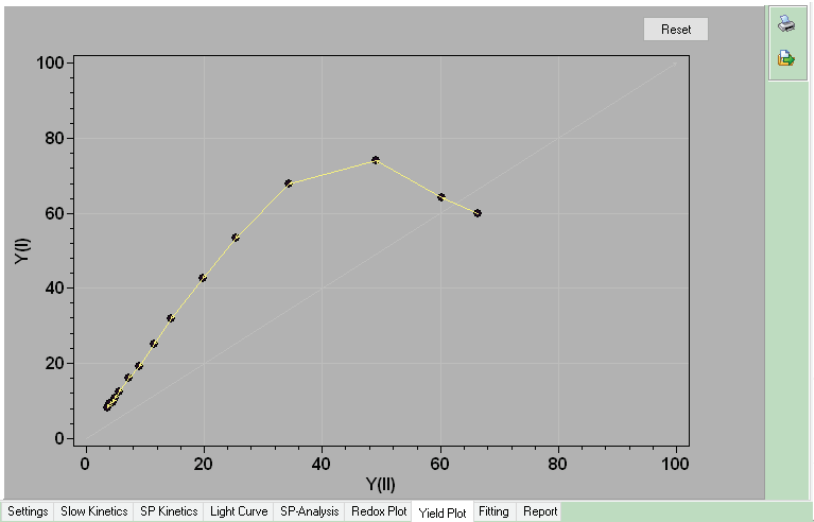


Fig. 66: Yield Plot.

There is a linear relationship at higher light intensities, but not exactly one to one. Differences in photosynthetic control (determining the rate of re-oxidation of PQH_2 by the cyt b6f complex) between plants are expected to affect the slope.

4.32 Get Fd-RCF, Get Pool Size and Get PC-RCF

Starting v65 of the software, one button with dropdown menu each can be found in the ‘Slow Kinetics’ and ‘SP Kinetics’ windows. In Fig. 67 the two buttons with their dropdown menus are shown.

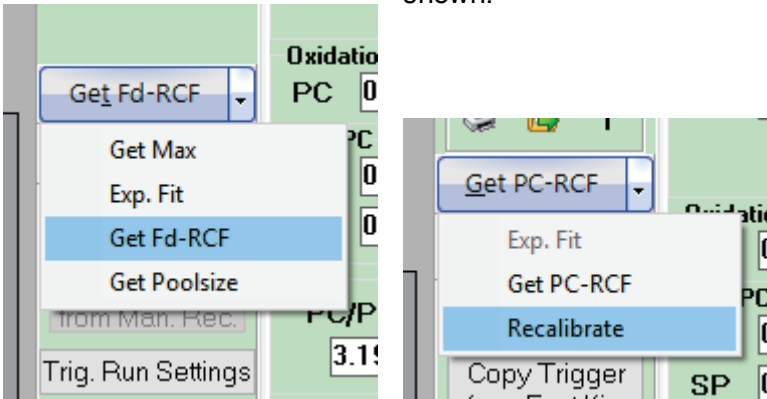


Fig. 67: Dropdown menus related to the Get Max button in the Slow Kinetics window (left) and the dropdown menu of a similar button found in the SP Kinetics window (right).

The <Get Max> option has been discussed above (section 4.4) and is used each time the <NIRmax> script is run to obtain the maximum P700, PC and Fd values. The <Exp. Fit> function has been discussed in section 4.14. The term RCF stands for Relative Concentration Factor and represents the conversion factor with which the NIRmax values can be turned into the concentration of molecules used for the calculation of the Fd/P700 and PC/P700 ratios. For the <Get Fd-RCF> determination a trigger file can be found in the KLAS-100\Slow Kin Trigger folder called Fd_RCF_ST_HR.STM. The same folder also contains a trigger file called Fd_RCF_TR_HR.STM, which can be used in combination with an external laser. The properties of the Fd_RCF_ST_HR.STM trigger file can be made visible by opening the file in the Setting/Trig Run window. To run this trigger file, it is for example possible to modify the NIRmax script. In the 'Slow Kinetics' window load NIRmaxHR.prg. Click on the Open Slow Kin. Trig File and replace NIRmaxHR.STM by Fd_RCF_ST_HR.STM. Delete the MT Pulse Width line, the MT line and the Wait(s)=27 line and save the result under a new name. Running this script file gives the measurement shown in Fig. 68, consisting of a 0.8 s pulse of actinic light followed by 6

STs given 100, 300, 600, 1000, 1400 and 1800 ms after the actinic pulse of light. The user should then click on the <%> button above the graph window to express the results as percentage signal.

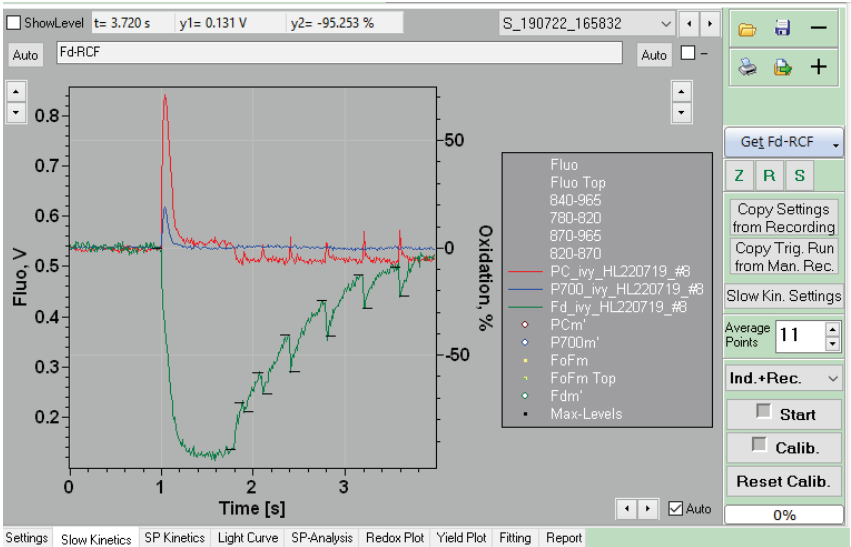


Fig. 68: The result of the Fd_RCF_ST_HR trigger file incorporated in a script file as described in the text.

Clicking on <Get Fd-RCF> following the measurement inserts the little black lines in the measurement window defining the flash-induced changes in the Fd-amplitude and opens a pop-up window (Fig. 69).

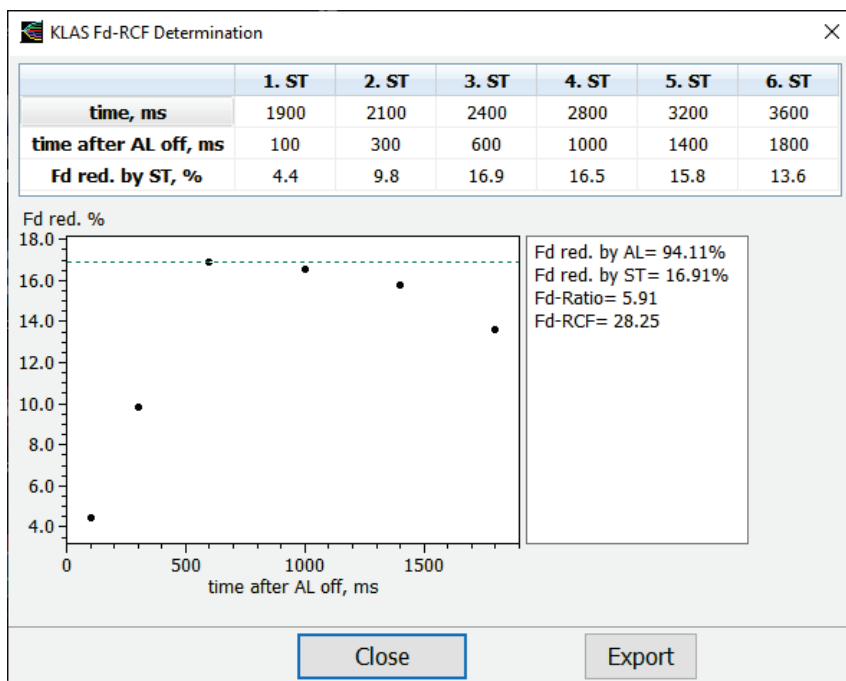


Fig. 69: Pop-up window opened by clicking on <Get Fd-RCF>. The pop-up window gives information on the position of the STs and the % Fd-reduction they induced. Flash 3 induced 16.91% Fd-reduction (the highest value) and the Fd-Ratio is $100/16.91 = 5.91$.

The pop-up window gives the positions of the STs relative to the actinic light pulse and the percentage of Fd-reduction each pulse induced. Dividing 100% by the maximum value (ST 3) gives the Fd-ratio, which is the Fd/P700 ratio.

The <Get PC-RCF> function works in the same way as the <Get Fd-RCF> but analyses a saturating pulse. In 'Settings/Mode' choose for example <Fluo SP>, go to the 'SP Kinetics' window and click the <Fluo SP> button in the bottom menu. The Fluo SP induced Fluorescence and NIR transients will be shown in the 'SP Kinetics' window. Select <Get PC-RCF> and click this but-

ton. A pop-up window shows up in which the PC redox state is plotted as a function of the P700 redox state. When clicking the <Calc. PC/P700 ratio> button the points around 50% are fit by linear regression and among other things PC-RCF is calculated.

4.33 Dual Channel fluorescence measurements

One of the features of the DUAL-KLAS-NIR is that it can measure two fluorescence channels (blue and green ML) simultaneously. It is important to note that the blue ML channel is invisible if the blue ML is 0 and this is the default value! Actinic illumination with red light (located in both emitter and detector units; intensity $0 \rightarrow 3400 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) from both sides of the leaf, that can be probed with green measuring light from the Emitter side of the leaf and blue measuring light from the Detector side of the leaf. Alternatively, it is possible to use blue actinic light (located in the detector unit; intensity $0 \sim 400 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) from the Detector side of the leaf that can be probed with blue measuring light from the same Detector side of the leaf and green measuring light from the Emitter side of the leaf.

The red-light source is the main actinic light source of the instrument. The user may want to choose the blue actinic light for special purposes (check in that case in the 'Blue Light' sub-window of 'Settings/Actinic Light' window the box <Use as Actinic Light>). Green light was chosen as ML based on the idea that green light is less well absorbed by the leaf, can penetrate deeper in the leaf and, therefore, is more representative for the fluorescence properties of the whole leaf. This would make the fluorescence measurements more comparable to the NIR-absorption measurements that also reflect the whole leaf.

It is important to consider as well that the saturating pulses (red) are also given from both sides of the leaf.

A comparison was made between the different optical configurations of the leaf and the instrument. The leaves were probed with green ML applied from either the abaxial or the adaxial side of the leaf (with the blue ML applied simultaneously to the other side of the leaf).

Light curves (2 min illumination per light intensity up to 1000 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$) were made and variables were the type of saturation pulse and the light intensity of the saturation pulse.

In most leaves there is a gradient of photosynthetic properties. The side of the leaf most exposed to the sunlight (adaxial side) behaves more like a sun leaf and the side of the leaf least exposed to the sun (abaxial side) behaves more like a shade leaf. The adaxial side of the leaf is characterized by palisade parenchyma cells that act as light guides for the incoming light, whereas the abaxial side of the leaf is characterized by spongy form parenchyma cells with many air spaces in between to facilitate the exchange of CO_2 . These cells have the tendency to disperse the light. The 'shade-type' chloroplasts on the abaxial side of the leaf have PS II reaction centers with larger antennae than chloroplasts on the adaxial side of the leaf. That makes it likely that the electron transport chains on the abaxial side of the leaf will saturate more quickly than the electron transport chains on the adaxial side of the leaf. If the green ML probes the whole leaf whereas the blue ML mainly probes one side of the leaf, there will already be a discrepancy between both types of measurements due to the fact that the green ML will probe also PS II with properties different from the ones probed by ML blue. A real one-to-one relationship between the green and blue ML will, therefore, not be achieved. As shown in Fig. 70, green ML applied to the abaxial side of the leaf and blue ML applied to the adaxial side of the leaf correlate much better than green ML applied to the adaxial side of the leaf and blue ML applied to the abaxial

side of the leaf. It could be argued that the difference between the two situations is completely due to the blue ML, but at least it indicates that abaxially applied green ML is probes most of the leaf.

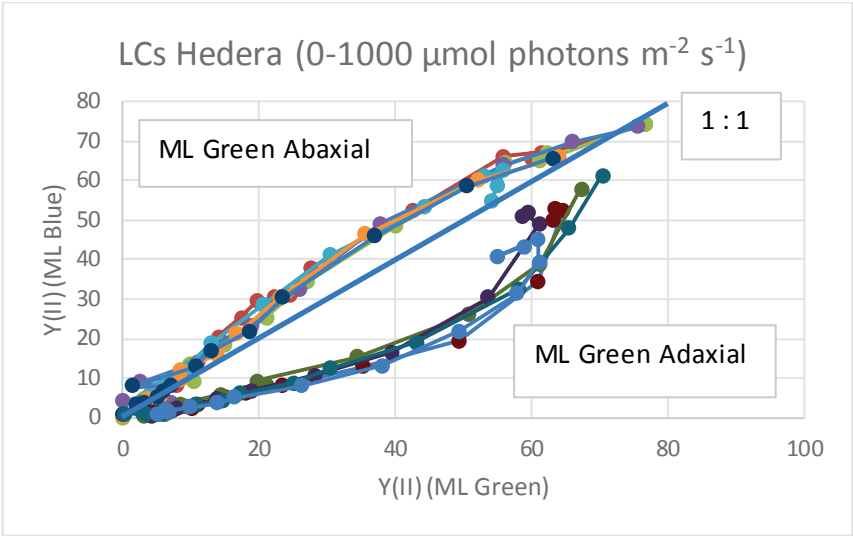


Fig. 70: Light Curves *Hedera helix* leaves, 2 min per light intensity, 15 levels between 0 and 1000 $\mu\text{mol photons m}^2 \text{s}^{-1}$ red light, saturation pulse intensity varied between 2300 and 15500 $\mu\text{mol photons m}^2 \text{s}^{-1}$.

The same observations can be made if one light intensity for the saturating pulses is chosen and the saturation pulse type is the variable (Fig. 71).

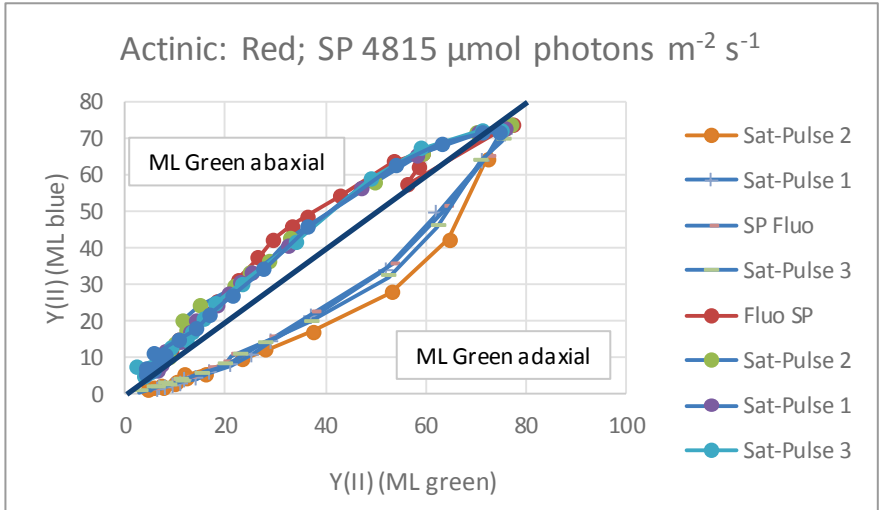


Fig. 71: Light Curves *Hedera helix* leaves, 2 min per light intensity, 15 levels between 0 and 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ red light; saturation pulse intensity set to 4815 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, type of saturation pulse varied.

Blue actinic light:

To use the blue light as actinic light, in 'Settings/Actinic light' <Use as actinic light> has to be checked in the 'Blue light' sub-window. The blue Actinic light can only be applied to one side of the leaf. Fig. 72 indicates that for leaves illuminated with blue actinic light from the adaxial side of the leaf an essentially linear correlation between Y(II) determined by the green and the blue ML is obtained. However, the Y(II) values determined by the green ML remain much higher than those determined with the blue ML, because the chloroplasts on the abaxial side of the leaf see only very low levels of blue actinic light, resulting in high Y(II) values for these chloroplasts. These chloroplasts are probed by the green ML, but not by the blue ML and this explains the large discrepancy in the Y(II) values. The fact that despite this, a linear correlation between both measurements is obtained seems to

demonstrate that the green ML also probes the chloroplasts in the palisade parenchyma cells on the adaxial side of the leaf

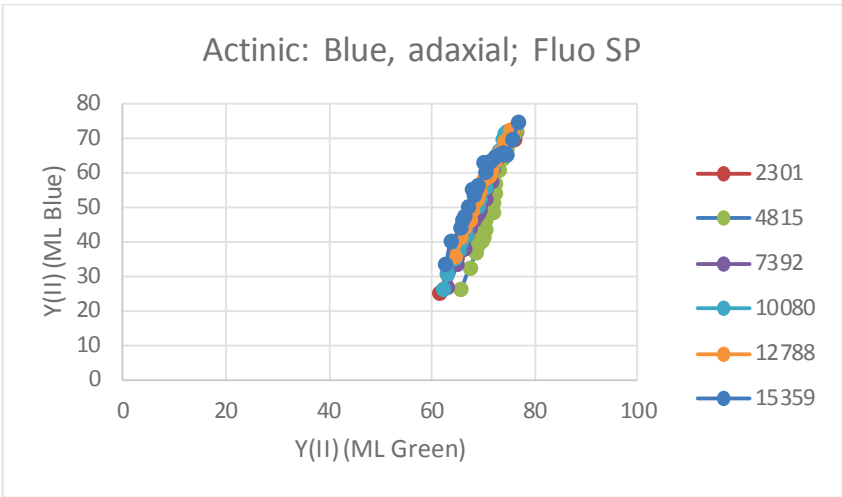


Fig. 72: Light Curves *Hedera helix* leaves, 2 min per light intensity, 15 levels between 0 and 1000 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ blue light, adaxial side of the leaf illuminated, saturation pulse intensity varied as indicated in the legend.

If the blue actinic light is applied to the abaxial side of the leaf (Fig. 73) the relation between Y(II) determined with the blue and green ML, respectively is no longer linear. It may also be noted that the Y(II) for the blue ML when the blue actinic light was applied on the abaxial side goes down to below 10 (= 0.1) compared to more than 20 (= 0.2) when the blue actinic light was applied to the adaxial side of the leaf demonstrating that the PSII's on the abaxial side saturate at lower light intensities than those on the adaxial side (i.e. shade versus sun-type chloroplasts).

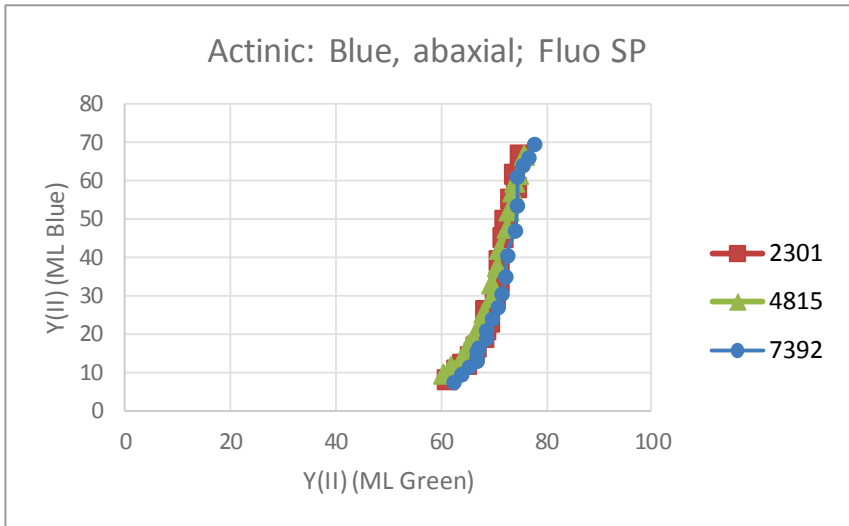


Fig. 73: Light Curves *Hedera helix* leaves, 2 min per light intensity, 15 levels between 0 and 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ blue light, abaxial side of the leaf illuminated, saturating pulse intensity varied as indicated in the legend.

Blue light penetration in the leaf is known to be limited and measurements of Light Curves with blue actinic light confirm this. The blue measuring light shows the typical decline of the $Y(II)$ as a function of the actinic light intensity, whereas the green measuring light probing the leaf from the other side detects a very limited decline of $Y(II)$. This indicates that the light intensity dependence of the PS II reaction centers excited by the blue actinic light and probed by the green measuring light is quite different from the light intensity dependence of the PS II reaction centers probed by the blue measuring light. In other words, that the green ML probes to a large extent other PS II reaction centers than the blue ML.

The above figures also show that the light intensity of the saturating pulse, and also the type of saturating pulse has limited effect on the measured PS II quantum yields.

4.34 Report window

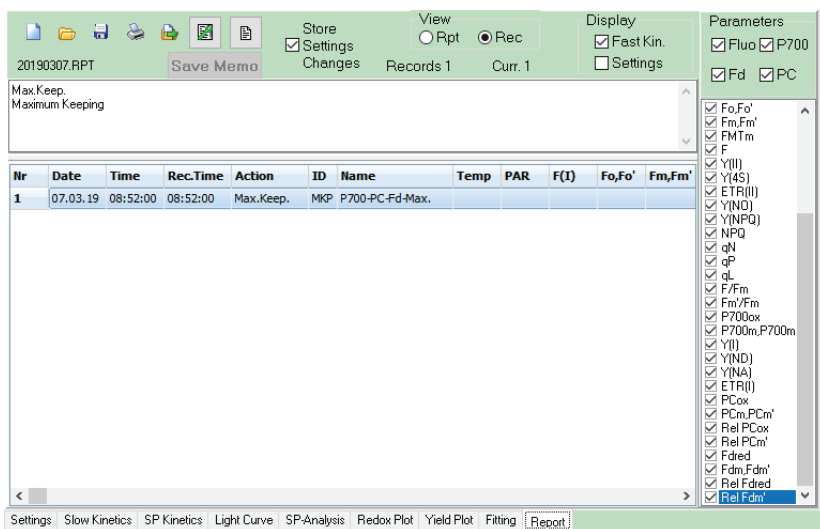


Fig. 74: Report Window.

On the right-hand side of the Report window (Fig. 74) the Parameters-section is found. In the present case the boxes of Fluo, P700, Fd and PC are checked. This means that all the parameters associated with these four components are included in the report file.

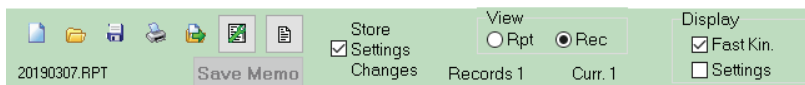
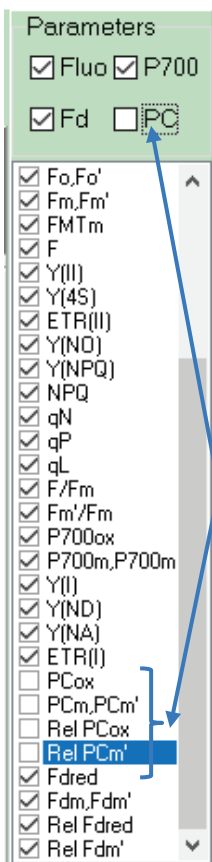


Fig. 75: Top Bar of Report Window.


Unchecking the PC box unchecks at the same time the boxes of the parameters related to PC and removes these as well from the Report file (Fig. 76).


In the top bar of the Report Window (Fig. 75) the user finds on the left-hand side several icons that were already discussed above. Clicking on the <empty leaf of paper> icon starts a new




report file is saved.

**Fig. 76: Side Bar
of Report Window.**

The next icon is the well-known Print icon . Clicking on it will trigger printing of the open/active record on the printer associated with the computer the which the data are sent directly, without first opening a print dialogue.

report. Clicking on the  icon opens the KLAS-100\KlasDualReport directory, where the dates on which reports were saved earlier are listed. If more report files were created on the same day, the second report file of the day gets the ending _01, the third report file of the day gets the ending _02, etc. Clicking on one of the dates will open the associated report. It is important to realize that in the present version of the software the instrument will add new measurements to an open Report, without warning. If the user would for example repeat a set of Light Curve measurements, these may get the same name as the older measurements in the same report and become thereby inaccessible. If the user

clicks on the  icon, it is with the intention to save the report file, however, the software first opens the associated comment window in order to allow the author, if necessary, to make some further notes defining the content of the file. And only after clicking <OK> the


Further to the right the Export icon  is found. Clicking on it will save the active record as a *.csv file that via the import text data option of Excel can be imported into Excel. In newer versions of Excel these files can also be opened by double clicking on them. When this is possible, this is the easiest way to access *.csv files.

Next to the <Export> icon there are two <comment icons>. Clicking on the one on the left will open the comment file for the Report as a whole and clicking on the one on the right will open the comment file of the active Record.

The next item on the bar is <Store Setting Changes>. It is probably always a good idea to check this box.

In the View section next to it, there are two buttons, one for report: <Rpt> and one for recording: <Rec.> If the <Rpt> button is black (clicked on) it means that the user sees an overview of all the Record files in the Report. By clicking on one of the record lines, this record is opened/activated and the data contained in it can be viewed in the relevant graph windows and the associated parameters can be seen in the 'Report' window as well as information on date, time, light intensity, type of action recorded, etc. Clicking on a Record means as well that the <Rec.> button turns black.

4.35 Printing

The print icon  can be found associated with several windows: 'Slow Kinetics', 'SP Kinetics', 'Light Curve', 'SP-Analysis', 'Redox Plot' and 'Yield Plot' windows in the 'Standard SP' mode and the 'Slow Kinetics', 'Fast Kinetics' and 'Redox Plot' windows in the 'Fast Acquisition' mode. Clicking on the print icon will in all

cases directly (without opening a print dialogue window) print the graph area on the printer associated with the computer on which the software is used.

It is, therefore, important to choose the printer to be used beforehand. The Printer dialogue (Fig. 77) can be found via <File> on the top bar, which will make a drop-down menu visible on which the option <PrinterSetup...> should be chosen:

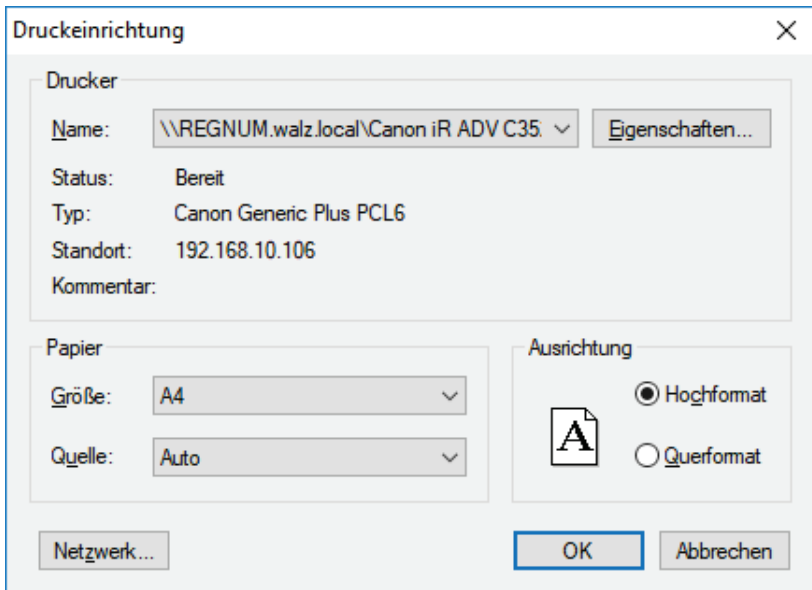


Fig. 77: Print Window.

4.36 File Menu

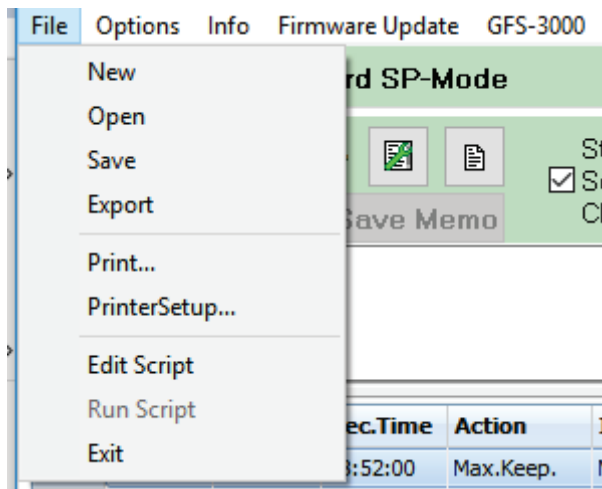


Fig. 78: File menu.

The first command on the top bar is <File>, where the user will find more or less the same options as in many other programs (Fig. 78). The <New> option will start a new report, deleting all unsaved data.

<Open> – Opens the KLAS-100\KlasDualReport directory. Compared to the <Open File> command in the 'Report' window, this one also gives information on the content of a report (Fig. 79).

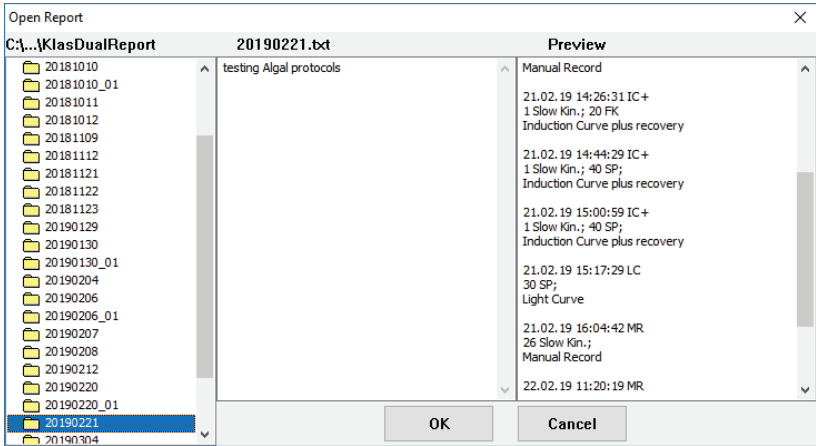


Fig. 79: Open Report Window.

The <Save> command saves the open report

The <Export> command...

The <Print...> command prints the whole active window including side and bottom bars.

The <PrinterSetup...> was discussed above. To avoid problems, the printer to be used for printing should be chosen here before any printing icon is clicked.

The <Edit Script> command opens the KLAS-100\Script Files window where a script file can be selected and opened. The <Load> command on the bottom bar has the same function.

The <Run Script> command is equal to the <Run> command on the bottom bar next to the <NIRmax> button.

The <Exit> command will first ask the user if the report should be saved (if that had not been done beforehand) and subsequently asks for permission to close the program.

4.37 Options Menu

The second choice of the top bar is <Options> (Fig. 80).

Clicking on <Options> will make a drop-down menu visible with a whole list of items. Quite a few of them are functions that can either be activated by checking them or inactivated by removing the check by clicking again on this item.

Functions that can be (in)activated are:

<Show Hints> – if <Show Hints> is activated, little messages giving short descriptions of the function associated with a particular icon are shown if the user holds the mouse over that icon.

<Automatic Signal Zero>

For the <Keep Scale of> option two further choices that can be (in)activated are associated: <Slow Kinetics> and <Fast Kinetics>.

<Off with Screensaver>

<Show both PAR>

<Batt/Temp Monitor off>

<Auto Report Backup off>

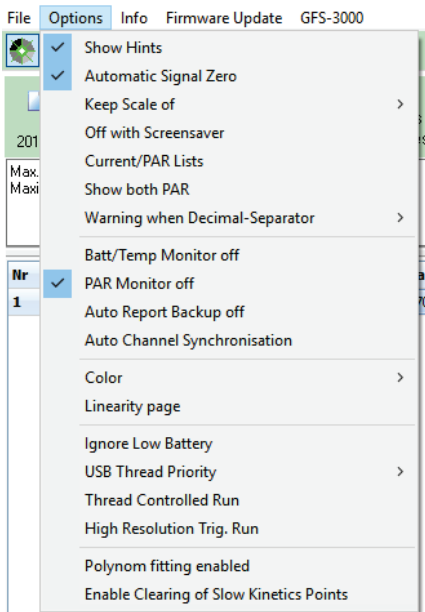


Fig. 80: Options Menu.

<Linearity page>

<Ignore Low Battery> – Here it can be noted that if the Battery voltage drops below a certain value (when running the instrument on the Battery) it starts to affect the measurements strongly and I experienced that the <Low Battery> warning comes too late. In this respect I am wondering if <Ignoring Low Battery> is a meaningful option when running the DUAL-KLAS-NIR on the Battery.

<Thread Controlled Run>

<High Resolution Trig. Run>

<Polynom fitting enabled>

<Enable Clearing of Slow Kinetics points>

Some of these options may save memory space or reduce Battery use, but most of these options are for the more advanced user and I am not there yet.

4.38 PAR lists

The <Current/PAR lists> item of the <Options> menu is practical if the user wants to consult the available light intensities of the different light sources. A special case is formed by the Red-LEDs. There is a Red-LED array in both the Emitter and Detector unit with which both sides of a leaf are illuminated. The Current/PAR lists give the values of only one of the two Red-LED light sources. The intensities of the other Red-LED light source have been determined separately. The values can be found in a Text-file (AL Red Light sources SN #...) that comes with the instrument and in the files: AL-MT-Detector-SN#....par and an associated text file.

For the instrument to detect a connected light meter it is important that <Options – PAR monitor off> is unchecked. The default state is the checked state. Therefore, to determine new PAR lists, click first on <PAR monitor off> to uncheck it. The light meter is connected to the AUX-port of the Control-Unit. In the Klas-100 script files directory routines are found that have to be run for each of the PAR lists. There are: Get_AL_intensities, Get_BL_intensities, Get_FML_intensities and Get_FTopML_intensities. To determine the PAR lists of the MT or SP intensities it is necessary to go to the 'Settings/Sat. Pulse' window (only found in the <Standard SP> mode) and click there on the <Get MT PAR> button.

The FML and FTopML light sources are in the Emitter and Detector unit, respectively, which means that for the leaf configuration the light meter has to be pointed towards either unit to determine the PAR-lists of both light sources.

4.39 Info, Firmware Update, GFS-3000

The other three options on the top bar are more limited in scope. The drop-down menu of <Info> contains two items: <Memory>, which when clicked on gives information on the available RAM memory and <About>, which gives the user the version number of the software, the copyright holders and the four components the instrument measures.

The <Firmware Update> option also contains two items: <Read Firmware Version> gives the version number of the installed firmware and the second item should be used to install firmware updates.

The GFS-3000 option also contains two items: <connect> and <disconnect> the GFS-3000 to/from the DUAL-KLAS-NIR.

5 Guarantee

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

5.1 Conditions

This guarantee applies if the defects are called to the attention of Heinz Walz GmbH, Germany, in writing within two (2) years of the shipping date of the product.

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.
- to damage caused from improper packaging during shipment or any natural acts of God.
- to batteries, cables, calibrations, fiberoptics, fuses, gas filters, lamps, thermocouples, and underwater cables.

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.

5.2 Instructions

To obtain guarantee service, please follow the instructions below:

- The Guarantee Registration form 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 warranty service are at customer expense. Duty and taxes are covered by Walz. Accompany shipment by the Walz Service and Repair form available at:

https://www.walz.com/support/repair_service.html

- 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.

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