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Dual - Modulation  
Kinetic Fluorometer  
**FL-3000**  
for Photosynthesis Research

User's Guide

2006



**Photon  
Systems  
Instruments**

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## 1. GENERAL DESCRIPTION

In fluorescence experiments light serves to drive photosynthetic reactions and, at the same time, to excite fluorescence emission. To separate these roles, two different light sources are frequently used. The actinic light delivers power that is sufficient to induce substantial change in the state of photosynthetic apparatus. On the contrary, the mean power of the measuring light is so low that the impact of the induced photosynthetic reactions can be neglected. In most of the presently used fluorometers, either the actinic or the measuring light is modulated, so that the detection system can separate the fluorescence excited by the measuring light.

Here, we present **the double-modulation fluorometer** that is based on a non-periodic, user-definable modulation of both the Actinic (F1 and F2) and Measuring Flashes (f). In addition, continuous Actinic Light (A1, A2, A3) supplied by separate sets of LEDs is available. The major novel feature of this fluorometer is the capacity of the instrument to generate, from an array of light emitting diodes, actinic flashes that can saturate  $Q_A$  reduction in cyanobacteria at duration as short as 25  $\mu$ s. In the configuration with two symmetrical LED units (Basic and Optional), the saturation can be reached even with green algae or thylakoid / chloroplast suspensions of higher plants. The leaf version of the instrument can produce single-turnover flashes that saturate  $Q_A$  reduction in higher plant leaves. The instrument is also distinguished by a highly accurate timing of the programmed events that is ensured by autonomous microprocessor controlling the execution of the experiment.

The fast version of the instrument (16 bit, 800 kHz) allows measuring the fluorescence induction **during** a single-turnover saturating flash. This technique allows assessing the Photosystem II antenna heterogeneity, the antenna size and the connectivity without disturbing the measured structure by herbicides as DCMU or atrazine (Nedbal et al., J.Photochem.Photobiol.1998).

The double-modulation fluorometer is most frequently used to measure  $Q_A^-$  reoxidation kinetics, fluorescence induction, antenna size or state-transitions. The wide dynamic range of the instrument is achieved primarily by the programmable (non-periodic) timing of the measuring flashes. In one transient, the fast processes can be covered by a sampling on the microsecond time-scale while the slow processes requiring minimal disturbance by the measuring light can be monitored by weak measuring flashes spaced several minutes apart. The software package includes a Wizard with frequently used experimental protocols. This allows easy and user-friendly operation. For an experienced professional, the instrument offers a sophisticated programming language that can be used to design novel timing and measuring sequences.

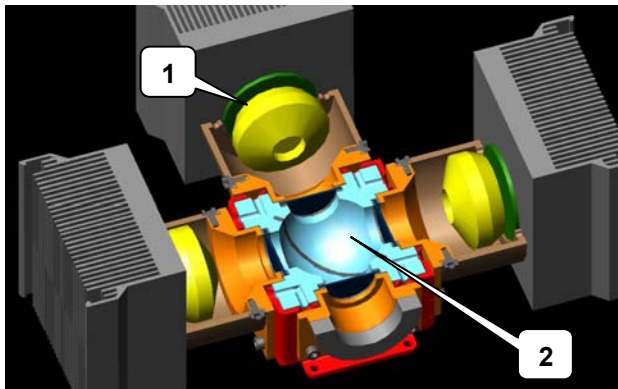
- - -

Free software upgrades and experimental protocols can be downloaded from our homepage (<http://www.psi.cz>). The homepage also offers mailing and E-mail addresses for inquiries and technical support as well as for pricing information and information on our newest products.

## 2. STANDARD CONFIGURATION OF THE INSTRUMENT

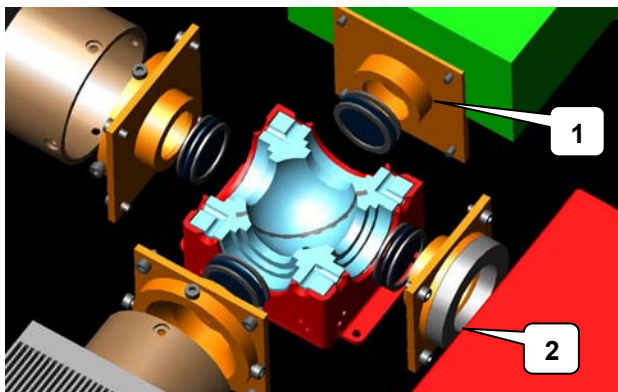
Pcs.	Item						
1	Unit FL3000X						
1	Power Supply						
1	<p><b>SuperHead contains:</b></p> <table border="1" data-bbox="300 562 951 902"> <tbody> <tr> <td data-bbox="300 562 951 613">Orange-red (625nm) – measuring light 1</td> </tr> <tr> <td data-bbox="300 613 951 665">Blue (455nm) – measuring light 2</td> </tr> <tr> <td data-bbox="300 665 951 716">Orange-red (625nm) – Actinic Flash 1</td> </tr> <tr> <td data-bbox="300 716 951 768">Orange-red (625nm) – Actinic light</td> </tr> <tr> <td data-bbox="300 768 951 819">Blue (455nm) – Actinic Flash 2</td> </tr> <tr> <td data-bbox="300 819 951 871">Blue (455nm) – Actinic light 2</td> </tr> </tbody> </table> <p>- <b>Two avalanche diodes</b></p> <p>- <b>Two sets of optical filters:</b></p> <p><b>1st Set:</b> 700 nm Corion (CFC)                    LG697 Corion                    690-730nm PSI band</p> <p><b>2nd Set:</b> 830nm Melles G.</p>	Orange-red (625nm) – measuring light 1	Blue (455nm) – measuring light 2	Orange-red (625nm) – Actinic Flash 1	Orange-red (625nm) – Actinic light	Blue (455nm) – Actinic Flash 2	Blue (455nm) – Actinic light 2
Orange-red (625nm) – measuring light 1							
Blue (455nm) – measuring light 2							
Orange-red (625nm) – Actinic Flash 1							
Orange-red (625nm) – Actinic light							
Blue (455nm) – Actinic Flash 2							
Blue (455nm) – Actinic light 2							
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**Fig.1:** Typical configuration of the Fluorometer FL3000X.

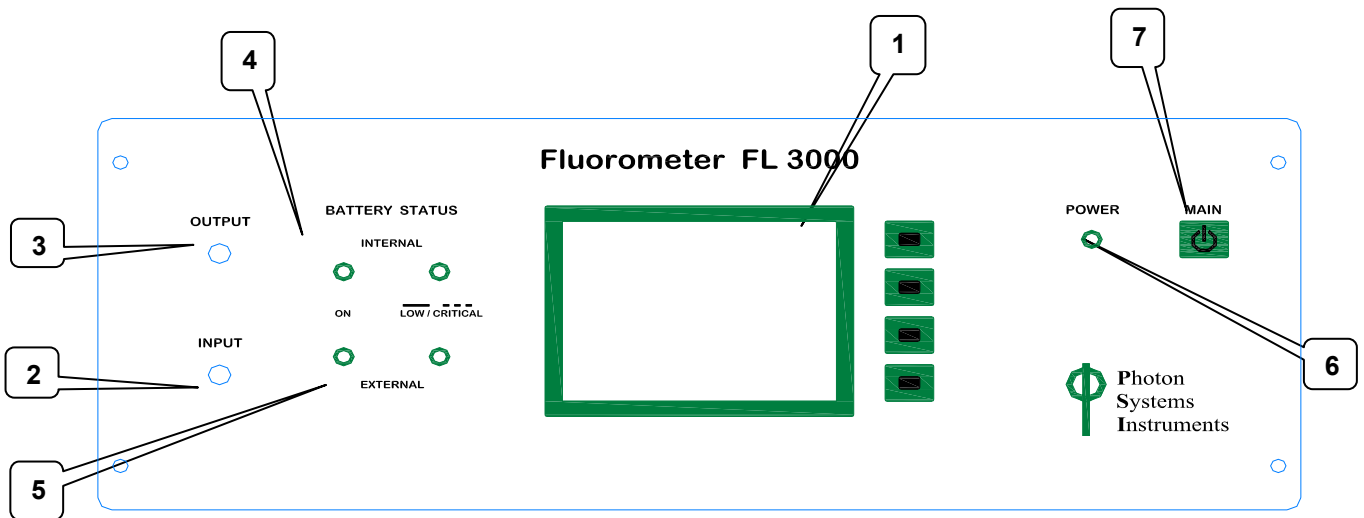


*(1) Light sources; (2) Integrating sphere.*

**Fig.2:** Optical configuration of the Fluorometer FL3000X.



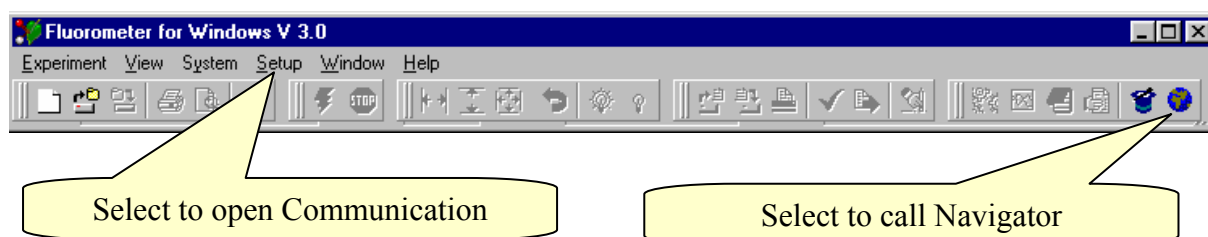
**Fig.3: Front Panel of the Fluorometer FL3000X Control Unit.**



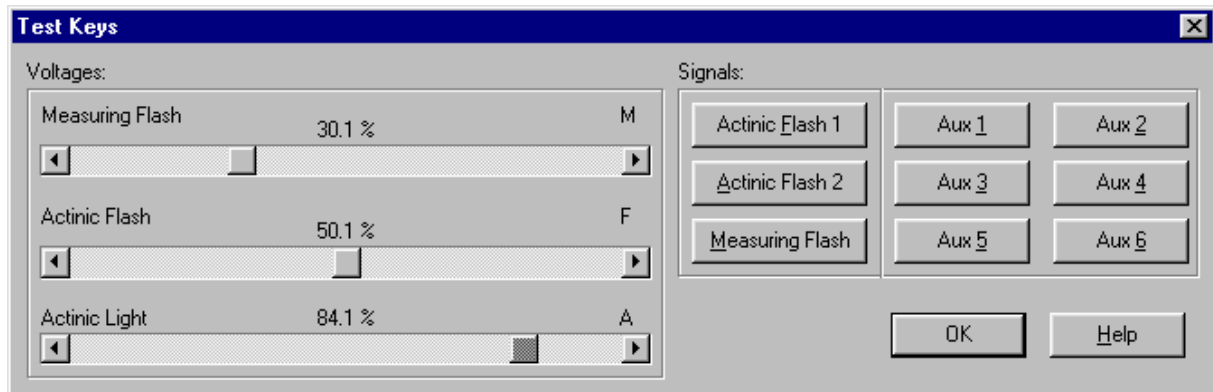
**Fig.3** (1) LCD touch screen; (2) Sample input; (3) Sample output; (4) Internal battery status- green light – battery on, red continuous light –low battery, red flashing light – critical battery; (5) External battery status- green light – battery on, red continuous light –low battery, red flashing light – critical battery; (6) Power LED; (7) Main power switch.

### 3. HARDWARE ASSEMBLY

- a) Check if the delivered components match the configuration of your instrument printed on the previous page.
- b) Place the Control Unit within 1 meter from the sample compartment and within 2 meters from your computer.
- c) Using the serial cable #2, connect the 9-pin connector (Serial) at the rear of the Control Unit to either COM1 or COM2 of your computer.
- d) Connect the AC/DC adapter to the plug at the rear of the Control Unit and to the 110-220V AC. The Control Unit contains an accumulator that, depending on the experimental protocol, allows up to 2 hours of operation without external power. The low power is indicated by flashing of the green LED on the main switch of the Power Supply.
- e) Connect the 8-pin connector of the SuperHead to the back of the Control Unit.
- f) For the Standard version of the Fluorometer: connect the 15-pin connector of the SuperHead to the Channel 1 signal input connector. For the Fast version of the Fluorometer: connect the 15-pin connector of the SuperHead to the Channel 2 signal input connector.
- g) Switch on the instrument and the computer and go to the Windows environment.
- h) Insert the installation CD into your computer. Start the FluorWin installation and install the software package to your computer. Click the FluorWin icon to start the program. Close the Wizard window if opened and select Setup / Communication from the menu. Select the serial port (COM1 or COM2) to which you have connected the fluorometer (see above) and open the communication (Ctrl-I). The status info in the lower left corner of the FluorWin window should read "Ready and waiting" and the device info in the lower right corner should give you the instrument name.



- i) Open the Navigator window (the globe button at the right edge of the icon bar). Select the Test Keys to perform the initial test. The Test Keys window allows manual setting of voltage for the Measuring Flashes, Actinic Flashes and for the Actinic Light. The selected voltage level is indicated by the respective horizontal array of the green LEDs on the front panel of the Power Supply. Pressing buttons in the Test Keys window may activate flashes of default duration (see top next page). The default duration of the flashes is in the microsecond range. Aux 1 button triggers a long pulse of light from the Actinic lights side.



- m) You can periodically measure signals at the Channel 1 and Channel 2 inputs by the System Monitor in the Navigator window (globe button). The measurement occurs with or without a measuring flash depending on the status of the „Measuring Flash“ control (ON/OFF).

*IF YOU WERE UNABLE TO ASSEMBLE THE INSTRUMENT OR IF THE INSTRUMENT IS NOT FUNCTIONING PROPERLY, PLEASE, CONTACT US FOR TECHNICAL SUPPORT ON <http://www.psi.cz>. WE SHALL BE HAPPY TO ASSIST YOU.*

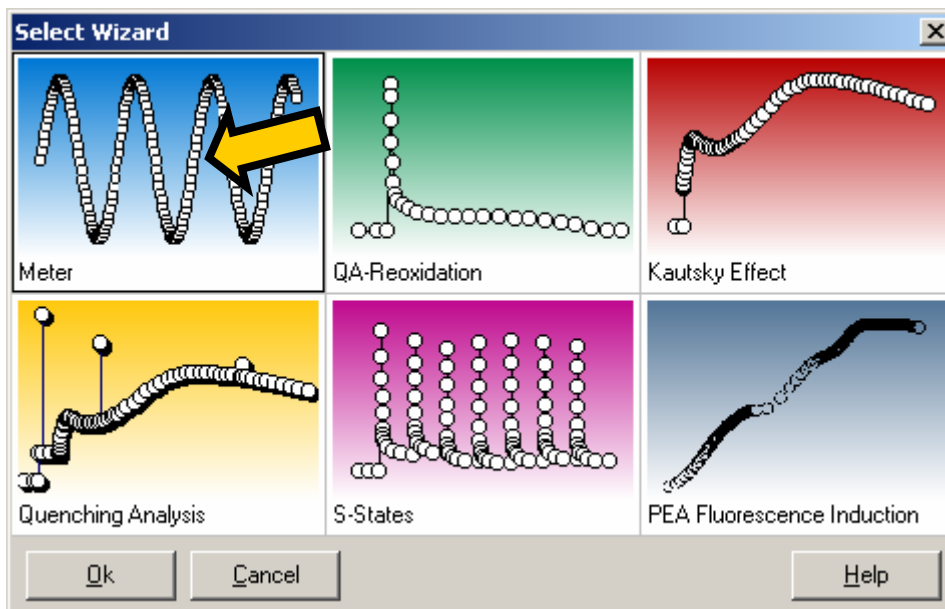
#### 4. FLUORWIN WIZARD AND APPLICATION EXAMPLES

The FluorWin software is designed primarily

- to define and archive the experimental protocols
- to download the experimental protocol into the Control Unit
- to initialize the experiment
- to retrieve the data from the Control Unit
- to visualize, archive, manipulate and export the data

The FluorWin software operates in the Windows graphical environment (Windows 3.1 or, preferably, Windows 9X, Windows 2000 or XP).

After the installation, start the FluorWin program. The Main Menu with the Wizard window will appear. The Wizard window can be called anytime either from the Window menu or by pressing the Wizard icon (see below).

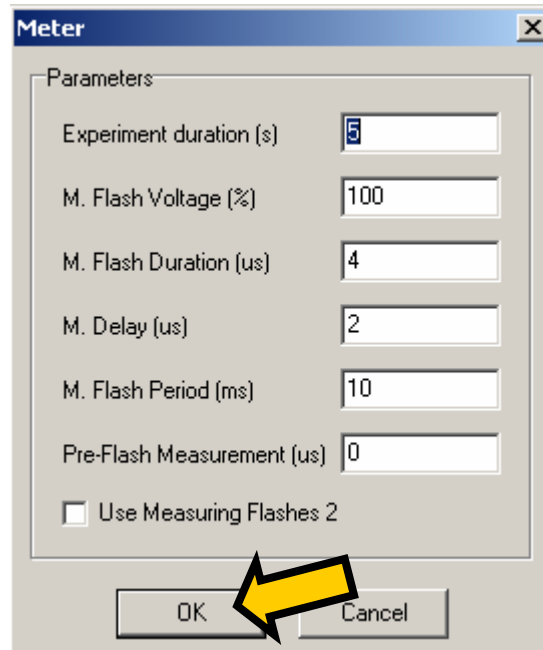


The Wizard will help you to write the first experimental protocols.

## 4.1. Meter

The simplest application Protocol can be prepared using the Meter option of the Wizard. It is only a periodic measurement of signal at the input channels. Press Next with Meter box checked (see arrows above).

The Meter Protocol Wizard allows modifying the protocol parameters.



*Experiment Duration* defines how long the measurement will last.

*M. Flash Voltage* defines the relative power of the Measuring Flashes. The fluorescence signal is proportional to the flash power. Care must be taken to avoid actinic effects of the Measuring Flashes. They must be either weak or given at a low frequency. Otherwise, the fluorescence emission will gradually increase due to  $Q_A$  reduction.

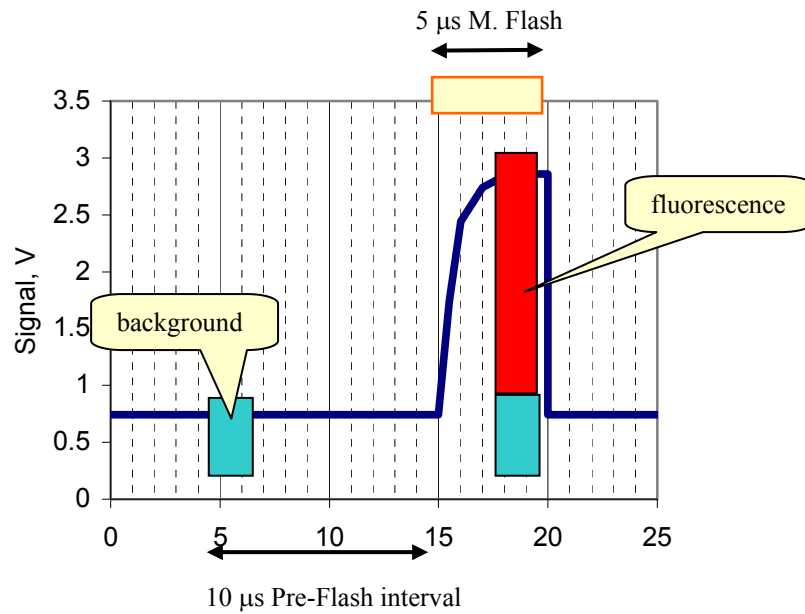
*M. Flash Duration* is mostly in the range 3 – 6 microseconds. The longer is the flash the more likely it is to cause actinic effects. However, if the flash is too short ( $\sim 2.5 \mu\text{s}$ ), the detector is unable to read full signal.

*M. Delay* is, in any standard application, by  $2 \mu\text{s}$  shorter than *M. Flash Duration*. Most users will not need to know more about this function. For special applications, it may be useful to know that the *Delay* defines the period between the Measuring Flash trigger and the  $2 \mu\text{s}$  long integration of the A/D converter. E.g. when measuring delayed luminescence, the *M. Flash Delay* will be longer than the measuring flash.

*M. Flash Period* defines how often the measuring flash is given and the measurement taken.

*Pre-Flash Measurement* defines if the background signal should be read prior each measuring flash (0 – NO) and, if so, how many microseconds before the flash. This function may help to eliminate artifacts originating, for example, from a strong background illumination or from a long tail of Xe-flashes.

The timing sequence of the `mfm` and `mfmsub` commands is explained in the following Figure.

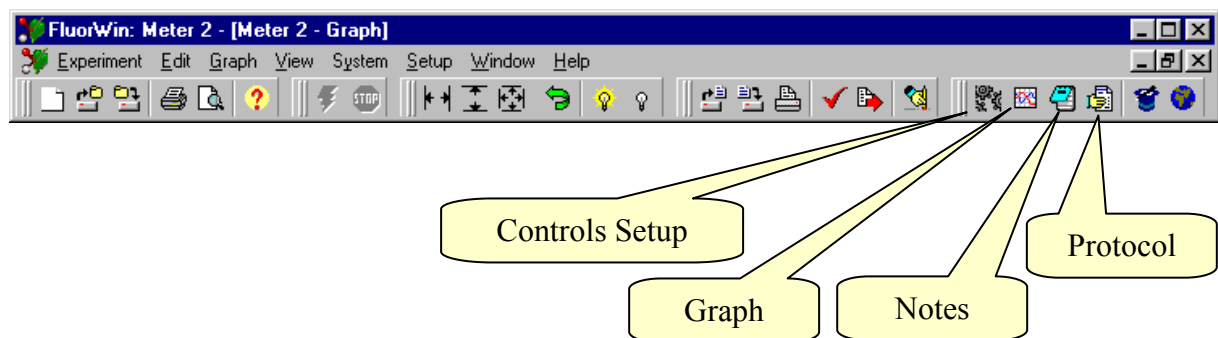


With a non-zero Pre-Flash parameter, a background measurement is done prior to the measuring flash trigger. The Pre-Flash parameter determines the interval between the background measurement and the M.Flash trigger (here 10  $\mu$ s). With `mfm` command, both measurements (background and background+fluorescence) are shown in Graph. Only the difference between the two measurements is shown with the `mfmsub` commands.

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Press Create to generate the Protocol (\*.p file) and to open a new Experiment that will use the Protocol.

Each Experiment (\*.exp file) consists of 4 information blocks: Protocol, Graph, Notes and Controls Setup. You may open the respective 4 windows of an Experiment using either the bottom part of the Window menu or the Navigator (globe icon) by choosing the Experiment name or by using the icons:



The *Graph* window presents the experimental data. This window is open in a new Experiment. Multiple pressing of this icon toggles the DataSet attribute info.

The *Protocol* window displays the current Protocol or any other Protocols used earlier in the Experiment.

The *Notes* window allows to make notes about the experiment and to save them with the data. This window works as a simple text processor. The Title of the Experiment specified in the Notes shows up also in the Graph window.

The *Control Setup* window allows setting manually the LED voltages. For advanced applications, it also allows to pre-define logical levels of the 15 pins at the rear panel of the Control Unit. These functions are rarely applied by most of the users.

#### 4.1.1. Protocol generated by the Meter Wizard

The Protocol may be read and edited (if needed) by opening the Protocol window (icon or Window menu or Navigator).

<pre> 1.      MeasuringFlash=5us 2.      ActinicFlash=0 3.      MeasurDelay=3us 4.      AuxDuration=0.2s 5.      PreFlash=0us 6.      include default.inc 7.      M_Voltage=75 8.      F_Voltage=0 9.      A_Voltage=0 10.     ;***** 11.     start=10ms 12.     stop=5s 13.     If (PreFlash GR 0) Then 14.     &lt;0,start..stop&gt;=&gt;mfm 15.     Else 16.     &lt;0,start..stop&gt;=&gt;fm 17.     End </pre>	
---	--

Line 1 – Measuring Flash (commands f, fm, mfm, mfmsub) will last 5 microseconds (= us =  $\mu$ s = mic). This will be a default setting that may be locally changed by a parameter in brackets: e.g., instruction f(2 $\mu$ s) anywhere in the Protocol will fire a measuring flash lasting 2 microseconds instead of default 5 microseconds.

Line 2 – Actinic Flash (commands F, F1 or F2) duration set to 0 will disable the function. This may be locally changed by a parameter in brackets: e.g. F1(30  $\mu$ s) will fire a 30 $\mu$ s-long Actinic Flash connected to F1 terminal of the Control Unit (usually Basic LED Unit).

Line 3 – Measuring Delay between the Measuring Flash trigger and start of the A/D integration is set to 3 $\mu$ s. In standard applications, this parameter is by 2 $\mu$ s shorter than the Measuring Flash duration.

Line 4 – Aux Duration sets the default duration of the pulses controlled by Aux 1-6 TTL outputs (commands A1, A2 ... A6) of the Control Unit to 0.2 second. Here the parameter is set arbitrarily because the A commands are not used in the Protocol. Otherwise, Aux Duration defines the duration of pulses of Actinic Light. The duration may be changed locally by a parameter in brackets, e.g., A1(20s) will switch on the light controlled by Aux 1 TTL output of the Control Unit for 20 s.

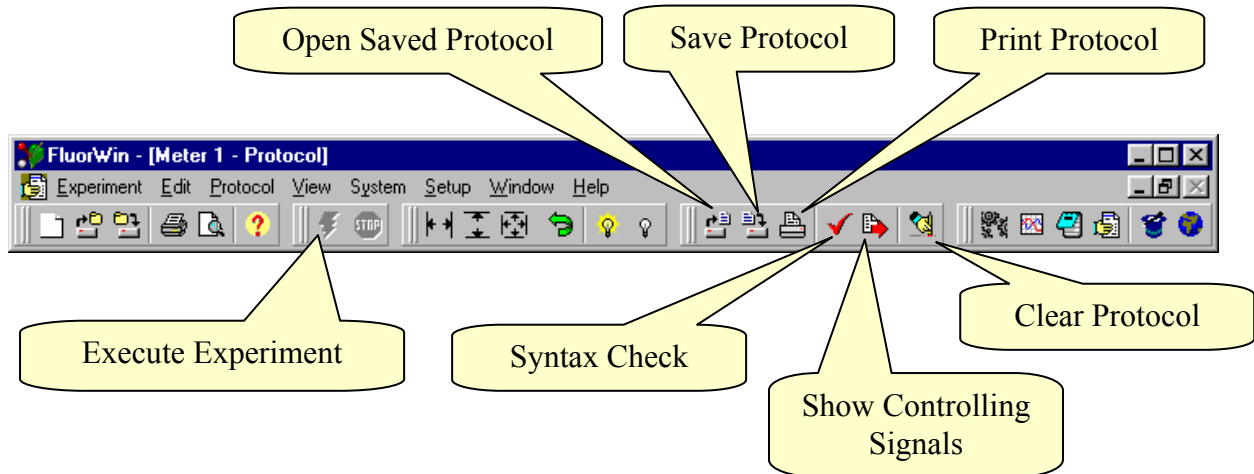
Line 5 – Pre-Flash parameter defines the background measurement prior to each fluorescence measurement (commands mfm, mfmsub). This function is disabled when Pre-Flash

is set to 0. When set for example to 10 $\mu$ s, a background measurement is taken 10 $\mu$ s before each measuring flash (recommended).

- Line 6 – File `default.inc` defines default functions of the software. Please, inquire at <http://www.psi.cz> before you attempt to modify your `default.inc` file.
- Line 7 – M. Voltage (commands `f`, `fm`, `mfm`, `mfmsub`) sets the relative voltage of the measuring LEDs. The signal is proportional to the voltage approximately in the range 20 – 100%.
- Line 8 – F. Voltage (commands `F`, `F1`, `F2`) sets the relative voltage of the Actinic Flash LEDs. The signal is proportional to the voltage approximately in the range 20 – 100%.
- Line 9 – A. Voltage (usually commands `A1`, `A2`, `A3`) sets the relative voltage of the Actinic Light LEDs. The signal is proportional to the voltage approximately in the range 20 – 100%.
- Line 10 – Any string subsequent to semicolon (;) is ignored by the compiler and serves only as a comment or separation line.
- Line 11 – Defines a parameter named `start` and gives it a value of 10ms. In the Meter protocol, it defines the period of measurements.
- Line 12 – Defines a parameter named `stop` and gives it a value of 5s.
- Line 13 – Defines conditional branching based on the value of PreFlash parameter defined in the Meter Wizard window. It decides if the background measurement should be taken prior to each measuring flash. If PreFlash is greater than (GR) 0, then Line 14 is executed. If equal to 0, then Line 16 is executed.
- Line 14 – `<0, start..stop>=>mfm`: Brackets `<>` define arithmetic (equidistant) timing series starting at time 0, second event (periodicity) at the time `start` (here 10ms) and the last event at the time `stop` (here 5s). Symbol `=>` means “execute” and the `mfm` command means “measure-flash-measure”: First, a background measurement is taken. Second, Measuring Flash is triggered. Third, measurement is taken usually 2 microseconds before the measuring flash is switched off. The background measurement is subtracted from the measurement during the Measuring Flash when the `mfm` command is replaced by `mfmsub`. The `mfm` and `mfmsub` commands read both Channel 1 and Channel 2. Only Channel 1 or 2 is read with commands `mfm1` (`mfm1sub`) and `mfm2` (`mfm1sub`), respectively.
- Line 16 – `<0, start..stop>=>fm`: The `fm` command means “flash-measure” and differs from `mfm` by skipped background measurement. Commands `fm1` and `fm2` would result in reading of the Channel 1 or Channel 2, respectively. Use command `fm` instead of `mfm` whenever there is no or little background signal. By doing so, the Protocol becomes faster and requiring less memory.

You may use the Meter Protocol for practicing the modifications of simple protocols, for instrument testing or for checking of actinic effects of measuring flashes given at different frequencies with different relative power and different flash duration.

The syntax of the Protocol may be checked prior to the experiment execution (Check mark icon). Protocols may be saved also separately from Experiments to generate a library.

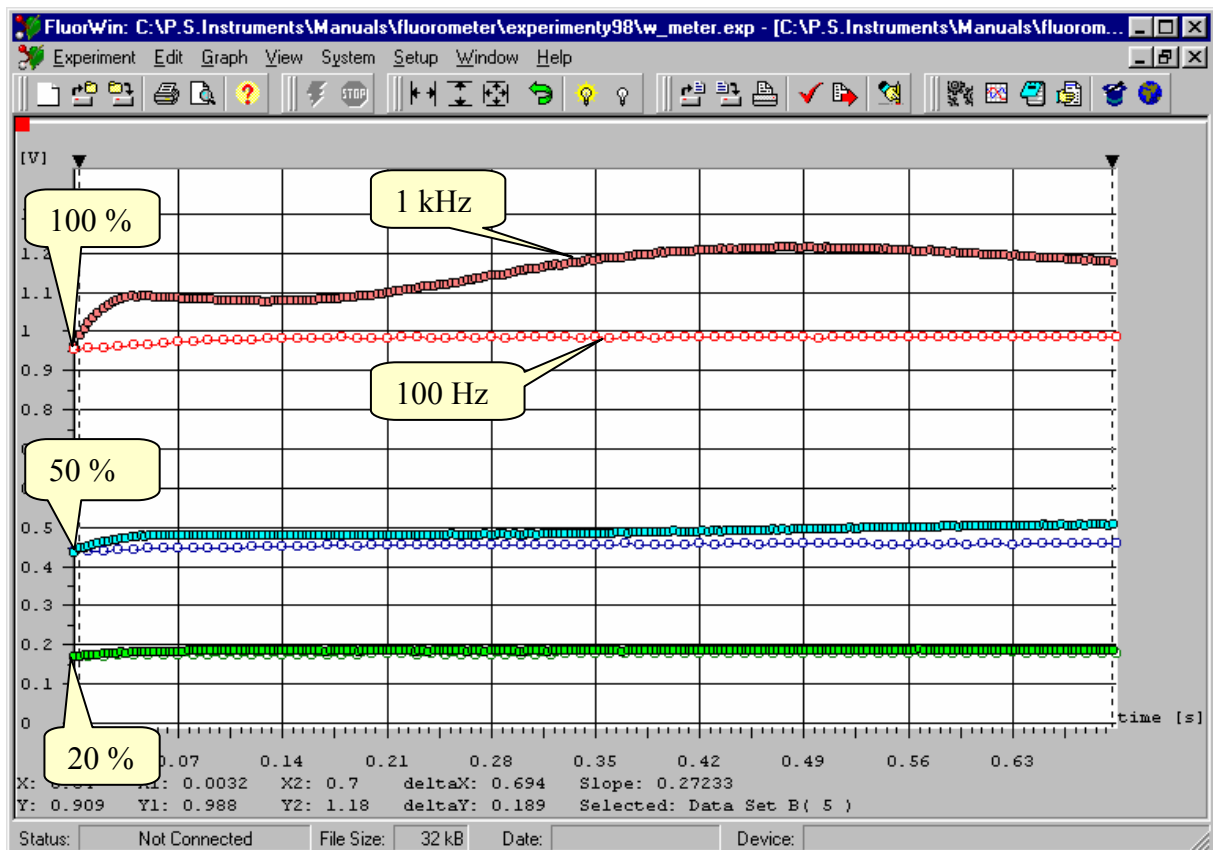


The experiment is executed by pressing the red “lightening” icon. The experiment cannot be executed if the icon is gray – signaling that either the instrument is not connected or that the present experiment is not active (Make Active in the Window menu).

#### 4.1.2. Application Example for Meter Wizard Protocol

The Meter application can be used, for example, to check the actinic effects of the measuring flashes.

Figure below shows fluorescence measurement (green alga *Scenedesmus quadricauda*) with 5  $\mu$ s measuring flashes of 20% (lowest two curves), 50% (middle two curves) and 100% power (upper two curves). The power of the measuring flashes is set by the Protocol line: M\_Voltage=20 (50,100). Please allow sufficient time between the Protocol application so that the LED capacitors can discharge to the newly set lower level.



The curves with open symbols show the measurement with 100 Hz frequency of measuring flashes while the closed symbols show measurements with 1 kHz frequency. Clearly, there are actinic effects at 1 kHz frequency even with measuring flashes of 20% power. At 1 kHz / 100%, the flashes elicit a strong fluorescence induction. The actinic effects are smaller with 100 Hz frequency compared to 1 kHz frequency.

The actinic effects of measuring flashes must be usually minimized to avoid distortion of the kinetic measurements. The actinic effects can be limited by using low power and short measuring flashes. The number of measuring flashes can be frequently minimized by using logarithmically spaced timing instead of periodic timing that was presented in this paragraph (see further). It is a good practice to check for the actinic effects with each new Protocol and each new organism.

## 4.2. $Q_A^-$ Reoxidation Kinetics

The Protocol measures  $F_0$ , executes a single-turnover flash to reduce  $Q_A$  acceptor and follows the subsequent decline of fluorescence that reflects the reoxidation kinetics. Press Next with QA- reoxidation box checked.

The QA- Reoxidation Protocol Wizard allows modifying the protocol parameters.

Protocol Wizard

QA- Reoxidation

Experiment Duration : 60 s      M. Flash Voltage : 20 %

No. DataPoints / Decade : 4      Pre-Flash Measurement : 0 μs

First DataPoint After F : 50 μs

A. Flash Voltage : 100 %

A. Flash Duration : 30 μs

Parallel Flash by :  Measur Flash  
 Aux 1  
 Aux 2  
 Aux 3

<< Previous    Next >>    Restore Defaults    Create    Cancel    Help

*Experiment Duration* defines how long the measurement will last.

*M.Flash Voltage* defines the relative power of the Measuring Flashes. The fluorescence signal is proportional to the flash power. Care must be taken to avoid actinic effects of the Measuring Flashes. They must be either weak or given at a low frequency.

*No. DataPoints / Decade*: The reoxidation is usually a multiexponential process with components as fast as hundreds of microseconds and as slow as many seconds. This dynamic range is well covered by logarithmic timing series that is defined by a constant number of datapoints per decade. This series always includes timing in decades ( $10^i$ ) and a constant number of measurements in each decade. The data looks equidistant on logarithmic time-axis. For example: with 4 datapoints per decade, the series may look like ....1, 1.78, 3.16, 5.62, 10, 17.8, 31.6, 56.2, 100 .... (=  $10^0$ ,  $10^{1/4}$ ,  $10^{2/4}$ ,  $10^{3/4}$ ,  $10^{4/4}$  ....)

*Pre-Flash Measurement* has the same function as in the Meter Wizard. Here it may be considered for reduction of flash artifacts in case of long-tail flashes.

*FirstDataPoint After F*: defines an approximate starting point for the logarithmic series used to measure reoxidation. The default value of  $50\mu\text{s}$  means that the first measurement will not be taken earlier than  $50\mu\text{s}$  after the Actinic Flash trigger. In case that 4 datapoints/decade are required, the first datapoint will be taken  $56.2\mu\text{s}$  after the Actinic Flash trigger. Please, note that for a  $30\mu\text{s}$ -long Actinic Flash the interval between its end and the first measuring flash is only ca.  $26.2\mu\text{s}$ .

*A.Flash Voltage* and *A. Flash duration* have an obvious meaning.

*Measuring Flash, Aux 1, Aux 2, Aux 3* checkboxes allow to trigger Measuring Flash and Actinic light LEDs controlled by Aux 1-3 and flash them in parallel to the Actinic Flash. This setting increases the power of the single-turnover flash.

Press Create to generate the Protocol (\*.p file) and to start a new Experiment.

#### **4.2.1. Protocol generated by the $Q_A^-$ Reoxidation Wizard**

The Protocol may be read and edited (if needed) by opening the Protocol window (icon or Window menu or Navigator).

```

1.     MeasuringFlash=2.5us
2.     ActinicFlash=30us
3.     MeasurDelay=500ns
4.     AuxDuration=ActinicFlash
5.     PreFlash=0us
6.     include default.inc
7.     M_Voltage=20
8.     F_Voltage=100
9.     A_Voltage=F_Voltage
10.    ;*****
11.    first=56.2341325190349us
12.    second=100us
13.    stop=60s
14.    bf=0s
15.    ba1=0s
16.    ba2=0s
17.    ba3=0s
18.    k=<200us,400us..800us>           ; Fo measurement
19.    i=1ms
20.    j=i+[first,second..i+stop]      ; Reoxidation Kinetics

21.    <i>=>F1,F2                       ; Actinic Flash

22.    ; measurements
23.    if(PreFlash GR 0) Then
24.    k|j=>mfmlsub
25.    else
26.    k|j=>fm1
27.    end

28.    if(bf GR 0)Then
29.    <i>=>f(ActinicFlash)
30.    end

31.    if(ba1 GR 0)Then
32.    <i>=>A1
33.    end

34.    if(ba2 GR 0)Then
35.    <i>=>A2
36.    end

37.    if(ba3 GR 0)then
38.    <i>=>A3
39.    end

```

- Line 1 – Measuring Flash (commands `f`, `fm` or `mfm`) will last 2.5 microseconds (=  $\mu\text{s}$  =  $\mu\text{s}$  =  $\mu\text{s}$ ).
- Line 2 – Actinic Flash (commands `F`, `F1` or `F2`) duration is set to  $30\mu\text{s}$ .
- Line 3 – Measuring Delay between the Measuring Flash trigger and the start of the A/D integration is set to  $500\text{ns}$  (=  $2.5\mu\text{s}$  –  $2\mu\text{s}$ ).
- Line 4 – Aux Duration sets the duration of pulses controlled by Aux 1-6 TTL outputs (commands `A1`, `A2` ... `A6`) to a value that is equal to Actinic Flash Duration ( $30\mu\text{s}$ ).
- Line 5 – Pre-Flash parameter defines the background measurement prior to each fluorescence measurement (command `mfm`).
- Line 6 – File `default.inc` defines default functions of the software. Please, inquire at <http://www.psi.cz> before you attempt to modify your `default.inc` file.
- Line 7 – M.Voltage (commands `f`, `fm`, `mfm`) sets the relative voltage of the measuring LEDs. The signal is proportional to the voltage approximately in the range 20 – 100%. Here it is set to 20% of the maximal power.
- Line 8 – F.Voltage (commands `F`, `F1`, `F2`) sets the relative voltage of the Actinic Flash LEDs. The signal is proportional to the voltage approximately in the range 20 – 100%. Here it is set to the full power.
- Line 9 – A.Voltage (usually commands `A1`, `A2`, `A3`) sets the relative voltage of the Actinic Light LEDs. The signal is proportional to the voltage approximately in the range 20 – 100%. Here it is set as F.Voltage to the full power.
- Line 10 – Any string subsequent to semicolon (;) is ignored by the compiler and serves only as a comment or separation line.
- Line 11 – Defines a parameter named `first` and gives it a value of  $56\mu\text{s}$ . Here, it defines the period between the Actinic Flash trigger and the first Measuring Flash. It is longer than  $50\mu\text{s}$  and conforms with the requirement of 4 datapoints / decade. It equals  $10^{7/4}\mu\text{s}$ .
- Line 12 – Defines a parameter named `second` and gives it a value of  $100\mu\text{s}$ . This is the second timing following  $56\mu\text{s}$  that conforms with the requirement of 4 datapoints / decade. It equals  $10^{8/4}\mu\text{s}$ .
- Line 13 – Defines a parameter named `stop` and gives it a value of 60s. This determines the end of the experiment. The last datapoint taken for the  $\text{Q}_A^-$  reoxidation will be ca. 56s ( $10^{31/4}\mu\text{s}$ ).
- Line 14 to 17 – Define a parameters named `bf`, `ba1`, `ba2`, `ba3` and give them value 0 or 1 depending if the checkboxes firing parallel `f`, `A1`, `A2`, `A3` flashes were selected in the Wizard window. These parameters are of a logical (YES/NO) meaning.
- Line 18 – `k=<200us,400us..800us>`: the variable `k` defines an arithmetic series (brackets `<>`). Every arithmetic series is defined by its first two datapoints and by the last datapoint. The variable `k` will be used to measure  $\text{F}_0$  every  $200\mu\text{s}$  during the first millisecond of the Experiment.
- Line 19 – `i=1ms`: The variable `i` defines timing of the Actinic Flash.
- Line 20 – `j=i+[first,second..i+stop]`: The variable `j` defines the logarithmic series for the reoxidation measurements. The logarithmic series

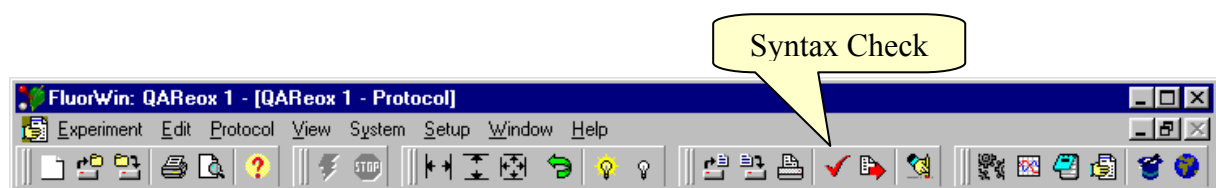
[*first,second...i+stop*] is identified by the square brackets [ ] and defined by the first two datapoints (*first, second*) and by the last datapoint (*i+stop*).

Line 21 –  $\langle i \rangle \Rightarrow F1, F2$  means: at the time  $i$  (=1ms) execute ( $\Rightarrow$ ) the Actinic Flash from both the Basic LED Unit (F1) and from the Optional LED Unit (F2).

Lines 23 to 27 – Define timing for fluorescence measurements. Both  $F_0$  ( $k$  variable) and reoxidation ( $j$  variable) are combined in conditional branching based on the value of PreFlash parameter defined in the Wizard window. Either  $k|j \Rightarrow mfmsub$  or  $k|j \Rightarrow fm$  commands are executed. Formula  $k|j$  combines timing defined by the arithmetic series ( $k$ ) and by logarithmic series ( $j$ ) into one mixed timing series ( $k|j$ ). Symbol  $\Rightarrow$  means execute. Command  $mfmsub$  means “measure-flash-measure” (as in the Meter Wizard) with subtraction of the background measurement. Command  $fm$  means “flash-measure”. Use  $fm1, fm2, mfm1, mfm2, mfm1sub$  or  $mfm2sub$  to read only one Channel.

Line 28 to 39 – Allow parallel flash at the time  $i$  by the Measuring Flash LEDs ( $bf$ ) and by A1-A3 LEDs ( $ba1-ba3$ ).

The syntax of the Protocol may be checked (recommended with all new protocols) by the Syntax Check function.

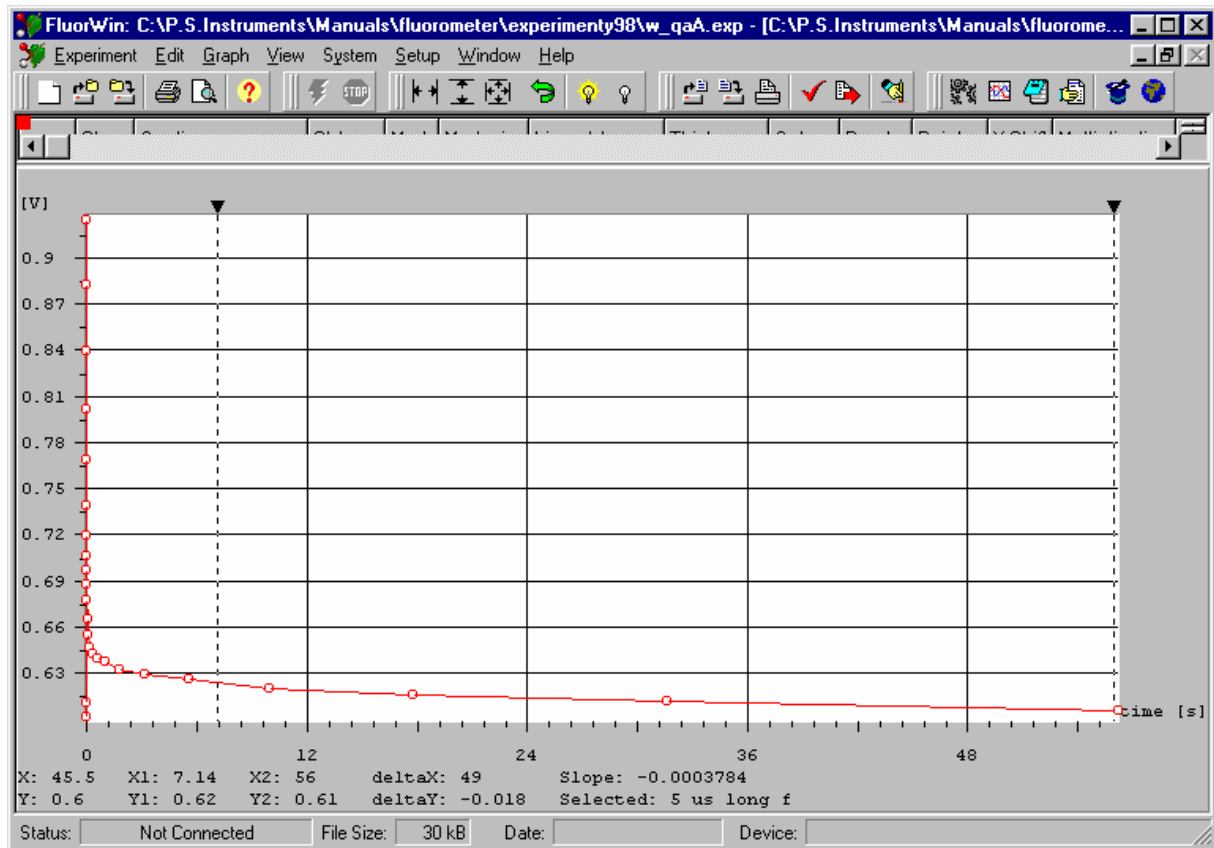


Upon successful syntax check, this function also generates a table of variables in the right-hand part of the Protocol window and downloads the protocol into the Control Unit. The horizontal LED indicators start showing the relative power set in the Protocol for LED Units.

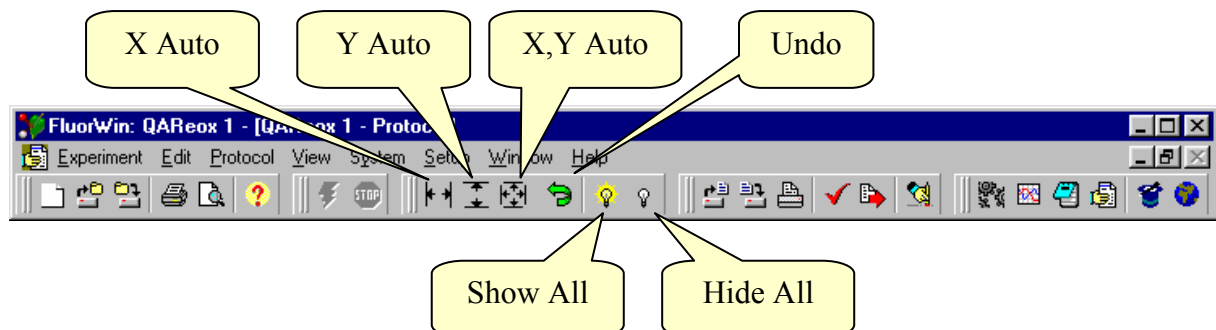
The lightning icon is used to trigger the experiment. The progress of the experiment is indicated in the Status info at the bottom left bar of the FluorWin window. Also the LED diodes on the front panel of the Control Unit are indicating what happens in the cuvette.

#### 4.2.2. Application Example for $Q_A^-$ Wizard Protocol

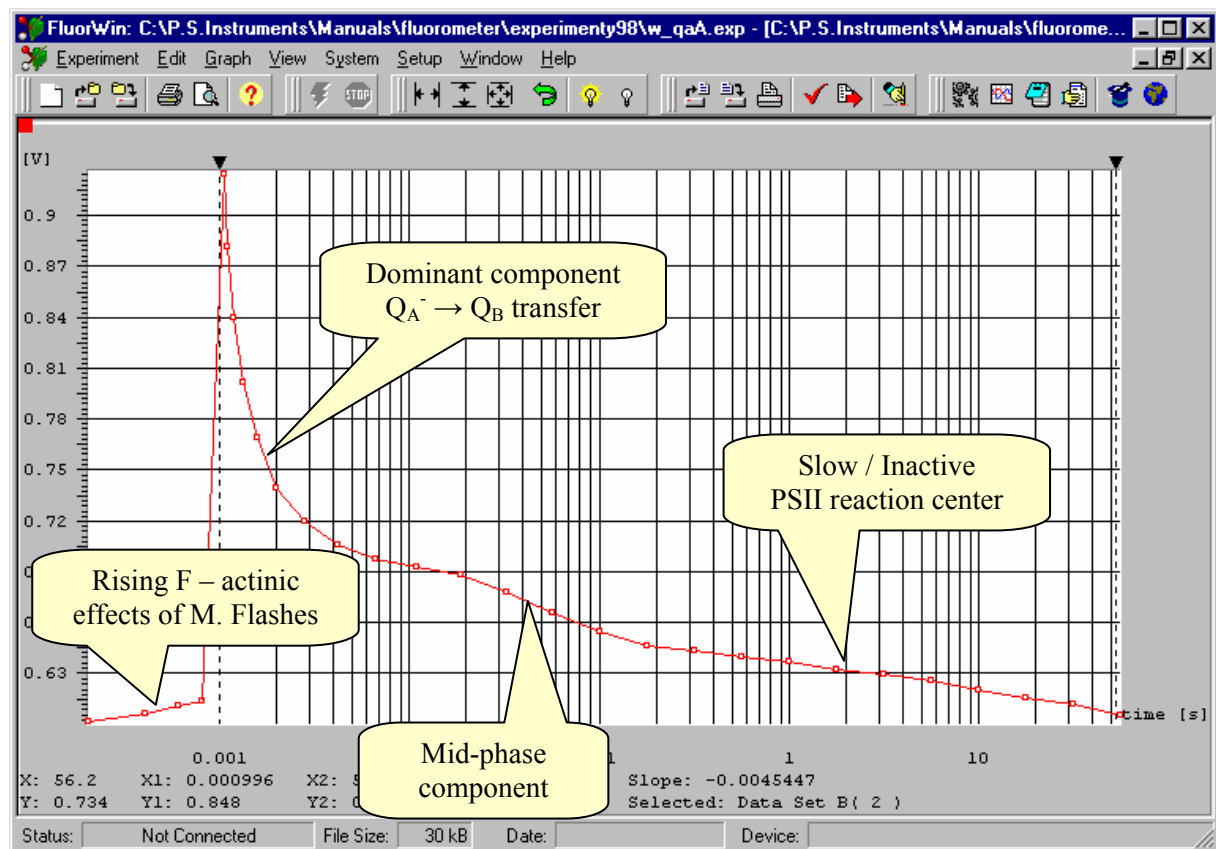
The resulting data may look as shown in the following Figure:



The data are shown on a linear time scale and it is difficult to estimate the reoxidation kinetics. The Graph menu, icons or zoom by cursor can be used to adjust the graphic presentation. The computer mouse can be used to zoom on the details of the graph. Press the left button and move the cursor on the screen to define the rectangular area for zoom. Any mistake can be corrected by the Undo icon on the menu bar. Autoscale buttons can be used to change X, Y or both axes to display all data points.

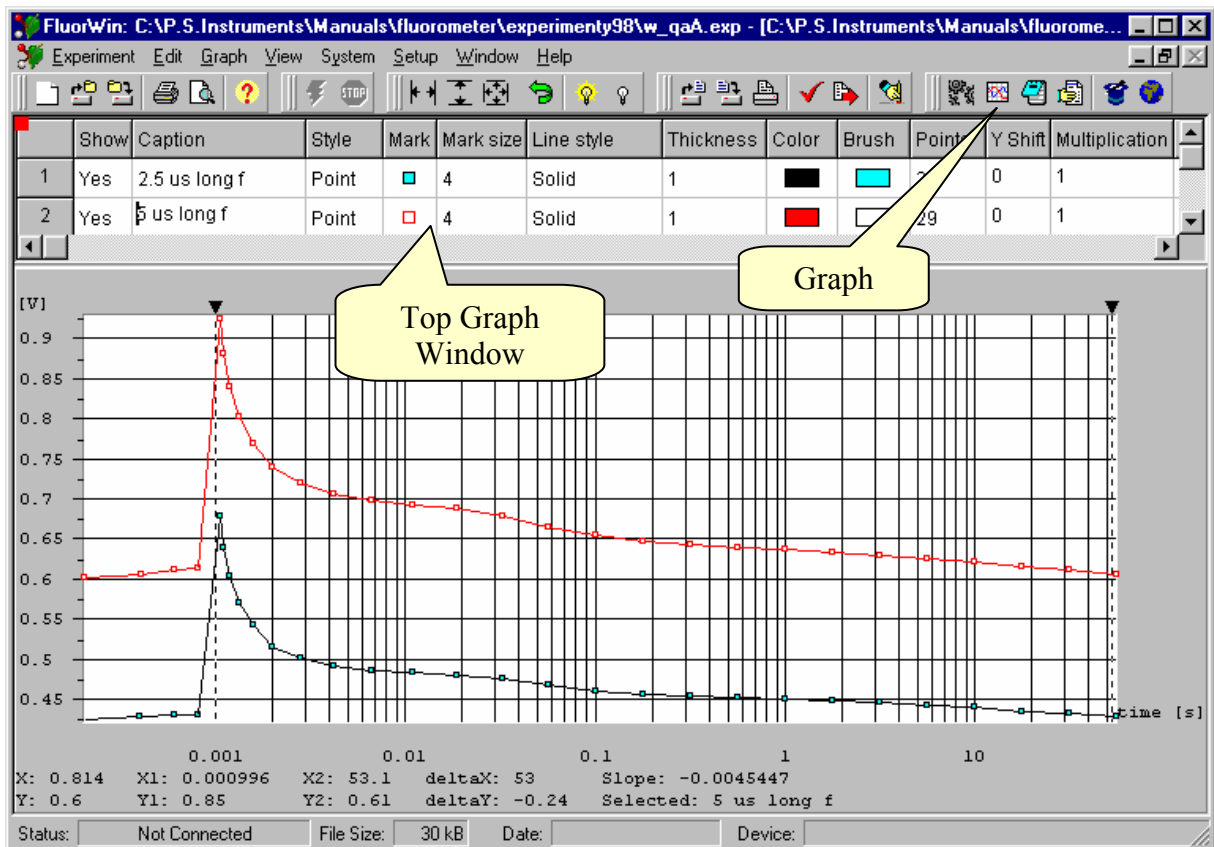


By selecting the Log scale in the Graph menu, the data are presented on a logarithmic time scale that is much better to display kinetics that spans over many decades. Here, 4 datapoints per decade were set in the QA- Wizard window.

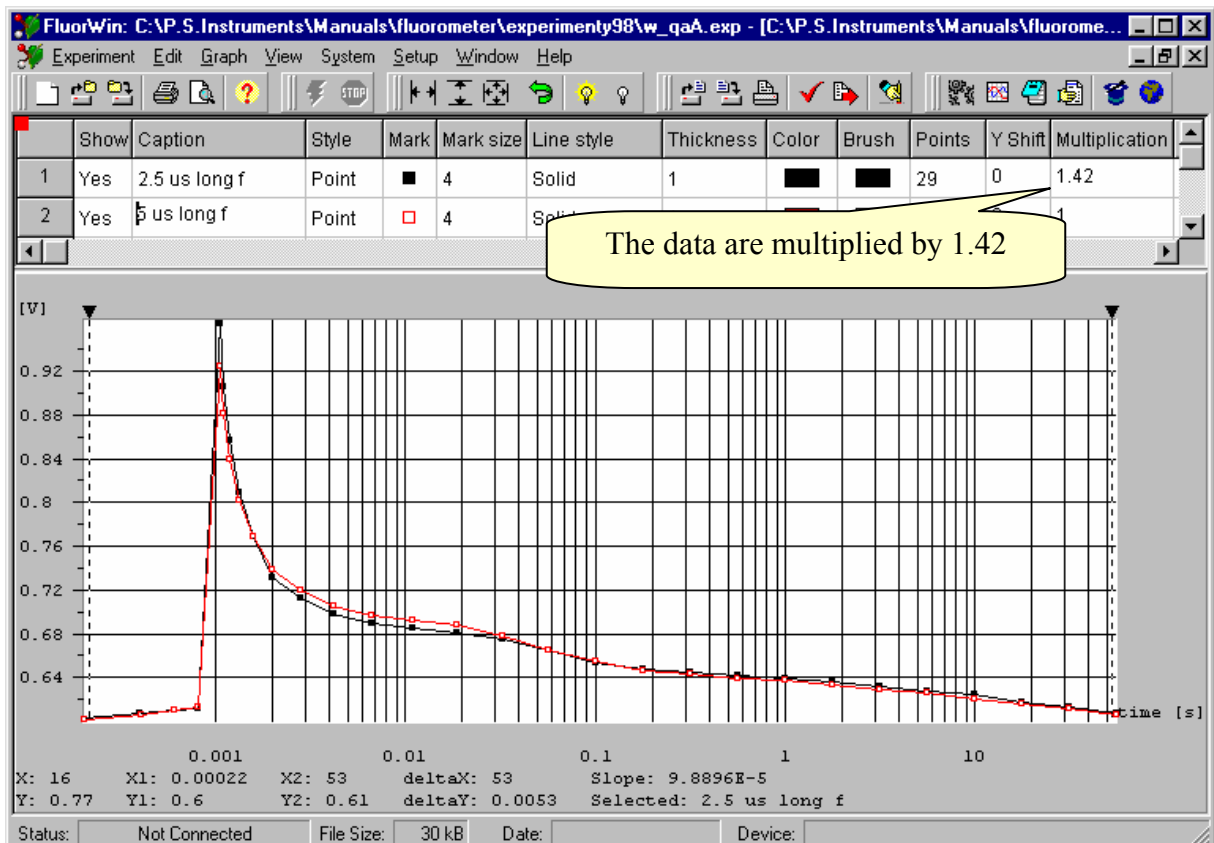


The fluorescence rising from  $F_0$  prior to the Actinic Flash indicates that the measuring flashes (100%, 5  $\mu$ s, 200  $\mu$ s period) have actinic effects. One way, how to test if these actinic effects of measuring flashes influence the measured kinetics is to lower the energy carried by the measuring flashes. The energy may be lowered by the Measuring Flash power (e.g.  $M\_Voltage=50$ ) or by shortening the Measuring Flash duration (e.g.  $MeasuringFlash=2.5\mu s$ ,  $MeasurDelay=500ns$ ). Both ways result in lower signals. The next Figure shows a comparison of fluorescence transients obtained with 5 and 2.5  $\mu$ s long measuring flashes.

The comparison is complicated by unequal signals that are caused by the response time of the detection that is longer than the 2.5  $\mu$ s measuring flash. The transients may be compared by normalizing the data using the top window of Graph where the data attributes are shown and simple data transformations can be achieved. The window is displayed / hidden by repeated pressing of the Graph icon.

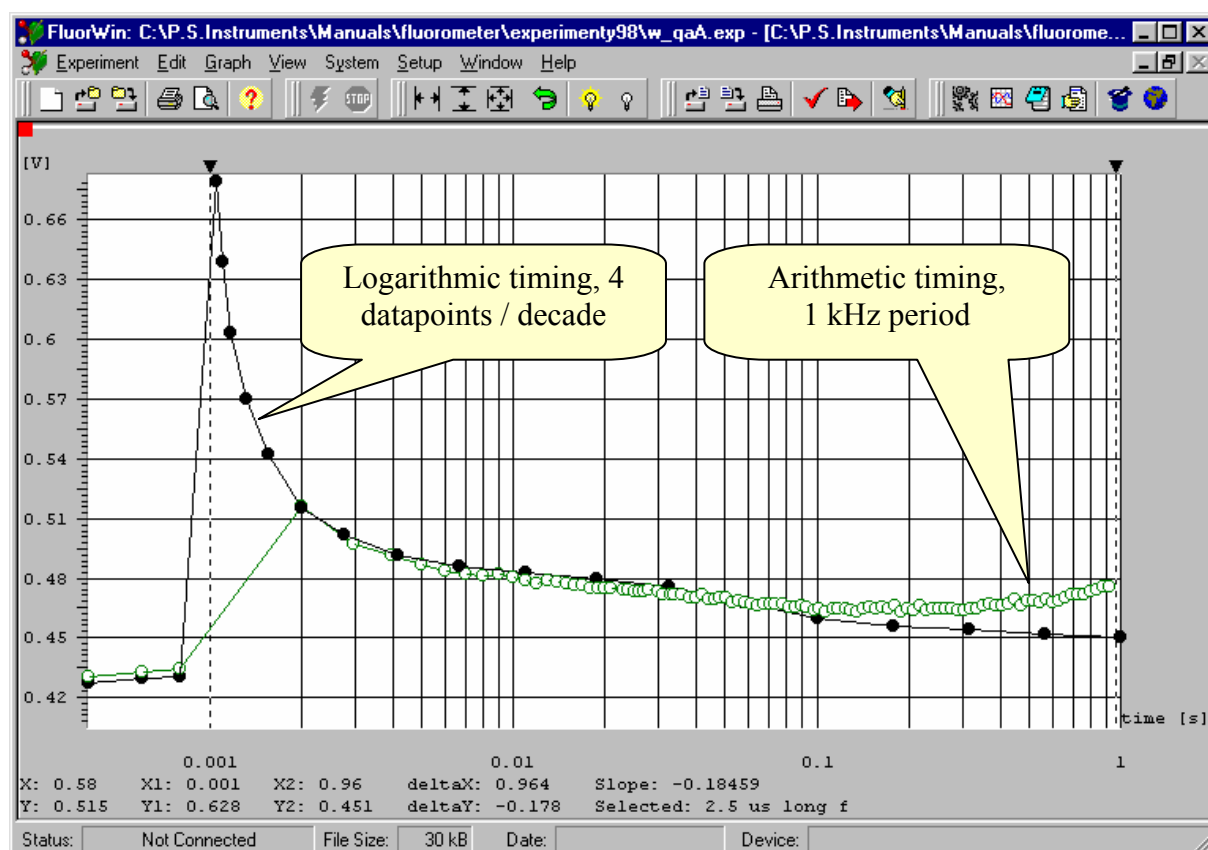


Multiplication of the lower signal transient results in re-normalization to an identical  $F_0$ .



The comparison of the two traces shows that the lowering of the Measuring Flash energy achieved by flash shortening did not cause any significant modification in the measured kinetics. It is made possible by the logarithmic timing series used for the measuring flashes. Only 29 measuring flashes (including 4 for  $F_0$ ) were enough to measure kinetics including the fastest (hundreds of microseconds) as well as the slowest (seconds) components.

For comparison, we show what would be the result if flashes of equal energy are used with periodic timing. In the Protocol, the logarithmic timing series ( $j=i+[first, second..i+stop]$ ) was replaced by arithmetic series (e.g.  $j=i+<1ms, 2ms..1s>$  for 1 kHz period).

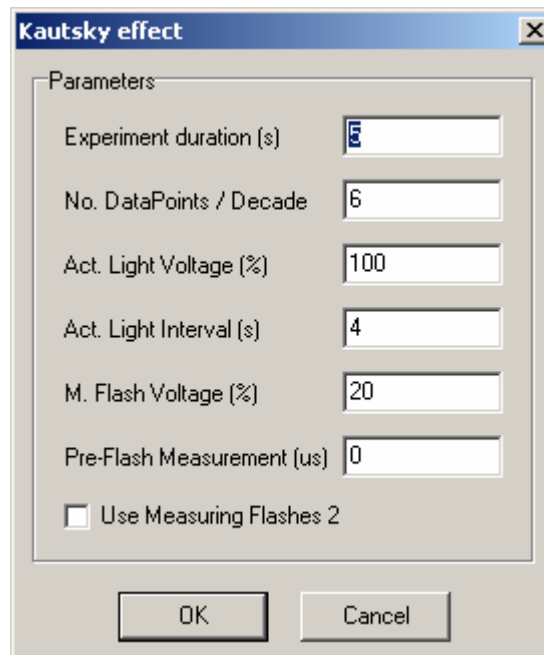


With arithmetic timing, the fastest components of the transient is not sufficiently covered and strong actinic effects of the too frequent Measuring Flashes are obvious in the long-time region that reflects the reaction centers with slow  $Q_A^-$  reoxidation.

### 4.3. Kautsky effect

Fluorescence induction (Kautsky effect) are another frequently measured phenomenon. The Protocol measures  $F_0$  and switches on Actinic Light that elicits the induction.

The Kautsky effect Protocol Wizard allows modifying the protocol parameters.



Most of the parameters have the same meaning as in the QA- Wizard window. Actinic Light Interval can be shorter than Experiment duration when dark relaxation after the induction is studied.

*Pre-Flash Measurement* has the same function as in Meter and QA- Wizards. Here it is however highly important. Fluorescence is excited not only by Measuring Flashes but also by Actinic Light. Usually the Measuring Flashes are much brighter (very short) so that the fluorescence excited by continuous Actinic Light may be neglected. If it is not the case, it is necessary to set the Pre-Flash Measurement to a longer than 0 interval (usually 10  $\mu$ s). Then, the fluorescence from the Actinic Light is measured (10  $\mu$ s) before the Measuring Flash trigger and subtracted from the fluorescence elicited by Measuring Flash and Actinic Light (mfmsub, mfm1sub, mfm2sub commands). This function makes it possible to eliminate or reduce artifacts from the Actinic Light or any other background signal.

#### ***4.3.1. Protocol generated by the Kautsky effect Wizard***

The Protocol may be read and edited (if needed) by opening the Protocol window (icon or Window menu or Navigator).

```

1. ;Kautsky Effect - This source was generated by the Wizard
2. MeasuringFlash=4us
3. ActinicFlash=0us
4. MeasurDelay=2us
5. AuxDuration=4s
6. PreFlash=10us
7. include default.inc
8. M_Voltage=20
9. F_Voltage=0
10. A_Voltage=100
11. ;*****

12. first=1ms
13. second=1.122018454302ms
14. stop=2s

15. ; Fo MEASUREMENT -----

```

```

16. h=<200us,400us..800us>
17. if(PreFlash GR 0) Then
18. h=>mfmlsub
19. else
20. h=>fm1
21. end

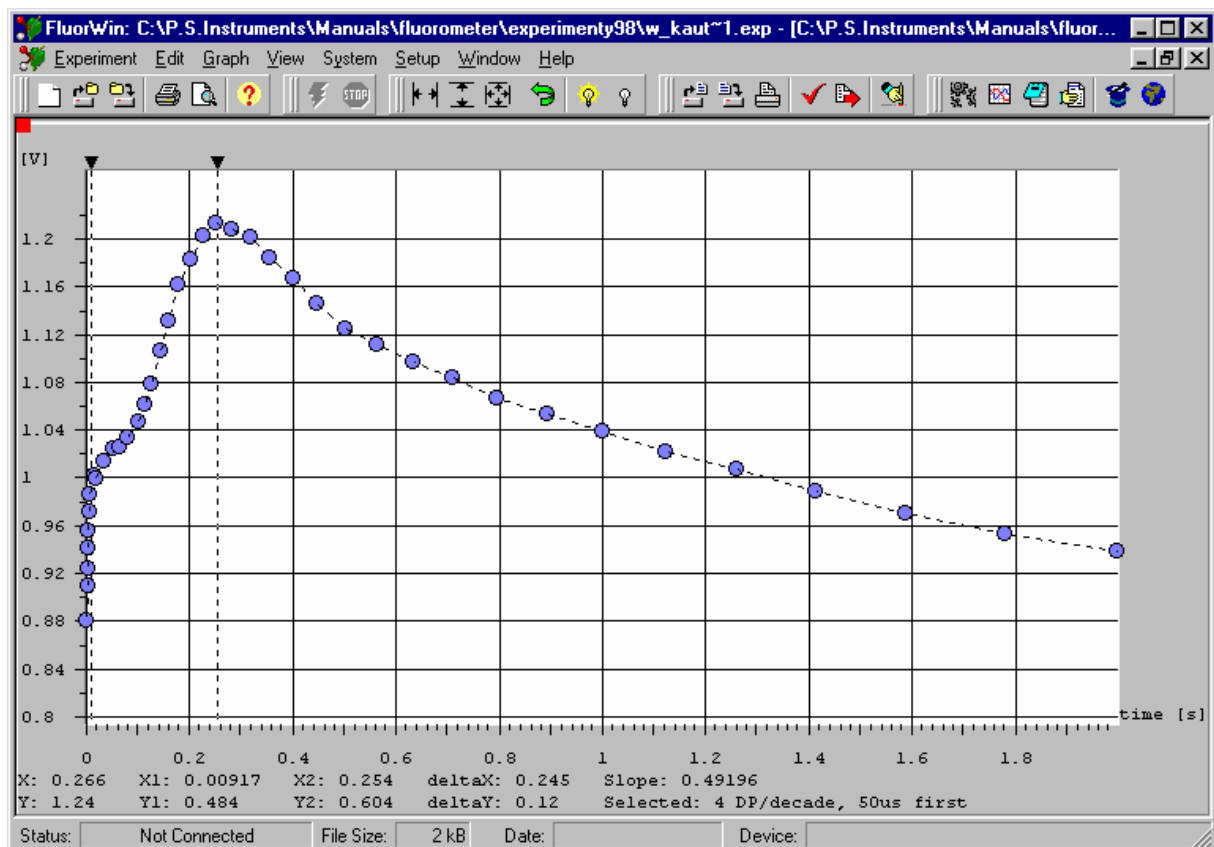
22. ; ACTINIC LIGHT -----
23. i=1ms
24. <i>=>A1

25. ; KAUTSKY EFFECT -----
26. j=i+[first,second..stop]

27. if(PreFlash GR 0)then
28. j=>mfmlsub
29. else
30. j=>fm1
31. end

```

The Kautsky effect Protocol resembles the QA- Protocol. Here, Actinic Light is switched on instead of the Actinic Flash trigger. Major difference between the two Protocols is in the relevance of the mfmsub (mfmlsub, mfm2sub) commands in Kautsky effect measurements. The continuous fluorescence from the background Actinic Light must be frequently subtracted and it is done by mfmsub (mfmlsub, mfm2sub) commands that measure the background signal before the Measuring Flash and fluorescence + background during the Measuring Flash. The background is subtracted from the measurement during the flash and the result is displayed in the Graph.



The Figure shows Kautsky effect in *Scenedesmus quadricauda* measured by the Wizard generated Protocol. It is obvious that again the logarithmic timing series for measurements is very convenient. The fast processes at the beginning of the induction are measured in detail while the later slower processes are measured much less frequently.

---

For those interested in details of the logarithmic timing used by Wizard:

The series [first, second..stop] are defined here so that always  $first = 10^{(n/N)}$  and  $second = 10^{((n+1)/N)}$ , where N is the number of DataPoints / decade and n is a whole number. Stop defines the end of the experiment. In the case that a faster sampling of the initial induction rise is required, you may modify the series in the Wizard generated Protocol.

#### 4.4. Quenching analysis

The analysis of fluorescence quenching is usually based on a comparison of the maximal fluorescence emission ( $F_{max}$ ,  $F_{max}'$ ) before and during an exposure to Actinic Light. The  $F_{max}$  ( $F_{max}'$ ) is measured as fluorescence emission that corresponds to the reduction of the primary quinone acceptor in all PSII reaction centers. This can be achieved in several ways:

- With added herbicide, the  $F_{max}^{DCMU}$  can be reached even in moderate light or in a short flash. The PSII reaction centers are in the state [ $Q_A^-$  / herbicide /  $PQ_{oxidized}$  state / S-states advanced by 1].
- In a single-turnover saturating flash ( $F_{max}^{flash}$ ), the PSII reaction centers are in the state [ $Q_A^-$  /  $PQ_{oxidized}$  / S-states advanced by 1].
- Frequently a long pulse ( $F_{max}^{long}$ ) of continuous light is used to bring the reaction centers to the state [ $Q_A^-$  /  $PQ_{reduced}$  state / S-states scrambled].

The  $F_{max}^{DCMU}$ ,  $F_{max}^{long}$  and  $F_{max}^{flash}$  parameters are measured in different states of the photosynthetic apparatus and are not necessarily equal. It is up to the user to decide on which parameter the quenching analysis must be based. Corresponding Protocols can be easily designed in FluorWin editor or generated by the Wizard.

The Quenching Analysis Wizard offers a Protocol that is generating well defined states of photosynthetic apparatus in short series of single-turnover saturating flashes. This technique minimizes the impact of actinic flashes ( $F_{max}$  measurements) on the experiment results and also allows an interpretation based on quantitative considerations (e.g. number of turnovers during the  $F_{max}$  measurement). The Quenching analysis Protocol is based on the Kautsky effect Protocol. The difference is only in added  $F_{max}$  ( $F_{max}'$ ) measurements. Prior to the Actinic Light period, the  $F_{max}$  is measured in a short series of single-turnover saturating flashes. The measurement of  $F_{max}'$  is repeated during the Actinic Light period when various quenching mechanisms are becoming effective.

The Wizard definition window allows to define experimental parameters similar to the other Wizard windows. In addition to parameters of the Kautsky effect Wizard, the Actinic Flash power is requested for the  $F_{max}$  measurements.

**4.4.1. Protocol generated by the quenching analysis Wizard**

```

1. ;Quenching Analysis - This source was generated by the Wizard
2. MeasuringFlash=2.5us
3. ActinicFlash=30us
4. MeasurDelay=500ns
5. AuxDuration=300s
6. PreFlash=10us
7. include default.inc ; Include standard options, don't remove it !
8. ;Delete semicolons for activate Auto set DACs and ignore visual
   settings
9. M_Voltage=20
10. F_Voltage=80
11. A_Voltage=50
12. ;*****

13. ; Fmax ACTION DEFINITION
14. Action Fmax(Count,Step) begin
15. <100us>=>mfmlsub
16. z=100us+<Step,2Num*Step..Count*Step>
17. z=>F1,F2
18. z#<100us>=>mfmlsub
19. end

20. ; Fo MEASUREMENT -----
21. a=<1s,2s..29s>
22. if(PreFlash GR 0) Then
23. a=>mfmlsub
24. else
25. a=>fm1
26. end

27. ; Fmax MEASUREMENT
28. <10s>=>Fmax(10Num,500us)

29. ; ACTINIC LIGHT -----
30. b=30s
31. <b>=>A1

32. ; KAUTSKY EFFECT -----
33. first=1ms
34. second=1.122018454302ms; 20 DataPoints / decade

35. j=b+[first,second..stop]

36. if(PreFlash GR 0)then
37. j=>mfmlsub
38. else
39. j=>fm1
40. end

41. ; QUENCHING ANALYSIS
42. x=b+[5.6234132519s,10s..stop]
43. x=>Fmax(10Num,500us)

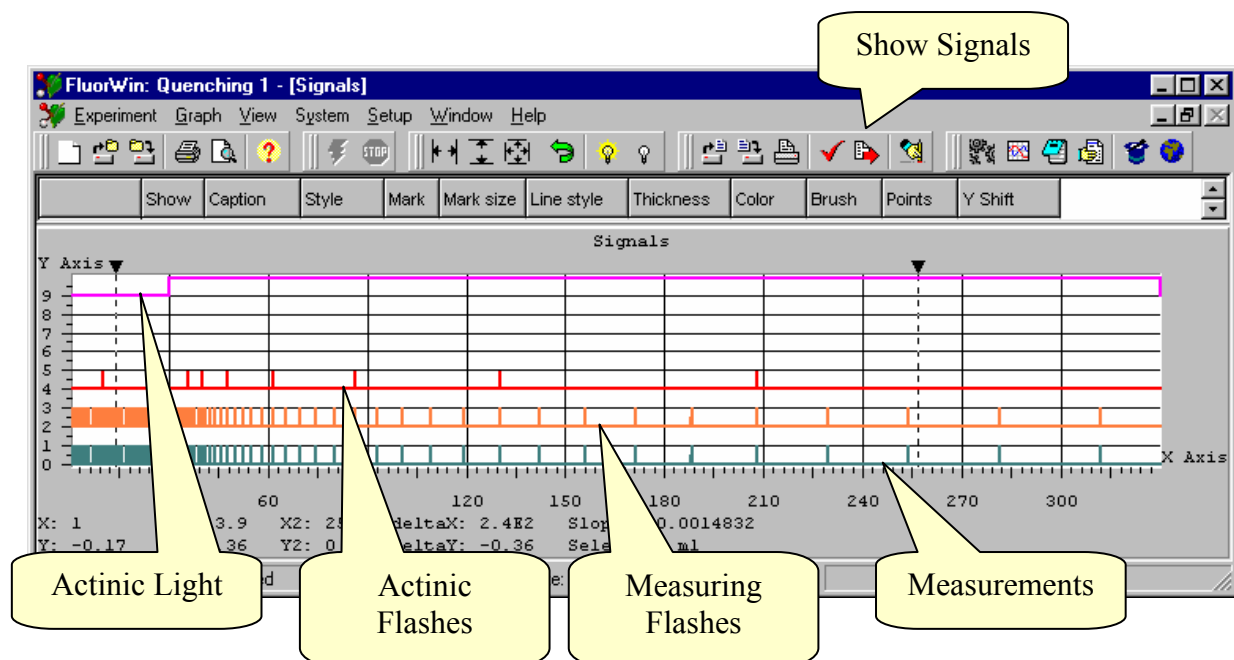
```

Lines 13 – 19 introduce a new feature called Action. Line 13 is a comment noting the name of the Action (Fmax). Line 14 specifies the format of the Action Fmax(Count,Step). There are two parameters: Count specifying how many Actinic Flashes (F) are used to measure Fmax and Step specifying the interval between the flashes. The Action Fmax starts

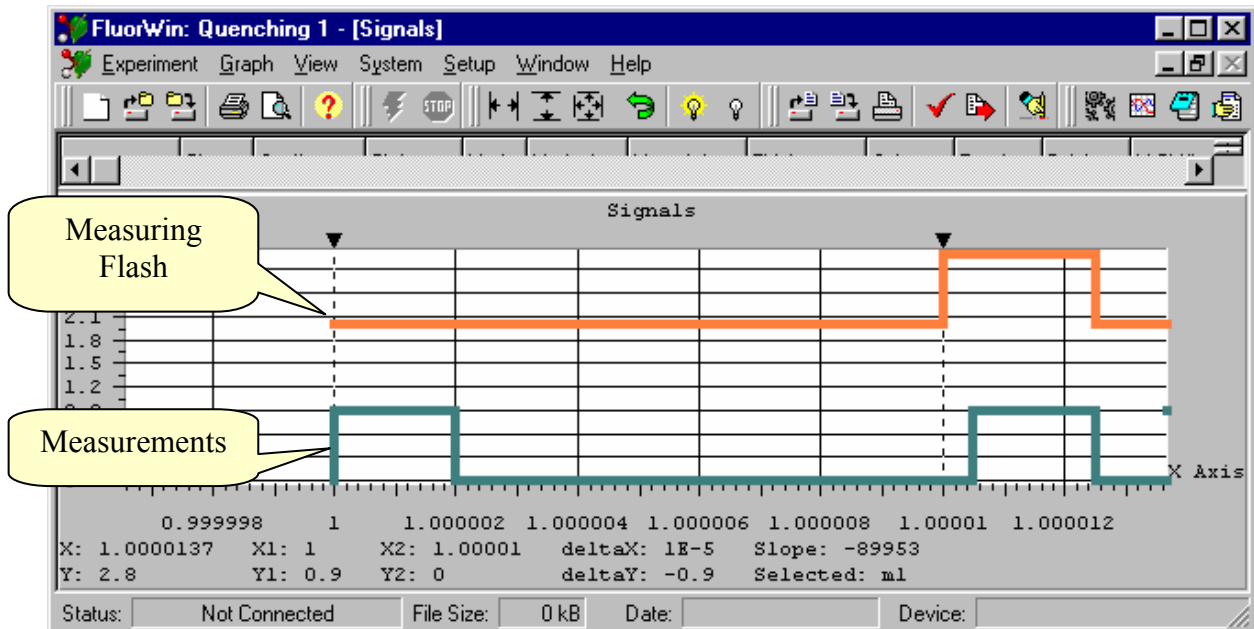
with `mfm1sub` command executed  $100\ \mu\text{s}$  after the Action is called in the Protocol (line 15). Line 16 defines a variable  $z=100\mu\text{s}+\langle\text{Step}, 2\text{Num}*\text{Step}.. \text{Count}*\text{Step}\rangle$  that serves to define timing of the Actinic Flashes. It is an arithmetic series shifted by  $100\ \mu\text{s}$  from the moment when the Action is called. Line 17 triggers the Actinic Flashes F1 and F2. Line 18 ( $z\#\langle 100\mu\text{s}\rangle\Rightarrow\text{mfm1sub}$ ) executes a fluorescence measurement  $100\ \mu\text{s}$  after each Actinic Flash. The sign # stands for the following timing series operation: with two timing series  $a=(a_1,a_2,a_3\dots)$  and  $b=(b_1, b_2, b_3\dots)$  the resulting series is  $a\#b = (a_1+b_1, a_1+b_2, a_1+b_3 \dots, a_2+b_1, a_2+b_2, a_2+b_3 \dots, a_3+b_1, a_3+b_2, a_3+b_3 \dots)$ .

Line 28 ( $\langle 10\text{s}\rangle\Rightarrow\text{Fmax}(10\text{Num}, 500\mu\text{s})$ ) executes the Fmax Action 10 seconds after the experiment was started. There will be 10 Actinic Flashes as  $\text{Count}=10\text{Num}$  (Num attribute specifies that the Count variable is numeric without physical dimension). The flashes will be separated by  $\text{Step} = 500\mu\text{s}$ . Similarly, the line 43 executes the Fmax measurement with logarithmic timing ( $x=b+[5.6234132519\text{s}, 10\text{s}.. \text{stop}]$ ) during the Actinic Light exposure.

Each Protocol can be visualized using the Show Signals icon.



The left mouse button can be used to zoom in the Signals window. The detail shows timing of a single mfm1sub command. At the time 1s, the first measurement (background) is executed. The integration time of the measurement (A/D converter) is 2  $\mu$ s. With a delay of 10  $\mu$ s, the measuring flash lasting 2.5  $\mu$ s is triggered. 0.5  $\mu$ s later, the second measurement (fluorescence + background) is executed. The two measurements are subtracted to get the fluorescence signal free of the background.



## 4.5. S-states

The fluorescence emission is dependent not only on  $Q_A$  redox state but, among other factors, also on the state of the Oxygen-Evolution Complex (S-states). There are five S-states:  $S_0 - S_4$ . A dark adapted organism has the PSII RCs statistically in a mixture of  $S_0$  and  $S_1$  states ( $S_0 / S_1$ ). By Actinic Flashes the S-states are advanced through  $S_1 / S_2$  (1<sup>st</sup> flash),  $S_2 / S_3$  (2<sup>nd</sup> flash),  $S_3 / S_4 \rightarrow S_0$  (3<sup>rd</sup> flash),  $S_4 \rightarrow S_0 / S_1$  (4th flash). This cycle explains periodicity of four in the oxygen evolution. A corresponding periodicity is observed also in the fluorescence quantum yield.

### 4.5.1. Protocol generated by the S-States Wizard

1. ;S-States - This source was generated by the Wizard
2. MeasuringFlash=2.5us
3. ActinicFlash=30us
4. MeasurDelay=500ns
5. AuxDuration=1us
6. PreFlash=0
7. include default.inc ; Include standard options, don't remove it !
8. M\_Voltage=20
9. F\_Voltage=100
10. A\_Voltage=F\_Voltage
11. ;\*\*\*\*\*
12. FlashPeriod=100ms
13. NFlashes=10
14. ; Fo measurement

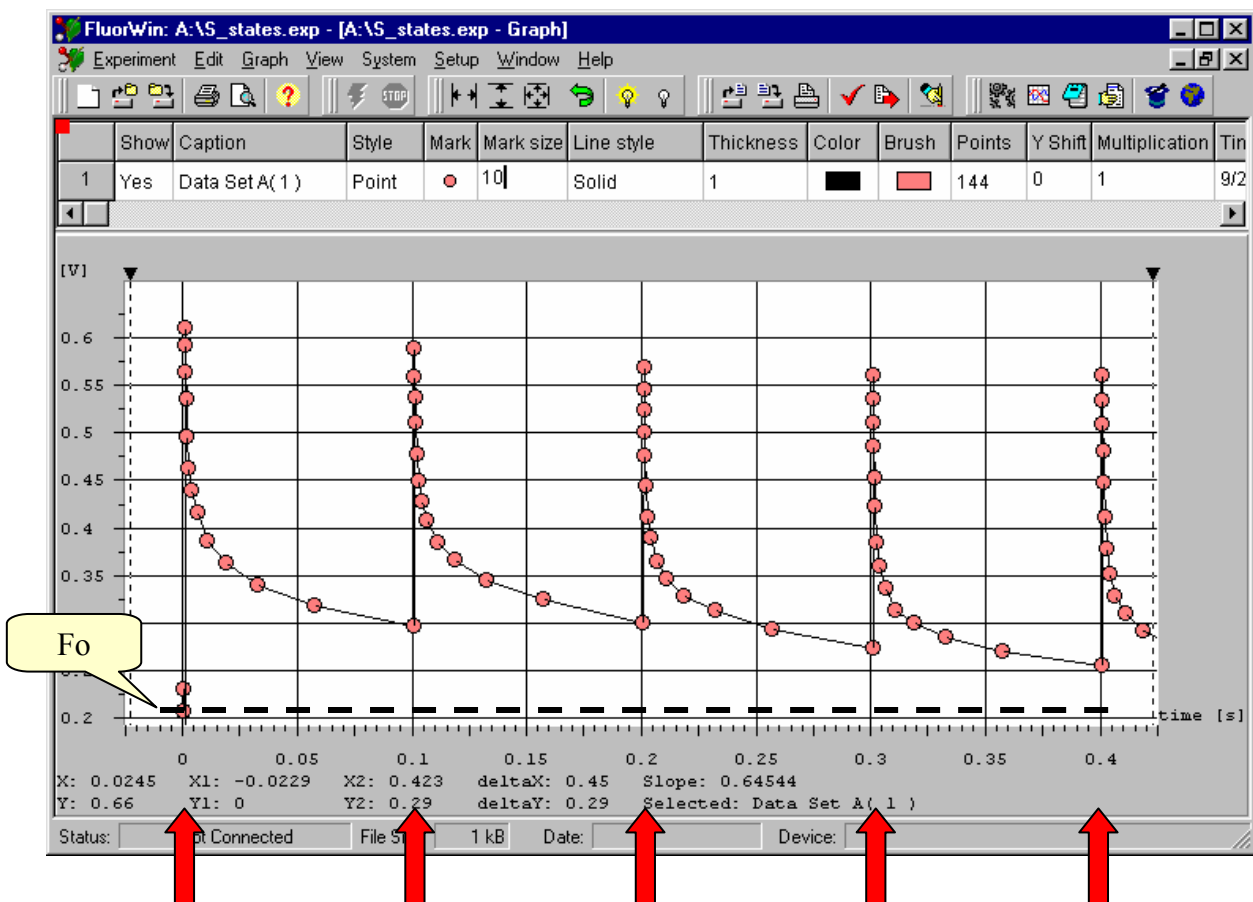
```

15. k=<200us,400us..800us>
16. if(PreFlash GR 0) Then
17. k=>mfml
18. else
19. k=>fm1
20. end

21. ; Actinic Flashes
22. i=lms+<0,FlashPeriod..NFlashes*FlashPeriod>
23. i=>F1,F2

24. ; F-decay after Actinic Flashes
25. j=[56us,100us..FlashPeriod]
26. i#j=>mfml

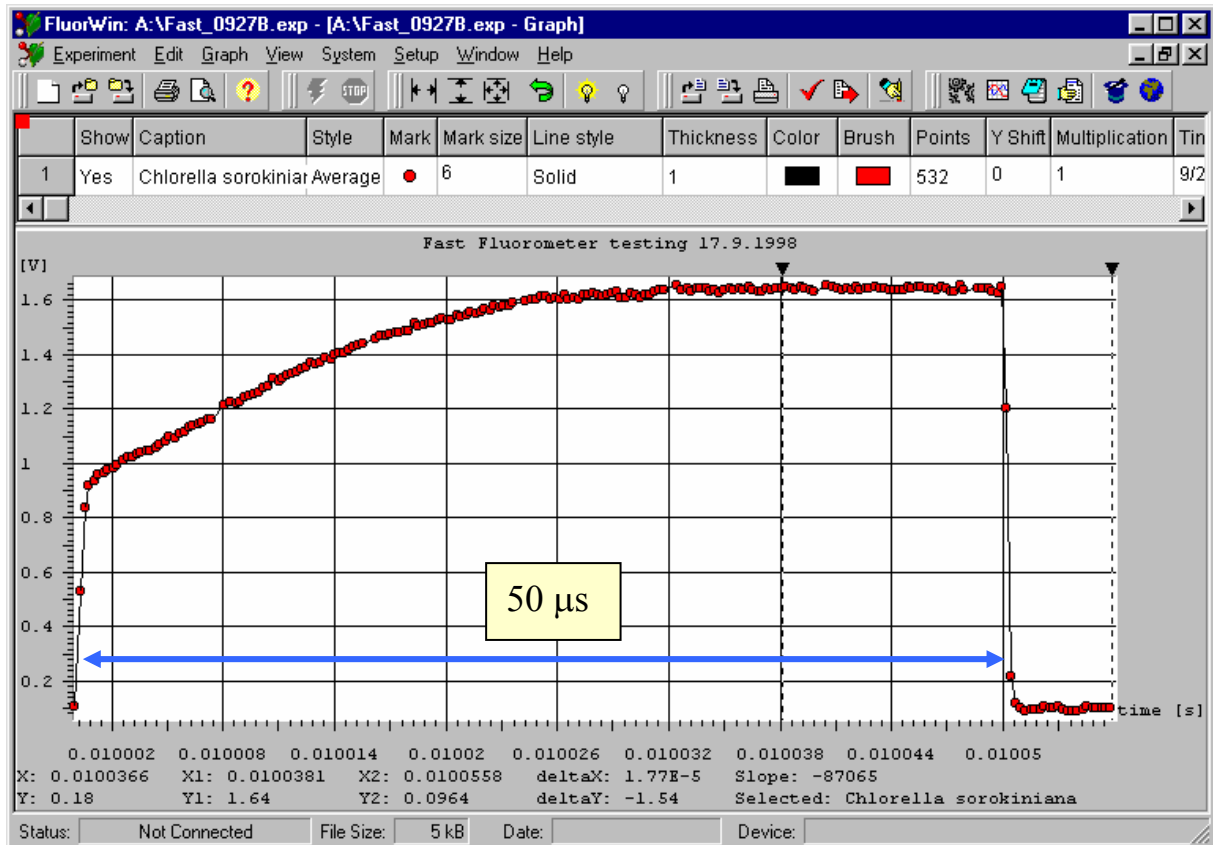
```



The  $F_0$  fluorescence is measured during the first 1 ms. 10 Actinic Flashes are fired 100 ms apart to advance the S-states. After each Actinic Flash, the fluorescence decay is measured. The fluorescence emission at the end of each 100 ms period is somewhat higher than  $F_0$  because a fraction of PSII reaction centers is unable to re-oxidize completely  $Q_A^-$  (non-periodic difference) and because the  $F_0$  quantum yield is S-states dependent. The S-states modulate the signal with the periodicity of 4.

#### 4.6. Preview of the Flash Fluorescence Induction Wizard

A new feature is added to the P.S.Instruments Fast fluorometer and FluorWin software. The 10 MHz / 14 bit A/D converter allows to measure fluorescence induction during a single-turnover, saturating flash. The software will allow to execute a new command (m2ex) that will trigger measurement series with 100 ns period lasting ca. 8  $\mu$ s. This measurement block can be repeated to cover longer measurement intervals.



Details are described in Nedbal et al. J. Photochem. Photobiol.: (in press 1998).

An example of a Protocol for Flash Fluorescence Induction:

```
;Default Inicialization for FLM DSP 56301 version
MeasuringFlash=50us
ActinicFlash=MeasuringFlash
MeasurDelay=500ns
AuxDuration=0.2
PreFlash=15us
include default.inc      ; Include standard options, don't remove it !
M_Voltage=80
F_Voltage=M_Voltage
A_Voltage=M_Voltage
;*****
time unit ms
a=<10>
a=>F1, f

time unit us
b=<10000>
c=<0, 8.1..50>
b#c=>mlex
```

## 5. BASIC SOFTWARE TOOLS

### 5.1. Summary of the PROTOCOL HEADER syntax

	INTERPRETATION	TYPICAL	UNITS
MeasuringFlash=	Duration of the Measuring Flash (f)	3-5 $\mu$ s	$\mu$ s
ActinicFlash=	Duration of the Actinic Flashes (F1, F2)	15-50 $\mu$ s	$\mu$ s
MeasurDelay=	Delay of the measurement after the Measuring Flash trigger	1-3 $\mu$ s	ns, $\mu$ s
AuxDuration=	Duration of the Actinic Light (A1, A2)	Seconds – minutes	ms, s, min, h
PreFlash=	Interval separating the Measuring Flash from the preceding Background Measurement	10-20 $\mu$ s	$\mu$ s
include default.inc	Default setting file		
M_Voltage=	Relative power of the Measuring Flashes	10-80%	
F_Voltage=	Relative power of the Actinic Flashes	60-100%	
A_Voltage=	Relative power of the Actinic Light	20-100%	

### 5.2. Frequently used elements of the PROTOCOL TIMING syntax

Here, only the most frequently used syntax elements are provided. More complete overview of the Protocol syntax is in the paragraph 7.

#### *Timing unit declaration:*

Time unit ns ( $\mu$ s, ms, s, min, hour)

For alternative formats of time units see Chapter 7. The timing unit can be changed several times in any Protocol. The declared time unit is attributed to any constant or variable of Value type (see Ch.7) that is without locally specified time unit and that is used after the present time unit declaration and before subsequent time unit declaration.

#### Example:

```
time unit  $\mu$ s      ; first declaration
<100> => fm      ; timing is 100  $\mu$ s for flash-measure (fm)
Action.
<200> => fm      ; timing is 200  $\mu$ s
<1ms> => F1      ; locally defined (ms) timing for Actinic Flash
overrides the previously declared timing ( $\mu$ s)
```

time unit hour ; second declaration changing the time unit for subsequent part of the Protocol (not shown here)

*Definition of a single timing variable:*

name = <value of implicit or explicit timing unit>

**Example:**

n = <100 $\mu$ s> ; a new variable named n is introduced and set to 100  $\mu$ s

*Definition of an arithmetic timing series:*

name = <first,second..last>

**Example:**

k = <100 $\mu$ s,200 $\mu$ s..0.5ms> ; an arithmetic timing series named k is introduced including 100 $\mu$ s, 200 $\mu$ s, 300 $\mu$ s, 400 $\mu$ s and 500 $\mu$ s.

*Definition of a logarithmic timing series:*

name = [first,second..last]

**Example:**

j = [10 $\mu$ s,100 $\mu$ s..10s] ; a logarithmic timing series named j is introduced including 10 $\mu$ s, 100 $\mu$ s,1ms, 10ms, 100ms, 1s and 10s.

*Combination of two timing series:*

Name1|Name2

**Example:**

k|j ; generates a new timing series combining all element of series k and all ellements of series j.

*Cartesian multiplication of two timing series:*

Name1#Name2

**Example:**

k#j ; generates a new timing series by Cartesian multiplication: e.g., for k=<1s,2s> and j=<10ms,20ms>, k#j=<1.01s,1.02s,2.01s,2.02s>.

*Execution of an Action:*

Name =&gt; ActionName

&lt;Timing&gt; =&gt; ActionName

**Examples:**

k => fml ; Action fml (flash-and-measure Channel 1) is executed at the timing defined by the variable k or by the timing series value k that were defined with parenthesis by k=<..> or k=[..] commands. For other ActionNames see section 7.

<i> => F1 ; Action F1 (Actinic Flash F1) is executed at the timing defined by the variable i that was set in a format without parenthesis, e.g. i=1ms.

<1ms> => A1(10s) ; Action A1 (Actinic Light A1) will be switched on 1 ms after the start of the experiment for a period of 10s.

**5.3. Overview of MOUSE controlled actions**

The mouse has all functions that are standard in the Windows environment. Here are the additional functions active in the Graph window.

*Positioning the cross cursor in Graph area on a particular curve*

Name of the data set shows up in a yellow rectangle. The name is set or can be modified in the top part of the Graph window that opens with a double click on the Graph icon..

*Left mouse button / single click on the yellow rectangle with the name of the data set.*

Given data set is selected for the readings in the bottom right part of the Graph area (X1, Y1 for the first cursor, X2, Y2 for the second cursor, delta X and delta Y for the differences between the cursors and corresponding mean slope for the interval defined by the cursors).

*Left mouse button / single click*

Cursor 1 (▼) goes to the specified position. The cursor 1 controls at which position the X2 and Y2 coordinates (see bottom of the Graph window) of the selected DataSet are read.

*Right mouse button / single click*

Cursor 2 (▼) goes to the specified position. The cursor 2 controls at which position the X2 and Y2 coordinates (see bottom of the Graph window) of the selected DataSet are read.

*Left mouse button / double click on the gray area around the Graph window*

Displays the list of DataSets with names (top part for definition). The list allows to select a particular DataSet for cursors reading (▼) of data (X1,Y1 and X2,Y2), of differences (deltaX, deltaY) and of slope.

*Left mouse button / drag in the Graph area*

Zoom in on Graph detail.

*Right mouse button / drag in the Graph area*

Zoom out.

#### **5.4. Overview of SHORTCUT KEYS and KEY-controlled actions**

***Alt + Backspace*** – Undo last Protocol modification

***Alt + X*** – Exit FluorWin

***Ctrl + 1*** – Trigger Aux1 TTL (A1) manually – usually Actinic Light 1

***Ctrl + 2*** – Trigger Aux2 TTL (A2) manually – usually Actinic Light 2

***Ctrl + 3 to 6*** – Trigger Aux3 to Aux6 TTL (A3 to A6) manually

***Ctrl + D*** – Description = Notes on the experiment

***Ctrl + Del*** – Delete in Protocol & Notes

***Ctrl + F1*** – Trigger Actinic Flash (F1) manually

***Ctrl + F2*** – Trigger Actinic Flash (F2) manually

***Ctrl + Ins*** – Copy in Protocol & Notes

***Ctrl + M*** – Trigger Measuring Flash (f) manually

***Ctrl + T*** – Test Keys window

***Ctrl + U*** – Undo last Graph modification

***Ctrl + W*** – Device ID. May be used to recover communication with the Control Unit

***Delete*** with selected DataSet in Graph – removes irreversibly the Data Set.

***F1*** – Help content

***F2*** – Save experiment file

***F3*** – Open existing experiment file

***F9*** – Start experiment

***F11*** – Navigator

***Shift + Ctrl + M*** – System monitor

***Shift + Del*** – Cut in Protocol & Notes

***Shift + Ins*** – Insert clipboard in Protocol & Notes

## 5.5. Overview of ERROR MESSAGES

PENDING

### APPENDIX I: FluorWin 3.x Help Index

#### Main Menu Items

<b>Experiment</b>	always on
<b>Notes</b>	Notes
<b>Edit</b>	Graph
<b>Edit</b>	Protocol & Notes
<b>Graph</b>	Graph
<b>View</b>	always on
<b>System</b>	always on
<b>Setup</b>	always on
<b>Window</b>	always on
<b>Help</b>	always on

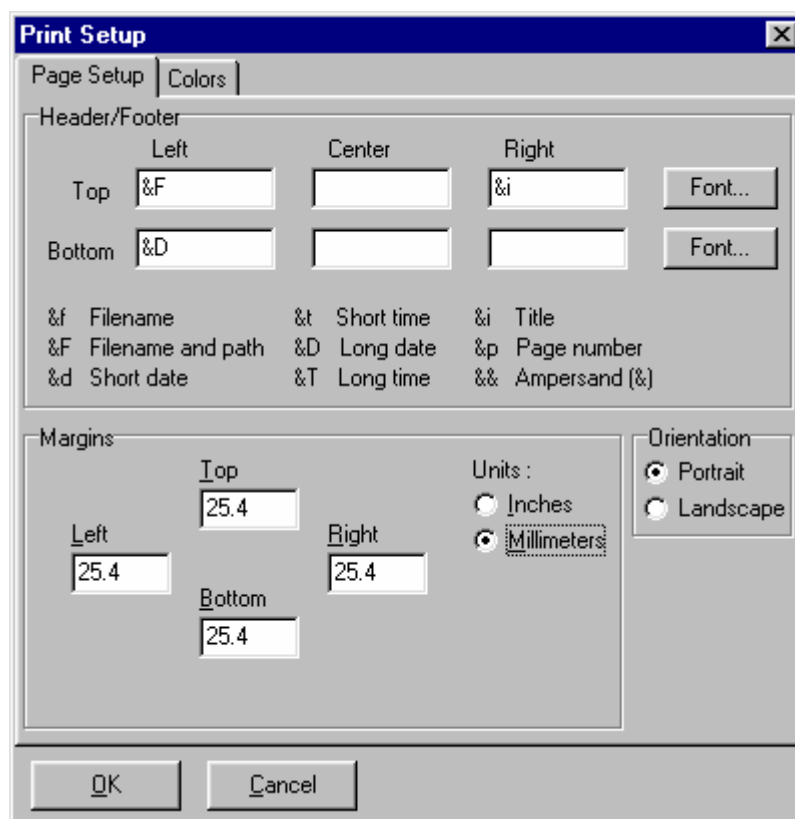
#### Experiment

- New**
- Open ...**
- Save**
- Save As ...**
- Close**
- Close All**
- Export ...**
- Page Setup ...**
- Preview ...**
- Print ...**
- Print Setup ...**
- Exit**

**New** - New experiment is open with four windows (Default Protocol, Graph, Notes, Controls). Any experiment opened earlier remains on. Only one experiment is active and ready for execution as indicated by the red square in the upper right corner of the respective Graph window.

It is possible to toggle between several open experiments using the Navigator or Window menu.

- Open** - The Experiment > Open command loads a previously saved experiment (\*.exp) file into the memory. The experiment file carries information on the used experimental Protocols, experimental results and graphical presentation of the data (Graph), user-written Notes and on the Controls set during the experiment.
- Save** - The Experiment > Save command saves the current experiment file under its current name.
- Save As** - The Experiment > Save As command allows you to save the current experiment file using a different name or directory.
- Close** - The Experiment > Close command terminates active experiment and closes the active graph window.
- Close All** - The Experiment > Close command terminates active experiment and closes the active graph window.
- Export** - The Experiment > Export command allows to export data from the active experiment into a text file that can be read by the Microsoft Excel or other spreadsheets. It is possible to limit the exported data to those that are just displayed or that are between cursors.
- Page Setup** - The Experiment > Print Setup command allows to modify the appearance of the Graph print. Orientation, margins, and info can be specified.



- Preview** – The Experiment > Preview command allows to see the Graph as it will be printed.

**Print** – The Experiment > Print command allows to print the Graph as defined in Experiment > Page Setup and in Setup > Environment Options.

**Print Setup** – The Experiment > Print Setup sets the Printer parameters.

**Exit** – FluorWin program is closed.

#### **Notes (only in the Notes window)**

**Open ...**

**Save As ...**

**Open** - The Notes > Open command is used to import any text file into the present Notes window.

**Save As** - The Notes > Save As command is used to save the present Notes as a text file.

#### **Edit (only in the Graph window)**

**Remove Data Set**

**Copy ...**

**Description ... will be removed in later versions**

**Remove Data Set** – Selected Data Set is removed from the Graph. Data are irreversibly lost.

**Copy** – The Graph window is copied into the clipboard and can be pasted in other programs as Word, Excel or other Windows compatible programs.

#### **Edit (only in the Protocol or Notes windows)**

**Undo (Protocol only)**

**Cut**

**Copy**

**Paste**

**Delete**

**Find ... (Protocol only)**

**Replace ... (Protocol only)**

**Select All**

**Copy to Current (Protocol only)**

**Clear**

**Font ...**

**Undo** – Last editing action can be canceled in the Protocol window.

**Cut** – Cut the selected text and transfer it into clipboard.

**Copy** – The selected text is copied into the clipboard.

**Paste** – Content of the clipboard is pasted at the position of the cursor.

**Delete** – The selected text is deleted.

**Find** – A string can be defined for a text search.

- Replace** – One string can be replaced by another string.
- Select All** – The entire text of Notes or Protocol is selected.
- Copy to Current** – Active only with an earlier used Protocol. Using this command, the Protocol is copied to the current window and can be executed.
- Clear** – The text in the Notes window is deleted. If used in a Protocol window, the current Protocol is replaced by the default Protocol.
- Font** – Fonts available in Windows environment can be used for editing.

### **Graph (only in the Graph window)**

- Undo**
- Log Scale X**
- Auto Scale X**
- Auto Scale Y**
- Auto Scale XY**
- Show All**
- Hide All**
- Toggle All**
- Manual Scale**

- Undo** – Last Graph editing action can be canceled.
- Log Scale X** – The data are shown with logarithmic time scale.
- Auto Scale X** – The limits of the time scale are changed to include all data of the experiment.
- Auto Scale Y** – The limits of the fluorescence scale are changed to include all data of the experiment.
- Auto Scale XY** – The limits of the time and fluorescence scales are changed to include all data of the experiment.
- Show All** – All data sets of the experiment are displayed.
- Hide All** – All data sets of the experiment are hidden.
- Toggle All** - All data sets of the experiment are hidden / displayed.
- Manual Scale** – Limits of the time and fluorescence scales are set manually.

### **View**

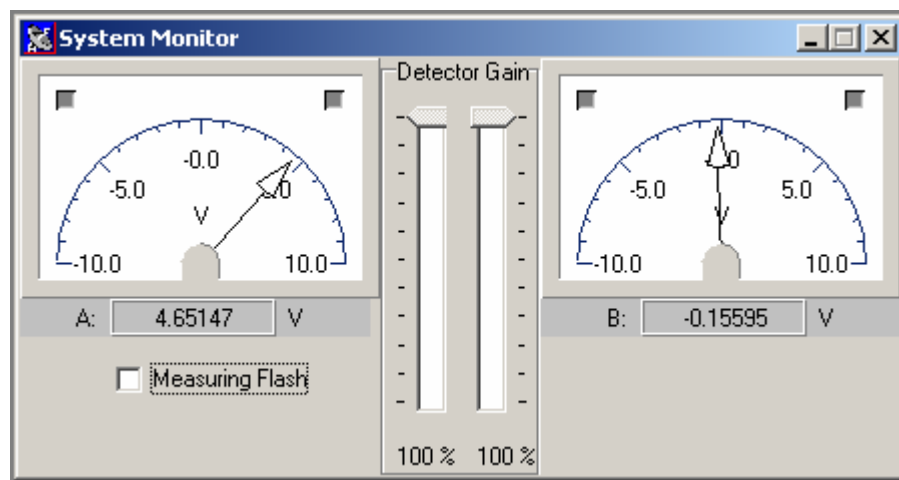
- Main** – show/hide the Experiment icon set.
- Graph** – show/hide the Graph icon set.
- Window** – show/hide the icon set of individual Experiment windows.
- Control** – show/hide Start / Stop icons

### **System**

- System Monitor**
- Start**
- Stop**

**Device ID****Test Unit****Download****Test Keys**

**System Monitor** – Two analog “displays” are presenting signals read by Channel A and Channel B. In standard configuration, the Channel A is the A/D converter 1 of the Fluorometer and the Channel 2 is the A/D converter 2. In special configurations, the Monitor can toggle several inputs showing e.g. temperature or irradiance. The measurements are done with or without the Measuring Flash (check required). The bar indicators show present relative voltage of the Measuring (M) and Actinic Flashes (F) and of the Actinic Light (A).



**Start** – The Protocol is compiled (if not done earlier during the Syntax Check) and executed in an Experiment.

**Stop** – Experiment is interrupted.

**Device ID** – ID of the Control Unit is returned. This command can be also used to re-establish communication between the Control Unit and the computer.

**Test Unit** – Testing routine to identify hardware problems as memory or processor defects.

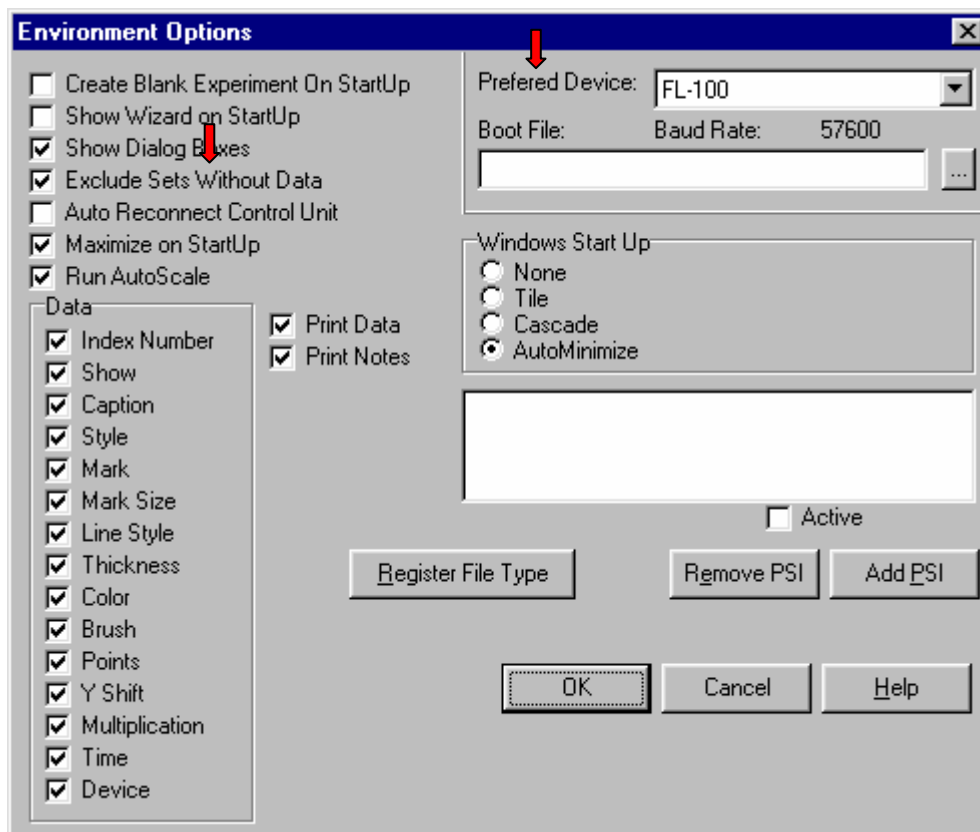
**Down Load** – Not active in the Standard configuration.

**Test Keys** – Manual triggers of Measuring Flash, Actinic Flash 1, Actinic Flash 2 and of A1 – A6 TTL outputs.

**Setup****Environment Options****Communication**

**Environment Options** – The Environment window allows to adjust the software to the needs of an individual user. The options are offered with a self-explanatory legend. The first block (upper right part) allows to customize the presentation and execution of the experiments:

- Create Blank Experiment* – The FluorWin generates a blank experiment with the default protocol after each Start.
- Show Wizard* – The Wizard window is offered after each Start.
- Show Dialog Boxes* – Warning messages are displayed.
- Exclude Sets without Data* – Data generated by a Protocol without measurements are not displayed.
- Auto Reconnect* – The Control Unit is automatically connected to the computer after any interruption.
- Maximize on Start Up* – FluorWin window is maximized.
- Run Auto Scale* – Autoscaling is performed after a new Data Set is collected.
- Data** – The selection defines what Data Set attributes are accessible for editing in the Graph window.
- Print Data* – Graph is printed to represent the data.
- Print Notes* – Notes are included in the printout.
- Preferred Device* – used to define version of the instrument that is primarily searched by the software.
- Boot File* – some instruments may have custom made boot program
- Windows Start Up** – The layout of the Graph, Protocol, Notes and Controls windows is defined for the FluorWin Start Up.
- Register File Type* – allows to extent applications by upgrade libraries of P.S. Instruments (details provided with special applications).
- Remove* – Plug In removed
- Add* – Plug In added (details provided with special applications)



**Communication** – The Communication window allows to identify the computer port that is used for communication with the Control unit.

## Window

### Navigator

### Protocol Wizard

### Test Keys

### Tile

### Cascade

### Arrange All

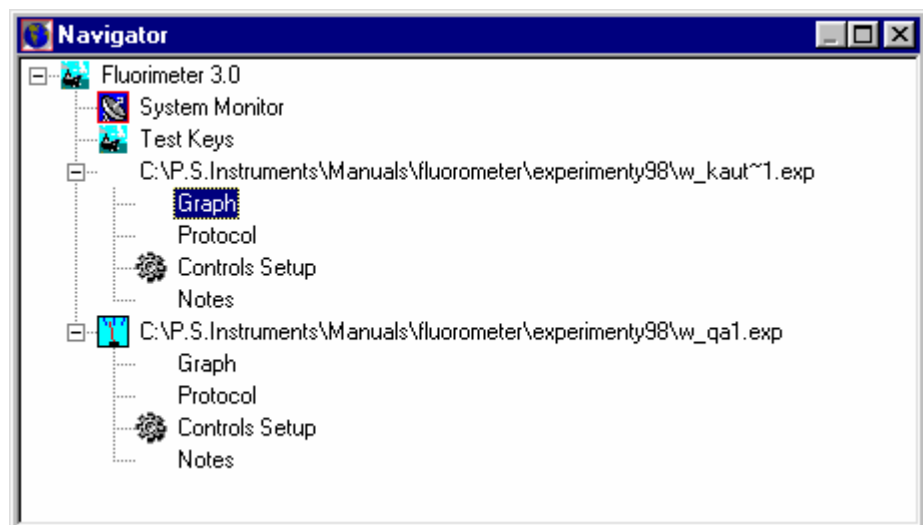
### Close

### Hide

### Show

### Make Active

**Navigator** – The Navigator window is offered to provide an overview of the currently available Experiments and to facilitate a rapid access to System Monitor and to Test Keys. The icon with “analog display” indicates which experiment is presently active (available for a new measurement). The other experiments can be open for data analysis or visual comparison.



**Protocol Wizard** – The Wizard window assists in designing Protocols of several typical applications.

**Test Keys** – The Test Keys window allows to set the relative power of the Measuring Flash, of Actinic Flash and of Actinic Light. Individual TTL outputs can be manually triggered.

**Tile, Cascade, Arrange All, Close, Hide / Show** – The commands facilitate operations in the Windows environment.

**Make Active** – The Make Active command activates the experiment that is currently selected. The activation means that the Experiment becomes available for a measurement. Inactive Experiments are open only for analysis or comparison.

## APPENDIX II: Selected details of the FluorWin 3.X Protocol Compiler

This paragraph is provided only for experienced users requiring specific applications. For most applications, use wizard to generate the protocols.

### 7.1. Lexical elements

**7.1.1. Dividers:** Space and tabulator are used to separate commands and keywords. E.g. in line 10ms => m, spaces are separating timing (at 10ms) from operator "execute" (=>) and action "measurement" (m). Interpreted as "at 10ms execute measurement".

**7.1.2. Comments:** Any string starting with semicolon (;) is interpreted as a comment and not compiled

**7.1.3. Keywords** are syntax elements recognized by the compiler without further specification. The compiler is case sensitive. Multiple representation of one keyword is allowed (e.g.  $\mu$ s, US or us always means microseconds).

#### TIME UNITS:

[Tt]ime [Uu]nit	...	Serves to declare implicit time unit for subsequent Protocol lines. Valid until next time unit declaration. Example: Time unit $\mu$ s
[Hh]our (s)	...	Hours
[Mm]in	...	Minutes
[Mm][Ss]	...	Milliseconds
[Nn][Ss]	...	Nanoseconds
[Ss]	...	Seconds
[Uu $\mu$ ][Ss]	...	Microseconds

#### ACTIONS:

[Bb]egin ...	...	indicates start of an action definition.
[Ee]nd ...	...	indicates end of an action definition.
[Pp]rim[Aa]ction ...	...	serves to declare a primitive action. Syntax: PrimAction Name (Hex1, Hex2, Value1) Example: Action F1 (Actinic Flash 1) is defined in the default.inc file as follows: PrimActionF1 (0x0008, 0x10000008, ActinicFlash) The first two numbers in the parenthesis define the start and stop codes for the microprocessor and the last parameter specifies the default duration of the action. The duration of the flash can be specified in the Protocol, e.g. F1(50us), when the default duration (ActinicFlash) needs to be changed.
[Aa]ction ...	...	serves to declare a simple action. Example: The action fm (flash & measure) is defined in the default.inc file as follows: Action fm begin <0>=>f <MeasurDelay>=>m end The first line declares the name of the action, second line says at time when the fm action is called (relative 0) execute (=>) „measuring flash“ (f). Third line requests that measurement at both Channels (m) are done (=>) with at the relative time <MeasurDelay>.
[Cc]omp[Aa]ction ...	...	serves to declare a name of a complex action. The complex action can define, for example, a subroutines written in the microprocessor sourcecode. Not required for typical Protocol design.
[Ii]nclude ...	...	used to include external *.inc file that may define user-written or default (default.inc) actions.

OTHER KEYWORDS ([Aa]ritm, [Bb]yte, [Tt][Ll]ine, [Tt][Vv]alue, [Ww]ord) are not required for typical Protocol writing

7.1.4. *Constants:*

- Timing: real number with time unit (e.g. 1.12345 ms or 1.12345E-3 s). Real number without time unit will be interpreted as timing of default time unit (usually second).
- Numeric value: Xnum (e.g. 100num means number 100 that can be used for mathematical operations as multiplication). Only numbers with num extension are not interpreted as a timing.
- Timing vectors (Sequence):
  - a) Listed, e.g. <1ms, 5ms, 100ms, 500ms, 1 s, 10s>
  - b) Arithmetic sequence <0ms, 2ms .. 10ms> stands for 0, 2, 4, 6, 8 and 10ms.
  - c) Logarithmic sequence [1s, 2s .. 64s] stands for 1, 2, 4, 8, 16, 32 and 64 seconds. More often logarithmic series with decade values are used, e.g. [1, 3.1622 .. 100] stands for 1, 3.1622, 10, 31.622, 100 default time units (usually seconds).
- Hexadecimal number

7.1.5. *Identifiers* represent a variable or an action. The identifiers start with lowercase or uppercase letter (a-z or A-Z) and continue by more letters or numbers (0-9).

7.2. **Source code structure.**

7.2.1. *Variables* can be of different types:

Value ... real number defining timing (e.g., x=1.25e-3s)  
 Num ... real number  
 Seqv ... timing sequence (e.g., y=<1,2..250>)  
 Hex ... hexadecimal number (0[xX][0-9a-fA-F])  
 Byte ... 0 - 255  
 Word ... 0 - 65535

It is not necessary to declare the type of the variable. When a variable is used several times in a Protocol, both content and type may change.

7.2.2. *Operator =* (equal)

Syntax: Identifier = Expression  
 Example: x = 5ms

7.2.3. *Operator -* (minus)

Syntax: - Expression (must be of Value type; real number)  
 Example: - 320 ns

7.2.4. *Binary operator +* (plus)

Syntax: Expression 1 + Expression 2 (must be of Value or Seqv type)

Example 1: 10 ms + 300 ns

Gives 10.0003 ms

Example 2: 10 ms + <0,300ns, 900ns>

Gives <10ms,10.0003ms, 10.0009ms>

7.2.5. *Binary operator -* (minus)

Syntax: Expression 1 - Expression 2 (must be of Value or Seqv type)

Example 1: 10 ms - 300 ns

Gives 9.9997 ms

Example 2: <10s,11s..14s>-100ms

Gives <9.9s,10.9s,11.9s,12.9,13.9>

7.2.6. *Binary operator \** (multiplication)

Syntax: Expression 1 \* Expression 2 (must be of Num, Value or Seqv type)

Example 1: 10num \* 300 ns  
Gives 3 us

Example 2: <0,FlashPeriod..NFlashes\*FlashPeriod>  
where FlashPeriod is of value - timing type (e.g. 100ms)  
and NFlashes is of Num type (e.g. 8num)

7.2.7. *Binary operator* \* (multiplication)

Syntax: Expression 1 \* Expression 2 (must be of Num, Value or Seqv type)

Example 1: 10num \* 300 ns  
Gives 3 us

Example 2: <0,FlashPeriod..NFlashes\*FlashPeriod>  
where FlashPeriod is of value - timing type (e.g. 100ms)  
and NFlashes is of Num type (e.g. 8num)

7.2.8. *Binary operator* / (division)

Syntax: Expression 1 / Expression 2 (must be of Num, Value or Seqv type)

Example: 300s / 10num  
Gives 30s

7.2.9. *Binary operator* # (Cartesian multiplication)

Syntax: Expression 1 \* Expression 2 (must be of Seqv type)

Example: for x=<1s,2s,3s> and y=<0.1s,0.2s>  
x#y= <1.1s, 1.2s, 2.1s, 2.2s, 3.1s, 3.2s>

7.2.10. *Binary operator* | (combination)

Syntax: Expression 1 | Expression 2 (must be of Seqv type)

Example: for x = <1s, 2s> and y=<5s, 6s>  
x|y = <1s, 2s, 5s, 6s>

7.2.11. *Operator* => (execute)

Syntax: Expression1 => Action1(...), Action2(...), ... ActionN(...)  
Expression1 must be of Seqv type

Example: <1s,2s..5s> => F  
Gives Actinic Flash F five times, every second.

Remaining elements of the syntax are not relevant for writing of standard experimental Protocols.