FluorCam
Instruction Manual

Version 1.5

Table of Contents

I. List of symbols and abbreviations

Symbol	Formula	Name	Description
F_0	Measured	minimum fluorescence in dark-adapted state	Q_A oxidized ($q_P=1$), non-photochemical quenching relaxed (NPQ=0)
F_0_Dn	Measured	minimum fluorescence during dark relaxation	Q_A oxidized ($q_P=1$), non-photochemical quenching relaxing (NPQ>0)
F_0_Ln	$\approx F_0 / ((F_M - F_0) / F_M + F_0 / F_M\_Ln)$	minimum fluorescence during light adaptation	Calculated estimate: Q_A oxidized ($q_P=1$), non-photochemical quenching induced (NPQ>0)
F_0_Lss	Measured	steady-state minimum fluorescence in light	Q_A oxidized ($q_P=1$), non-photochemical quenching at maximum (NPQ max)
F_M	Measured	maximum fluorescence in dark-adapted state	Q_A reduced ($q_P=0$), non-photochemical quenching relaxed (NPQ=0)
F_M_Dn	Measured	instantaneous maximum fluorescence during dark relaxation	Q_A reduced ($q_P=0$), non-photochemical quenching relaxing (NPQ>0)
F_M_Ln	Measured	maximum fluorescence during light adaptation	Q_A reduced ($q_P=0$), non-photochemical quenching induced (NPQ>0)
F_M_Lss	Measured	steady-state maximum fluorescence in light	Q_A reduced ($q_P=0$), non-photochemical quenching at maximum (NPQ max)
F_P	Measured	peak fluorescence during the initial phase of the Kautsky effect	local F-maximum resulting from rapid reduction of plastoquinone pool and slower activation of re-oxidation mechanisms and of non-photochemical quenching
F_t_Dn	Measured	instantaneous fluorescence during dark relaxation	instantaneous F-level during dark relaxation that results from a dynamic equilibrium of plastoquinone reducing and re-oxidizing processes and from non-photochemical quenching
F_t_Ln	Measured	instantaneous fluorescence during light adaptation	instantaneous F-level during light adaptation that results from a dynamic equilibrium of plastoquinone reducing and re-oxidizing processes and from non-photochemical quenching
F_t_Lss	Measured	steady-state fluorescence in light	steady-state F-level that results from a dynamic equilibrium of plastoquinone reducing and re-oxidizing processes and from non-photochemical quenching
F_V	$F_M - F_0$	variable fluorescence in dark-adapted state	variable fluorescence increment that is due the transition from dark-adapted state with all-open reaction centers to the all-closed state during saturating flash of light.
NPQ_Dn	$(F_M - F_M\_Dn)/ F_M$	instantaneous non-photochemical quenching during dark relaxation	non-photochemical quenching relaxing in dark
NPQ_Ln	$(F_M - F_M\_Ln)/ F_M$	instantaneous non-photochemical quenching during light adaptation	non-photochemical quenching induced in light
NPQ_Lss	$(F_M - F_M\_Lss)/ F_M$	steady-state non-photochemical quenching	steady-state non-photochemical quenching in light
q_P_Dn	$(F_M\_Dn - F_t\_Dn) / (F_M\_Dn - F_0\_Dn)$	photochemical quenching during dark relaxation	estimate of the fraction of open PSII reaction centers $PSII_{open} / (PSII_{open} + PSII_{closed})$
q_P_Ln	$(F_M\_Lss - F_t\_Lss) /$	photochemical quenching during light	estimate of the fraction of open PSII reaction centers $PSII_{open} / (PSII_{open} + PSII_{closed})$

	$(F_M\_Lss - F_0\_Lss)$	adaptation	
q_P_Lss	$(F_M\_Lss - F_t\_Lss) / (F_M\_Lss - F_0\_Lss)$	steady-state photochemical quenching	estimate of the fraction of open PSII reaction centers $PSII_{open} / (PSII_{open} + PSII_{closed})$
QY_Dn	$(F_M\_Dn - F_t\_Dn) / F_M\_Dn$	instantaneous PSII quantum yield during dark relaxation	PSII quantum yield relaxing in dark
QY_Ln	$(F_M\_Ln - F_t\_Ln) / F_M\_Ln$	instantaneous PSII quantum yield during light adaptation	PSII quantum yield induced in light
QY_Lss	$(F_M\_Lss - F_t\_Lss) / F_M\_Lss$	steady-state PSII quantum yield	steady-state PSII quantum yield in light
QY_max	F_V / F_M	maximum PSII quantum yield	maximum PSII quantum yield in dark-adapted state
R_{fd}	$(F_P - F_t\_Lss) / F_P$	fluorescence decline ratio	empiric parameter used to assess plant vitality

II.   Introduction to chlorophyll fluorescence techniques

II.A.   Chlorophyll fluorescence

Kinetics of chlorophyll fluorescence emission is the most important optical signal reporting on the dynamics of photosynthesis in intact plants, algae, and in cyanobacteria.
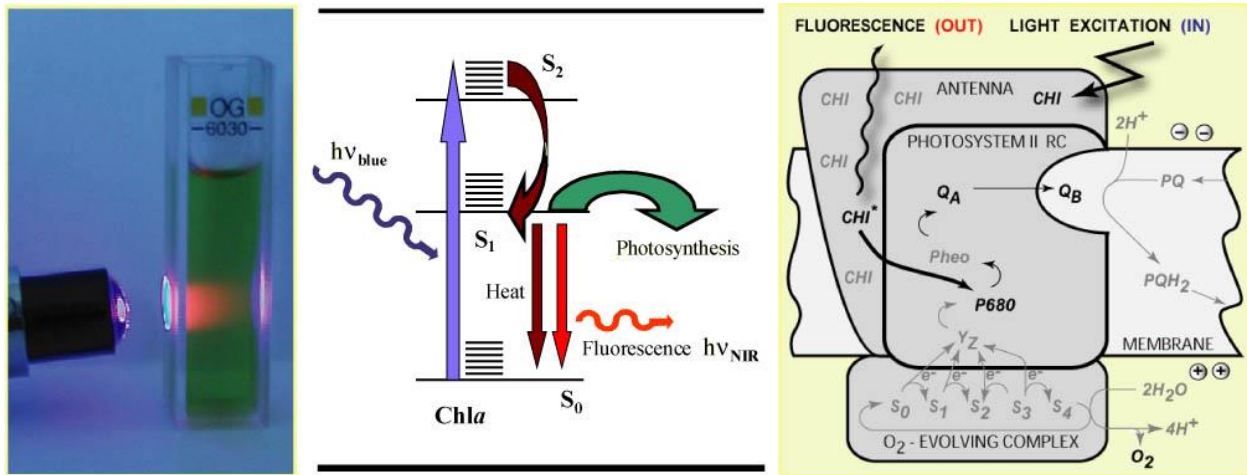

FIGURE 1. The left panel shows a cuvette containing chlorophyll solution that is illuminated by a beam of blue light. The red light emanating from the solution is the chlorophyll fluorescence emission. The change from the absorbed blue light to the emitted red fluorescence is explained by the Jablonski scheme shown in the middle panel. The energy of the blue absorbed photon brings the chlorophyll molecule into the upper excited singlet state, here S_2. Part of the energy is rapidly lost to heat changing the energetic state to the lowest excited singlet state, here S_1. The S_1 energy is, in photosynthetic organisms, largely used for photosynthetic energy conversion in the reaction centers (right panel). Only a fraction of the S_1 energy is lost to either heat dissipation or to fluorescence emission.

Chlorophyll fluorescence emission competes with photosynthesis for the excitation energy (Fig.1). The more effective is the photosynthetic energy conversion, the lower is the chlorophyll fluorescence yield and *vice versa*. In healthy, dark-adapted plant, the photosynthetic capacity is available at its maximum and, thus, the chlorophyll fluorescence yield is minimal (F_0). The photosynthetic capacity can be reduced to null by an herbicide or by a pulse of strong light that transiently congests the photosynthetic electron transport pathway. With the photosynthetic yield at zero, the fluorescence emission reaches a maximum (F_M). An exhaustive review of all aspects of chlorophyll fluorescence emission is provided in [Govindjee and Papageorgiou 2004]. Several models of photosynthetic apparatus and of fluorescence emission dynamics are available at www.e-photosynthesis.org.

II.B.   Oxygenic photosynthesis

Oxygenic photosynthesis starts with light absorption in photosynthetic antenna complexes (Fig.2). The antenna complexes deliver the captured energy to the Photosystem II and Photosystem I reaction centers. The effectiveness of the energy capture and of its transfer to the reaction centers is described by the effective antenna cross section σ_{PSII} and σ_{PSI}.[1]

The excitonic energy delivered to the reaction centers is largely used for primary charge separation. A fraction of the energy is lost to fluorescence and heat dissipation. The fluorescence yield is low (F_0) when the photochemical yield is maximal and it is high (F_M) when the photosynthesis is blocked, e.g., by congestion of the plastoquinone pool that mediates the

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[1] Irradiance of 2000 µmol(photons).m^{-2}.s^{-1} that is a typical sunny summer day noon light in Central Europe leads with an effective antenna cross section of 100 Å^2 to ca. 1200 excitons arriving to the reaction center per second.

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electron transport from Photosystem II to Cyt b/f complex (Fig.2). The variability of chlorophyll fluorescence yield originates from Photosystem II as the fluorescence yield of Photosystem I does not depend on the photochemical state of its reaction centers.
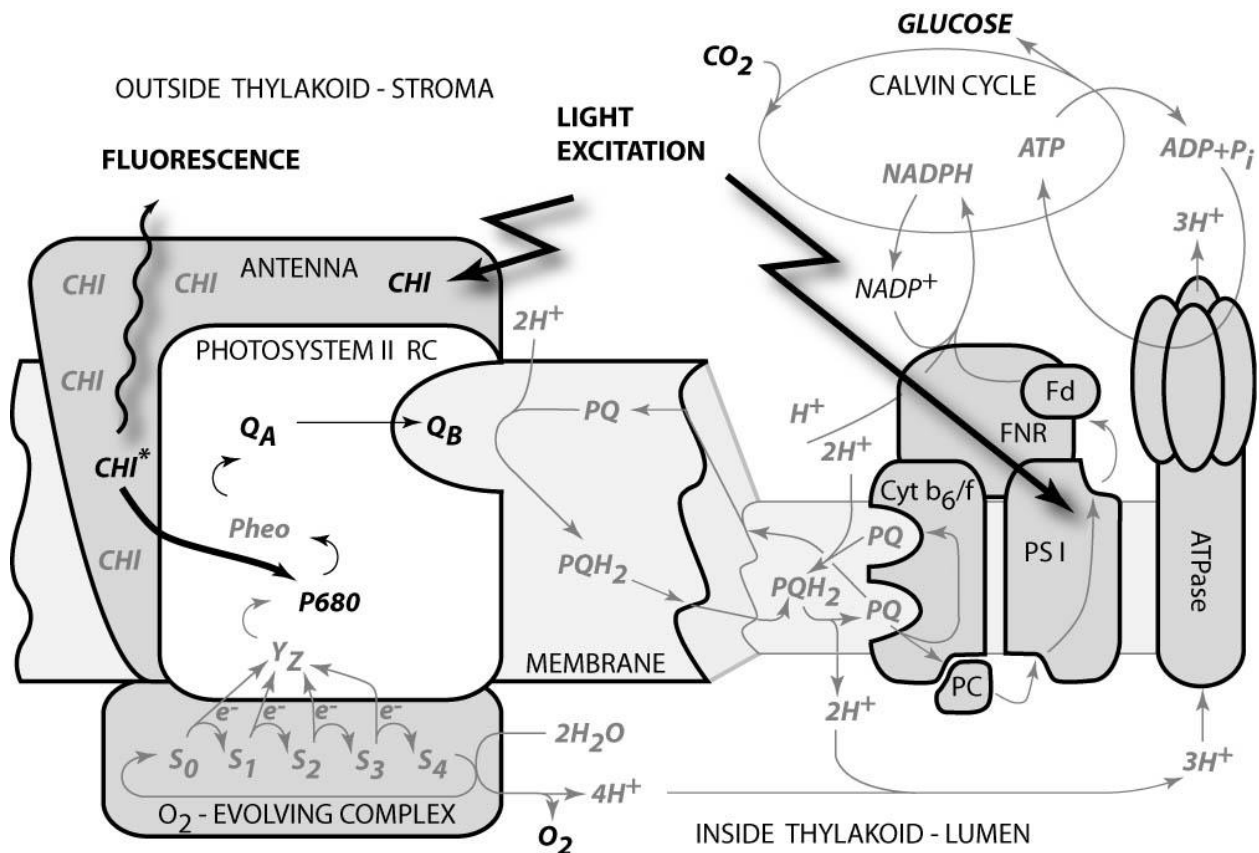

FIGURE 2. Schematic presentation of principal photosynthetic modules in plants and green algae. Light is absorbed by antenna pigments of Photosystem II (in front) and of Photosystem I (in back). The excitons generated in the antennae are rapidly transferred to the reaction centers where their energy serves to drive the primary charge separation. In PSII, the primary charge separation to P680+-Pheo- is followed by secondary charge transfer processes: the electrons are extracted by the oxidized primary donor P680+ from water by the O2-evolving complex and by the YZ donor. On the acceptor side, the electron is rapidly stabilized by a transfer from pheophytin (Pheo) to the primary quinone acceptor QA. A mobile plastoquinone pool shuttles two electrons sequentially taken from QA- and two protons taken from the stromal side of the membrane to the lumenal side of cytochrome b6/f complex where the protons are released and electrons are sent to PSI. The PSI is using the excitonic energy to generate reducing NADPH.H+. The charge transfer reactions in the thylakoid membrane result also in accumulation of protons on the lumenal side and depletion on the stromal side of the thylakoid. The difference in electrochemical potentials is used by ATPase to generate ATP that is used together with NADPH.H+ in Calvin-Benson cycle to assimilate inorganic CO2 into sugars.

II.C.   Essentials of chlorophyll fluorescence technique

II.C.1 Kautsky effect measured in continuous light mode

The complex transient of the chlorophyll fluorescence emission that reflects the dynamics of plant photochemical yields during transition from dark-adapted to light-adapted state is called Kautsky effect (Kautsky and Hirsch 1931, Govindjee 1995). Kautsky effect is one of the most investigated transients in photosynthesis research.

It is elicited by a continuous, non-modulated actinic light that drives photosynthetic reactions at physiological rates (typically hundreds of $\mu mol(photons).m^{-2}.s^{-1}$). The measured fluorescence emission can be excited using the same light. However, one must remember that, in this continuous light mode, the fluorescence signal is proportional to the actinic light. Increasing

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irradiance means not only that the dynamics of the fluorescence transients will be faster but also the signal will proportionally rise. The other aspect of the continuous light measurement is that the fluorescence signal cannot be discriminated from the background light. Last not least, the fluorescence rise during the initial phase of the Kautsky effect can be so rapid that the typical integration time of imaging fluorometers (ca. 20 ms) will obscure the true value of F_0 fluorescence emission. The advantage of this technique is that the measured signals as well as signal-to-noise ratio are higher than in the pulse modulation mode (see below).

The Kautsky effect measurement in continuous light mode is a very simple and attractive method to demonstrate and probe dynamics of photosynthetic reactions during transition from dark to light. The method yields:

- an upper estimate of F_0 fluorescence emission of dark-adapted plant;
- F_P peak fluorescence attained during the first second(s) of the transient when the plastoquinone pools is largely congested by the light-driven electron flux (Fig.2) and neither the photoprotective quenching mechanisms nor the CO_2 assimilating machinery are fully activated.
- F_t_Lss steady-state fluorescence in the terminal light-adapted phase that reflects a balance between the various fluorescence quenching mechanisms.

There is a large difference between F_P and F_t_Lss in healthy plants whereas it is much smaller in stressed plants. This observation led Lichtenthaler et al. [Lichtenthaler and Miehé 1997] to propose R_{fd} fluorescence ratio ($R_{fd}=(F_P - F_t\_Lss) / F_P$) as an empirical parameter to quantify plant fitness. The R_{fd} parameter is requiring only very simple imaging instrumentation and can be measured with a low noise. The measurement is relatively time consuming as it requires light adaptation that can take tens of seconds or up to few minutes depending on the measured system and on incident irradiance.

II.C.2 Kautsky effect measured in pulse-amplitude-modulated (PAM) mode

Alternatively, the transitions from the dark-adapted state to the light-adapted state and back can be investigated in great detail by pulse-amplitude-modulated (PAM) technique that was first introduced to chlorophyll fluorescence imaging by Photon Systems Instruments, spol. s r.o. in 1996 [Nedbal et al. 2000]. Similarly to the earlier non-imaging application [Schreiber et al. 1986], the fluorescence excited by short flashes of measuring light is dynamically discriminated from the fluorescence excited by continuous light and from any potential background light. The measuring light flashes are so short that they turn over only a small fraction of reaction centers leading to a negligible perturbation of the plant from the dark-adapted state. The measuring flashes are of constant amplitude so that the measured fluorescence signal $F(t)$ is a good relative measure of fluorescence yield $\Phi(t)$ that is multiplied by the effective antenna size $\sigma_{PSII}(t)$.

- The measurement starts with dark-adapted plant that is characterized by low, minimum fluorescence emission signal F_0 (Fig.3). F_0 is interpreted as the fluorescence signal from open PSII reaction centers with their primary quinone acceptor Q_A fully oxidized (Fig.2). The open reaction centers have the maximum q_P photochemical quenching and the minimum Φ_0 fluorescence yield. At the same time, the NPQ non-photochemical quenching is fully relaxed with the effective antenna size at its maximum $\sigma_{PSII}(dark)$.
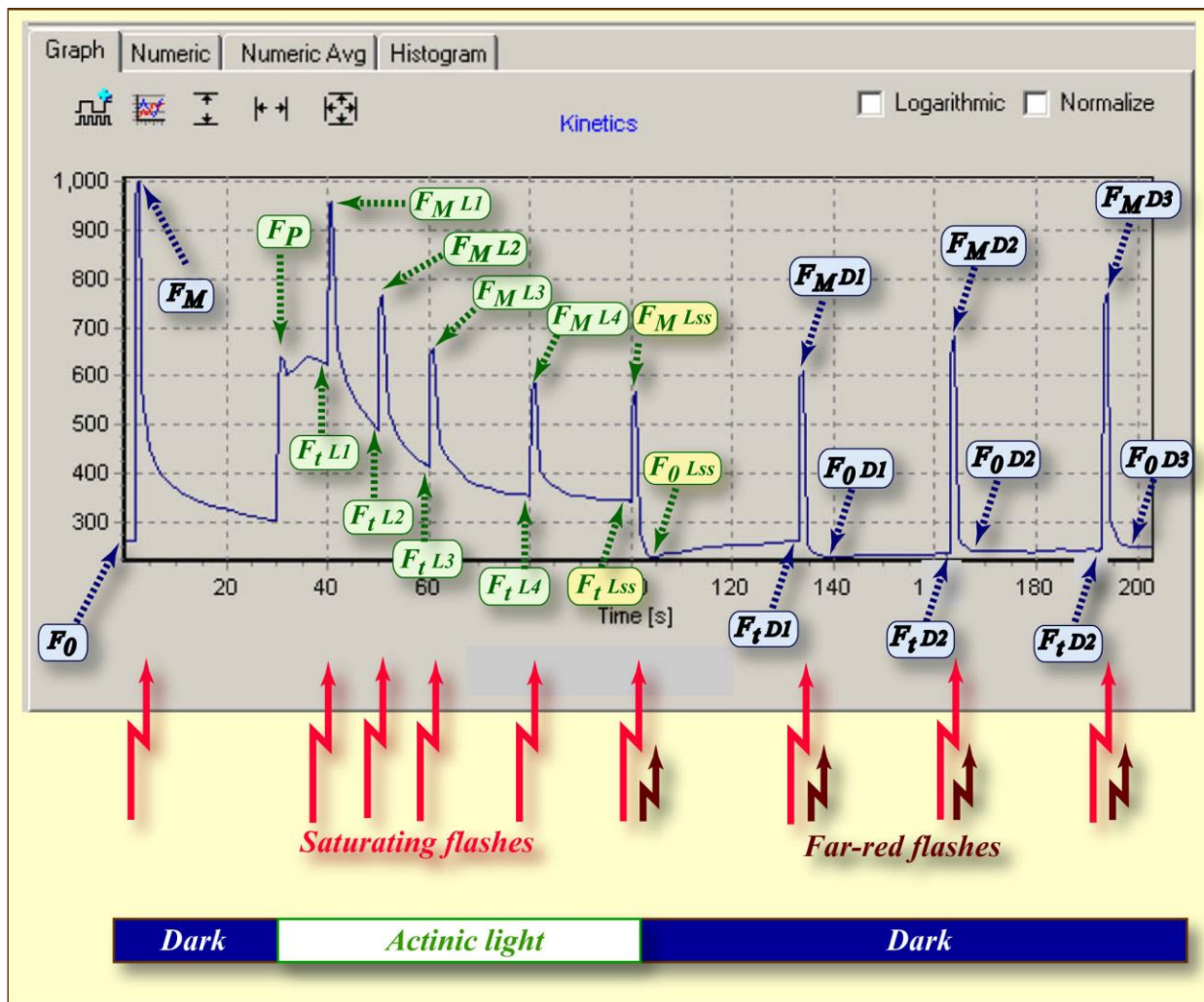
FIGURE 3. *FluorCam window showing a result of induction experiment measured in PAM-mode. The blue labels mark the fluorescence levels measured either in dark (F_0, F_M) or during dark relaxation (Ft_Dn, F_M_Dn, F_0_Dn). The green labels mark the fluorescence levels measured during light adaptation (Ft_Ln, F_M_Ln). The yellow labels show the steady-state fluorescence levels attained in continuous light (Ft_Lss, F_M_Lss, F_0_Lss). The bright-red arrows indicate timing of flashes that transiently saturate the electron transport chain, reduce plastoquinone pool and Q_A acceptor. The closed reaction centers do not quench fluorescence photochemically and the fluorescence signal reaches local maximum that is modulated only by the non-photochemical quenching: F_M in dark, F_M_Ln during light adaptation, F_M_Lss in light-adapted steady-state, and F_M_Dn during dark adaptation. The dark-red arrows indicate far-red flashes that selectively excite Photosystem I and facilitate effective re-oxidation of the plastoquinone pool and of the Q_A acceptor. The oxidized Q_A acceptor in all PSII reaction centers means maximum quenching by photochemical charge separation and minimum fluorescence signal F_0_Dn that is modulated only by residual non-photochemical quenching.*

- The measurement continues with dark-adapted plant that is exposed to a strong flash of light (marked by the red arrow in Fig.3) that transiently reduces plastoquinone pool and the primary quinone acceptor Q_A (Fig.2). The quenching by photochemistry of the reaction centers (q_P) is eliminated and the fluorescence yield reaches its maximum (Φ_M). The NPQ non-photochemical quenching remains relaxed because the illumination is transient and the quenching mechanisms cannot respond on such a short time-scale. The effective antenna size thus remains at its maximum $\sigma_{PSII}(dark)$. The measured fluorescence signal F_M ($\approx \sigma_{PSII} \cdot \Phi_M$) is at its maximum. This signal is often called F_M maximum fluorescence (yield).
- The variable fluorescence F_V signal defined as the difference $F_M - F_0$ combined with the maximum fluorescence F_M are used to calculate the maximum quantum yield of Photosystem II photochemistry $QY\_max = F_V / F_M$.

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- The dark relaxation following the saturating flash leads to a re-oxidation of the plastoquinone pool and of the primary quinone acceptor Q_A^-. The decline of fluorescence signal from the F_M level back to F_0 dark-adapted level (Fig.3) reflects kinetics at which the reaction centers re-open.

- The Kautsky effect is induced by actinic light (marked by the green-white horizontal bar in Fig.3) that drives the primary charge separation in the reaction centers of both photosystems (Fig.2). First, the signal rises to the F_P peak fluorescence level as the plastoquinone pool becomes largely reduced by Photosystem II activity (Fig.2). Later, the signal declines to the Ft_Lss steady-state fluorescence level (Fig.3). The decline is due to re-oxidation of the plastoquinone pool by Photosystem I that transfers the electrons to the activated Calvin-Benson cycle. In parallel to the activated electron transport that relaxes the congestion of the charge transport intermediates, the fluorescence decline is further enhanced by the photoprotective non-photochemical quenching that diminishes the maximum fluorescence from the high level F_M in dark to much lower level F_M_Lss in light adapted steady-state. The steady-state non-photochemical quenching is quantified by $NPQ\_Lss = (F_M - F_M\_Lss)/ F_M$. Another parameter introduced to characterize the Kautsky effect is the R_{fd} fluorescence decline ratio ($R_{fd} = (F_P - F_t\_Lss) / F_P$) that is used to assess plant vitality [Lichtenthaler and Miehé 1997].

- The saturating flashes (bright-red arrows in Fig.3) eliminate transiently photochemical quenching by reducing the plastoquinone pool and the Q_A acceptor (Fig.2). The fluorescence signal reaches local maxima that reveal dynamics of non-photochemical quenching during light adaptation (F_M_Ln). The non-photochemical quenching is quantified by $NPQ\_Ln = (F_M - F_M\_Ln)/ F_M$.

- Instantaneous fluorescence signal, Ft_Ln, is measured before any of the saturating flashes. Minimum fluorescence levels during the light adaptation are estimated by the formula $F_0\_Ln \approx F_0 / ((F_M - F_0) / F_M + F_0 / F_M\_Ln)$. This estimate is needed to assess the fraction of PSII reaction centers that are open at given time. This fraction is characterized by the photochemical quenching q_P_Ln ($\equiv$ PSII$_{open}$ / (PSII$_{open}$+ PSII$_{closed}$) = $(F_M\_Ln - F_t\_Ln) / (F_M\_Ln - F_0\_Ln) \approx (F_M\_Ln - F_t\_Ln) / (F_M\_Ln - F_0 / ((F_M - F_0) / F_M + F_0 / F_M\_Ln))$.

- Finally, the saturating flashes serve to estimate the instantaneous quantum yield of PSII photochemistry $QY\_Ln = (F_M\_Ln - F_t\_Ln) / F_M\_Ln$.

- The instantaneous signal F_t_Lss and the maximum signal F_M_Lss in light-adapted steady-state are measured before and during the saturating flash at the end of actinic light period (yellow labels in Fig.3). This measurement is used to asses the steady state non-photochemical quenching ($NPQ\_Lss = (F_M - F_M\_Lss)/ F_M$), the fluorescence decline ratio ($R_{fd} = (F_P - F_t\_Lss) / F_P$), and the steady-state quantum yield of PSII photochemistry ($QY\_Lss = (F_M\_Lss - F_t\_Lss) / F_M\_Lss$).

- The minimum fluorescence signal in light-adapted steady-state F_0_Lss is measured immediately after the actinic light is switched off and the plastoquinone pool and Q_A acceptor are re-oxidized by a far-red flash (dark-red arrow in Fig.3). The measured F_0_Lss signal is used to calculate the steady-state fraction of open reaction centers estimated from the level of photochemical quenching q_P_Lss ($\equiv$ PSII$_{open}$ / (PSII$_{open}$+ PSII$_{closed}$) = $(F_M\_Lss - F_t\_Lss) / (F_M\_Lss - F_0\_Lss)$).

- The dark relaxation (dark-blue horizontal bar in Fig.3) following the actinic light period is monitored by measuring fluorescence signal before the saturating flashes (F_t_Dn), during the saturating flashes (F_M_Dn) and during the far-red flashes (F_0_Dn).

- The fluorescence signals measured during the dark period are used to calculate the relaxation of non-photochemical quenching $NPQ\_Dn = (F_M - F_M\_Dn)/ F_M$, the re-opening of PSII reaction centers q_P_Dn ($\equiv$ PSII$_{open}$ / (PSII$_{open}$+ PSII$_{closed}$) = $(F_M\_Dn - F_t\_Dn) / (F_M\_Dn - F_0\_Dn)$), and the relaxation of PSII quantum yield $QY\_Dn = (F_M\_Dn - F_t\_Dn) / F_M\_Dn$.
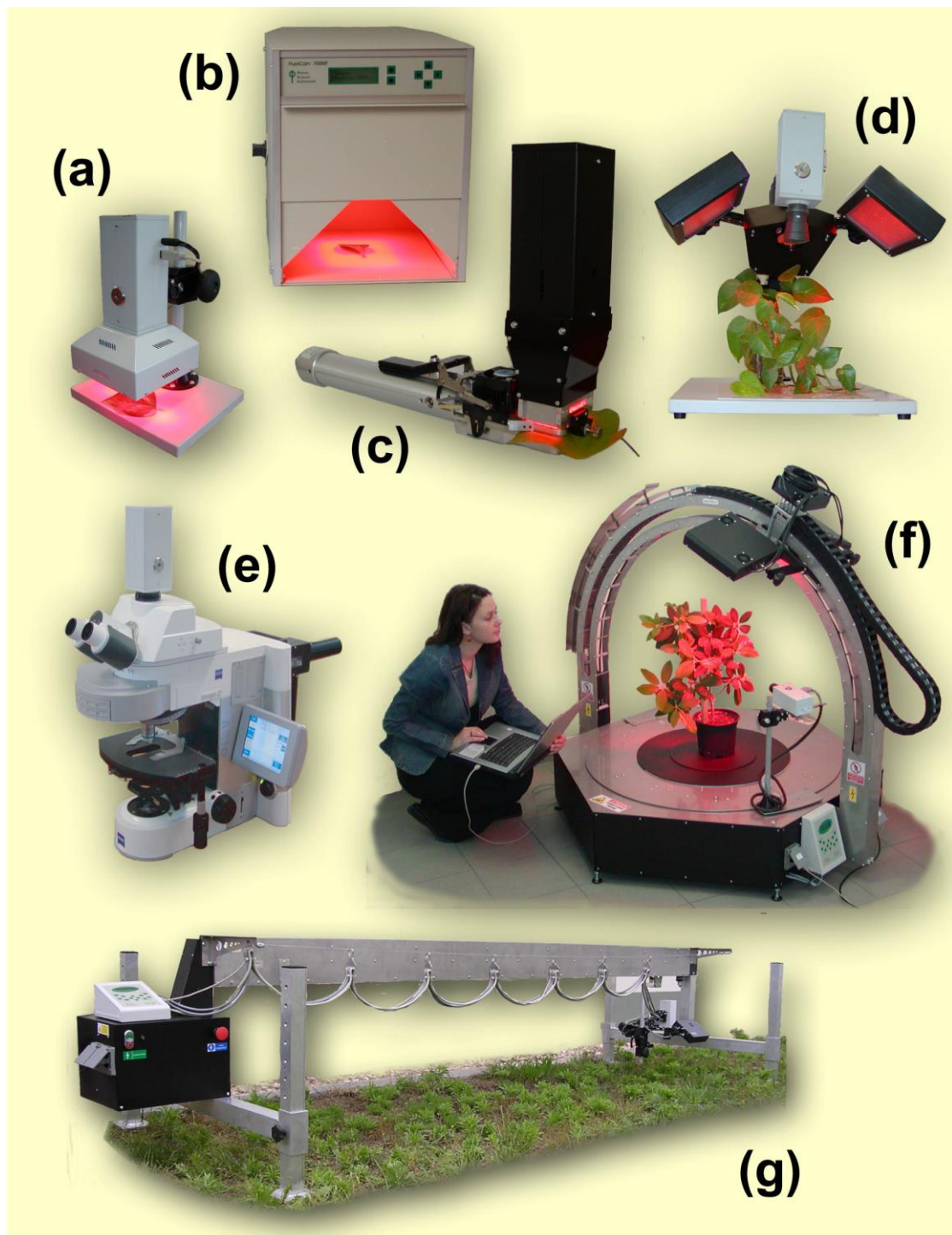
FIGURE 4. FluorCam hardware versions. (a) Portable HandyCam for single leaf / Petri dish measurements. (b) Closed FluorCam with fixed working distance for small plant / Petri dish measurements. (c) FluorCam combined with cuvette for gas-exchange measurements. (d) Open FluorCam with variable working distance and angles for small plant / Petri dish measurements. (e) μ-FluorCam with Zeiss Axio Imager. (f) Custom-made arch FluorCam for multi-angle fluorescence imaging of small and medium-size plants. (g) Custom-made large transect FluorCam for 2m (x 1m) area fluorescence imaging.

III.   FluorCam instrument

FluorCam is a name for a family of kinetic imaging fluorometers developed by Photon Systems Instruments, spol. s r.o. (Fig.4). Every FluorCam version contains a camera with standard modules serving to capture and grab images and modules required to control the measuring light

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flashes, actinic light and saturating flashes that are generated by version-dependent light sources. Both the camera and the light sources are fed by a high-capacity power supply. The operation of the camera and of the light sources is controlled according to a protocol that is designed in the computer FluorCam software (see section IV) and that is transmitted to the camera *via* USB-2 cable. The acquired data are transmitted from the camera to the computer by the same cable.

III.A. HandyCam

HandyCam is the compact portable version of FluorCam instrument for measurements on a single leaf level or on a level of a single Petri dish (Fig.5). The positions of the LED panels and of the camera objective are fixed and do not need any adjustment. The only manual controls are focusing and zoom of the objective.
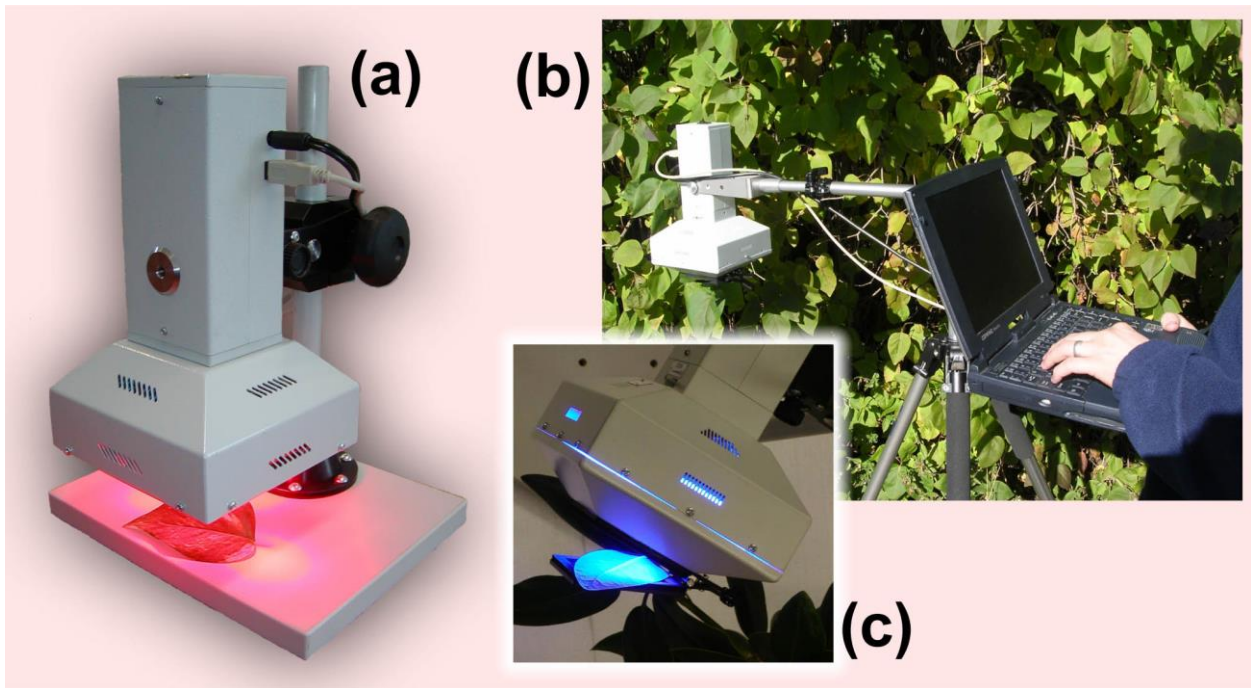
FIGURE 5. HandyCam. (a) Portable HandyCam for single leaf / Petri dish measurements in laboratory. (b) HandyCamfield operation. (c) Close-up of blue-color Handycam with leaf clip open.

Fig.6 shows a scheme of FluorCam macroscopic systems (HandyCam, closed FluorCam, open FluorCam, FluorCam with gas exchange and custom-made large-scale systems). The scheme shows a full configuration. In some versions, the spectrum of light sources can be limited to measuring flashes and actinic light source. Also, the control unit can be integrated in the camera in some versions or can be in form of a separate hub.
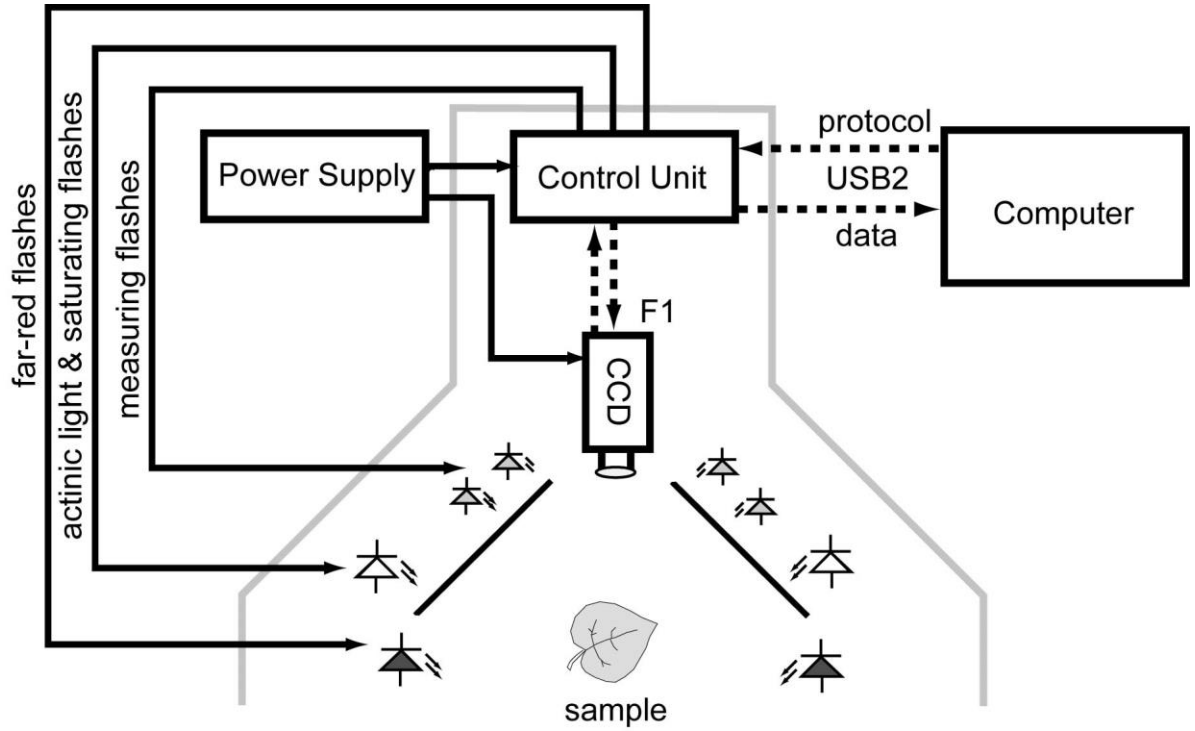
FIGURE 6. Scheme of macroscopic FluorCam versions.

III.B. Closed FluorCam

Closed FluorCam is a compact laboratory version of FluorCam instrument for measurements on a single leaf level or on a level of a single Petri dish (Fig.7). The positions of the LED panels and of the camera objective are fixed and do not need any adjustment. The only manual controls are focusing and zoom of the objective. Scheme of typical configuration is shown in Fig.6.
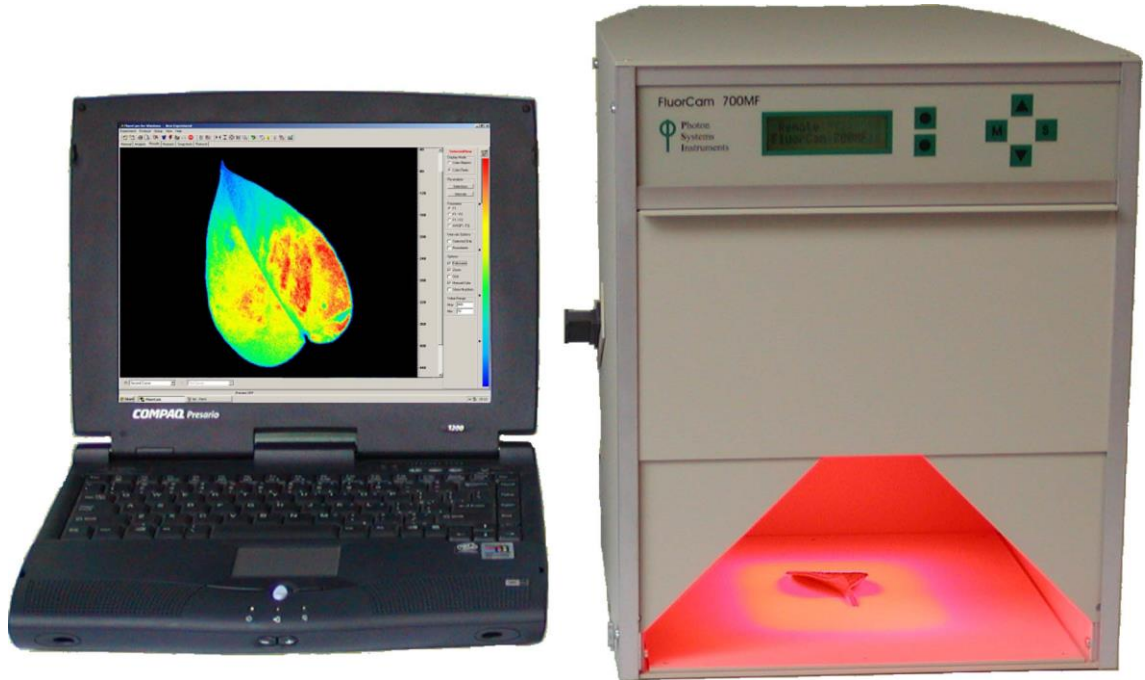
FIGURE 7. Closed FluorCam. The saturating flash is generated by a halogen lamp equipped with a mechanical shutter.

III.C. FluorCam mounted to gas-exchange cuvette

FluorCam can be combined with a gas-exchange measuring systems of all major producers (Licor, PP Systems and others). The miniaturized LED panels fit the upper window of the cuvette leaf-clip (Figs. 6 and 8).
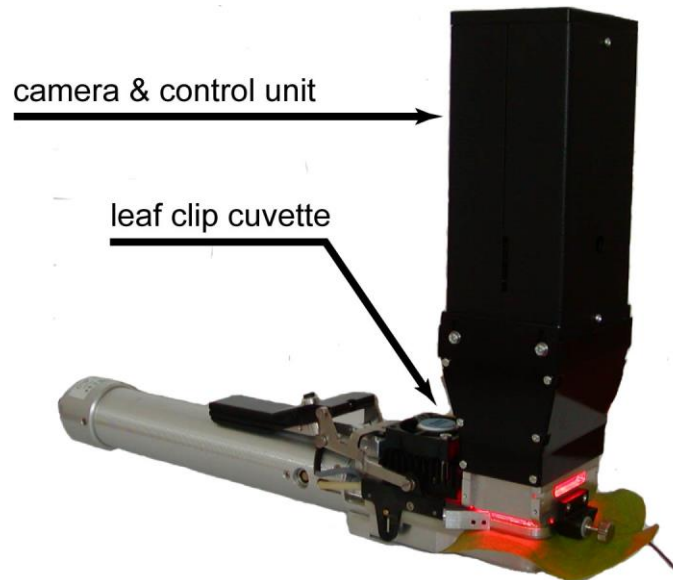
FIGURE 8. FluorCam with gas-exchange measuring cuvette.
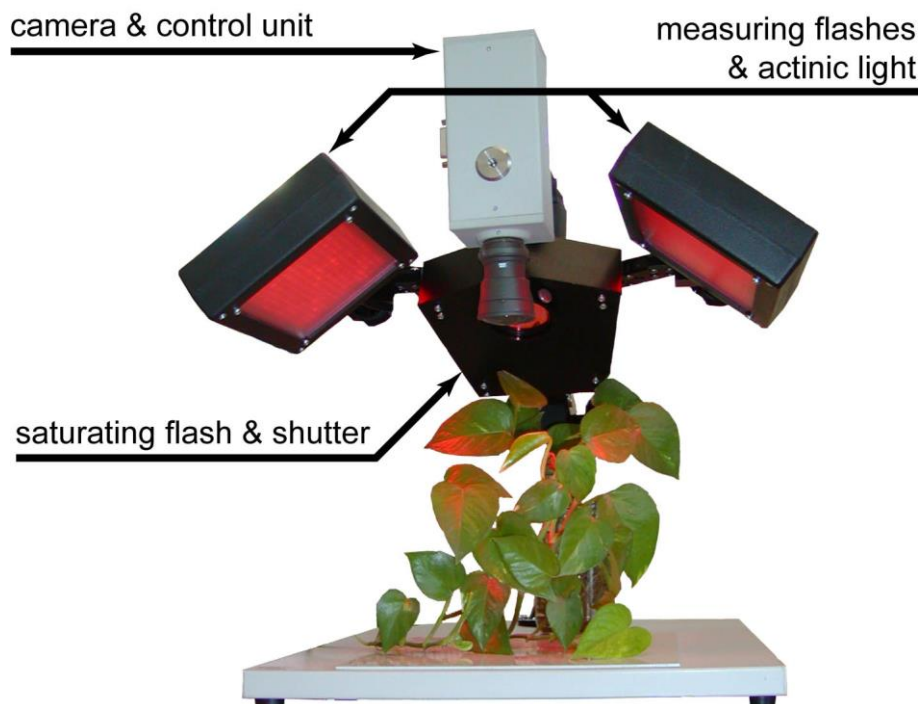
FIGURE 9. Open FluorCam. The positions and angles of light sources and of the camera can be manually adjusted making this instrument version highly flexible. The user finds the optimum fitting best the spatial characteristics of the sample and the requirement for irradiance homogeneity.

III.D. Open FluorCam

Open FluorCam is the most versatile version of FluorCam instrument for measurements on a single leaf level or on a level of a small plant. The light sources as well as the camera can be arranged in various configurations. The FluorCam hub can support up to 4 LED panels.

III.E. µ-FluorCam

µ-FluorCam can be combined with any wide-field fluorescence microscope satisfying the needs of the user. The major difference from the macroscopic FluorCam versions is in the light sources. Up to three LED systems with two dichroic mirrors can be integrated into the module that fits the microscope condensor port. Measuring flashes, actinic light, saturating flashes and 'fluorescence protein' exciting source are typically supplied in this module. The Far-Red source is mounted separately in the transmission direction. Scheme in Fig.9 shows a typical configuration of µ-FluorCam.
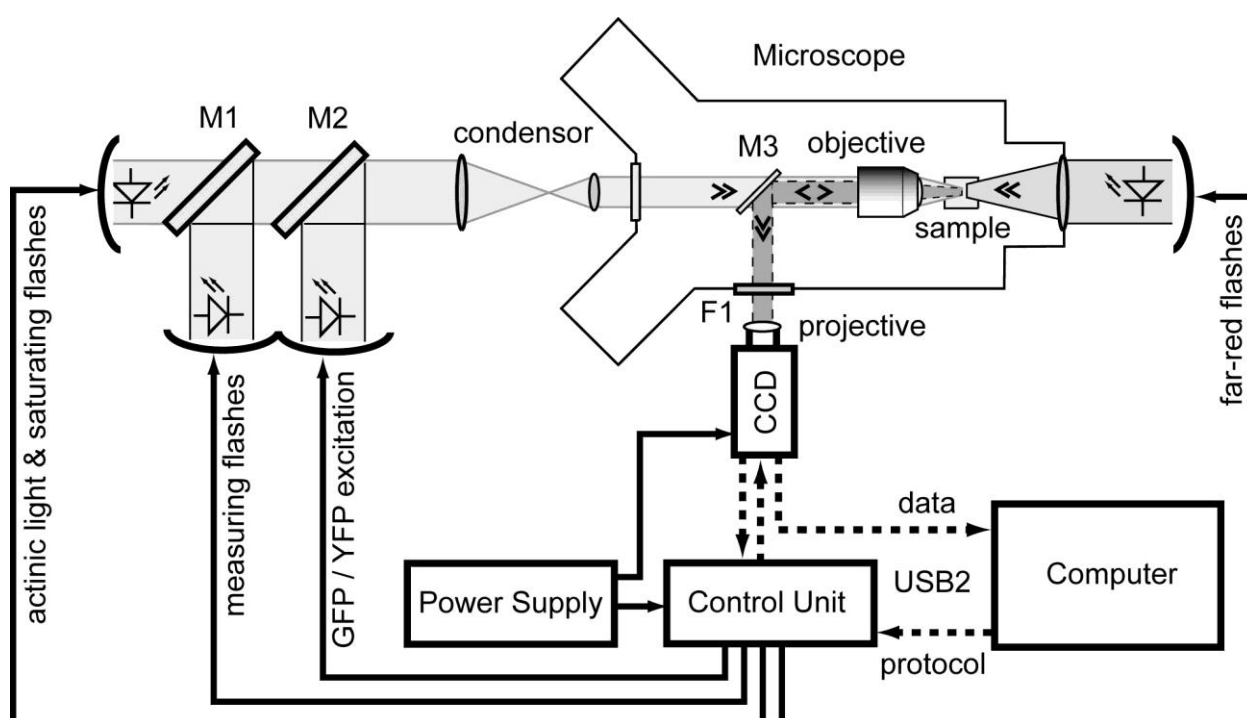
FIGURE 10. Scheme of µ-FluorCam.

III.F. Custom-made FluorCam versions ('arch', 'large-transect')

Open FluorCam can be mounted to various robotic systems with motorized positioning of light sources and of the camera. Fig.11 shows the Arch FluorCam that can monitor plants from exactly controlled angles. An area up to 2 x 1 meter can be surveyed by large-transect FluorCam.
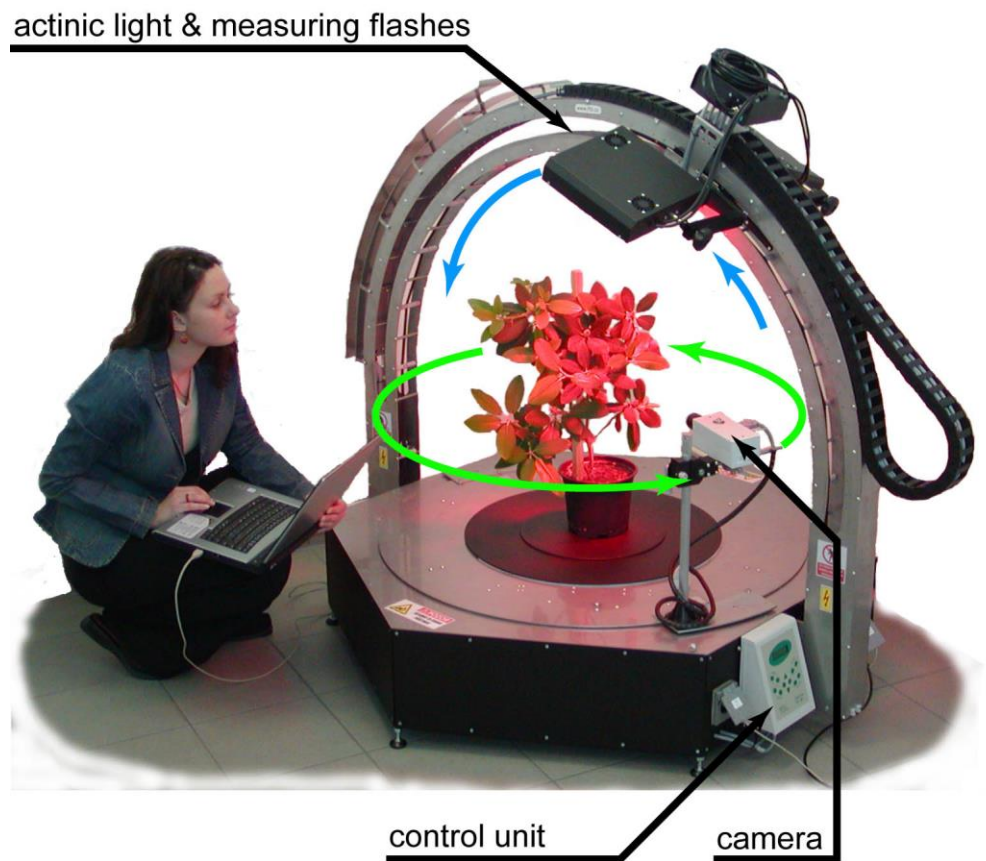
FIGURE 11. Arch FluorCam.
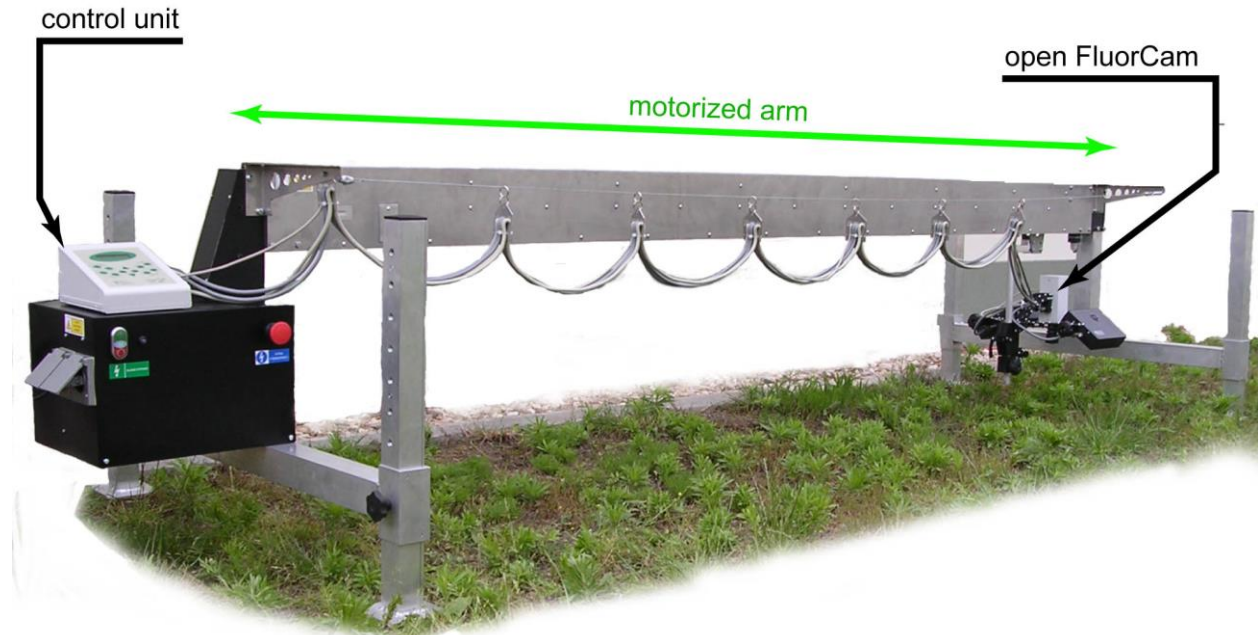
FIGURE 12. Large Transect FluorCam. Another motorized arm can be added transversally so that the covered area is 2 x 1 meter.

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IV.   FluorCam software

IV.A.   Installation and start

- Connect the instrument to power supply.
- Connect the power supply to 110/230V outlet or to battery pack.
- Connect the instrument to the computer by enclosed USB2 cable.
- Switch on the power supply, camera and the computer.
- Install the software: The software is delivered on a CD in form of a self-extracting file. Insert the installation CD into the mechanics of the FluorCam computer and follow the instructions appearing on the computer screen. Alternatively, the installation files can be downloaded from www.psi.cz. To start the program, click the FluorCam icon on the desktop of the computer.
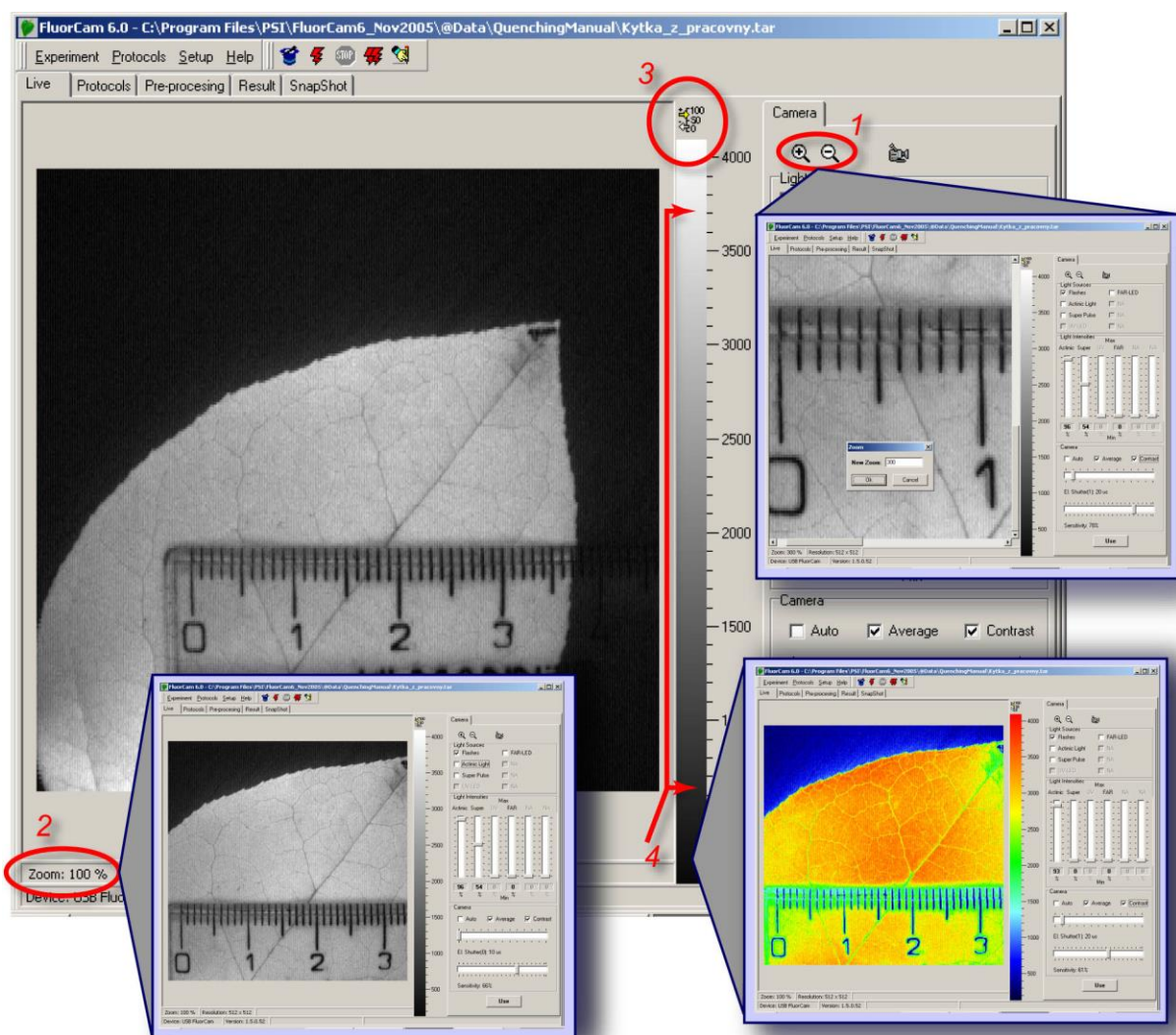
FIGURE 13.   Initial adjustment with the Live FluorCam window. By clicking the magnifying and de-magnifying icons (1), one can increase and decrease the size of the image window on the computer screen. By clicking the zoom field (2), one can zoom while preserving the size of the image window. By clicking the numeric scale field (3), the scaling bar is supplemented by numeric scale. By clicking anywhere on the color / gray scale bar (4), one can change color scheme of the image.

IV.B. Testing the instrument in live window

Properly started FluorCam and FluorCam software show live window in the auto mode that adjusts the camera electronic shutter and the sensitivity to ambient light level. Place, in a dim lit room, a plant leaf in front of the camera objective and adjust the zoom and focus of the camera objective so that you see a sharp leaf image. What you see is an image transmitted by ambient light photons scattered on the surface of the leaf or table. The brighter is the ambient light the lower is the automatic shutter setting and the camera sensitivity.

IV.B.1 Live visualization features tested in scattered ambient light

The live window shows an on-line stream of images taken by the camera. FluorCam generates 512 x 512 pixels images with 12-bit resolution, i.e., discriminating maximum 4096 light levels. 25 images are taken per second, one every 40 milliseconds.

Fig.13 shows the live window with the fields serving to control the visualization features marked by red labels. The size of the image window can be adjusted using (de-)magnifying icons - (1) in Fig.13.   Details of the image can be viewed by setting higher zoom - (2) in Fig.13.   The numbered scale can be viewed after clicking the corresponding field - (3) in Fig.13. The color scheme can be changed by clicking the color / gray shade scale bar (4) in Fig.13.
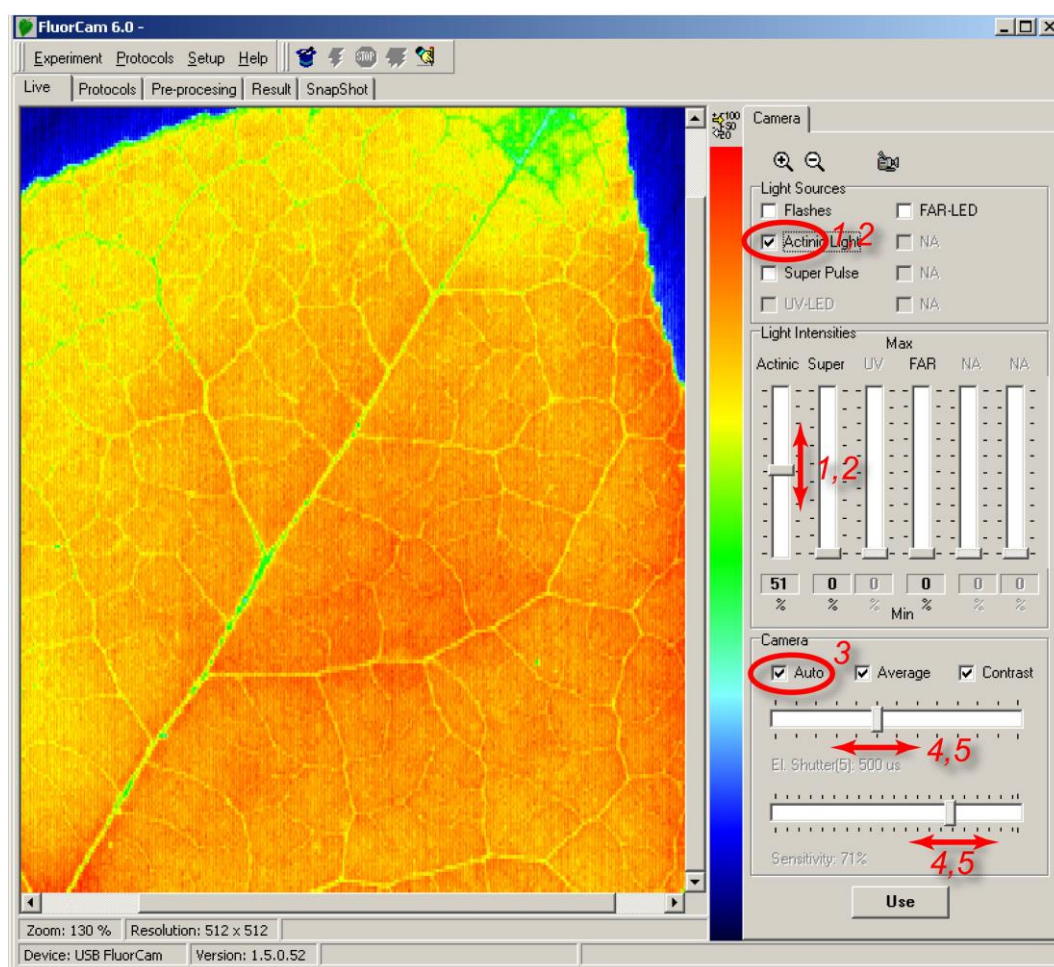
FIGURE 14. Testing continuous actinic light mode with the live FluorCam window. The actinic light is switched on and off and its power is set by the controls (1) and (2). In the 'Auto' mode, the electronic shutter and the camera sensitivity are adjusted automatically. The Auto mode can be switched off (3) and the shutter and sensitivity can be adjusted manually using the controls (4) and (5).

IV.B.2 Live imaging with continuous light excitation

The live window can be used to reproduce the famous Kautsky and Hirsch experiment [Kautsky and Hirsch 1931]. First, the right actinic light irradiance, electronic shutter and camera sensitivity must be adjusted by controls described in Fig.14. With the actinic light off and with the auto mode off, the plant must be dark adapted for a minute or more. Then, switch on the actinic light and follow the changes in the brightness of the leaf image on the computer screen. The fluorescence signal will rise during the first second and decline thereafter. The hand-drawn recording of this transient was the essence of the famous paper by Kautsky and Hirsch.

IV.B.3 Live imaging with excitation by measuring flashes

The live window can be used to test also the PAM mode of the instrument. In this mode, the measured fluorescence that is excited by very short flashes can be discriminated from fluorescence due to saturating pulses or due to continuous light. Also, the signal originated from ambient light that has the same wavelength as the chlorophyll fluorescence can be subtracted. The operation controls are shown in Fig.15. The PAM mode can be tested by switching on the flashes (*1*) with auto mode on (*2*). The auto mode is switched off thereafter by (*2*). Shutter opening time and camera sensitivity can be adjusted by (*3*) and (*4*). The shutter opening controls also the duration of the measuring flashes and that cannot be longer than XX μs. The apparently brighter measuring light with longer shutter opening time is due to the longer on-time rather than to an increased photon flux. The brighter fluorescence signal with longer shutter opening is due to longer integration in the CCD chip. The higher signal and the better signal-to-noise ratio with longer shutter opening time is unfortunately traded off for increased actinic effects of the measuring light. The measured signal deviates from the true F_0 if the measuring light is too strong. The problem can be alleviated by low repetition frequency of the measuring flashes (see the Protocol description below).
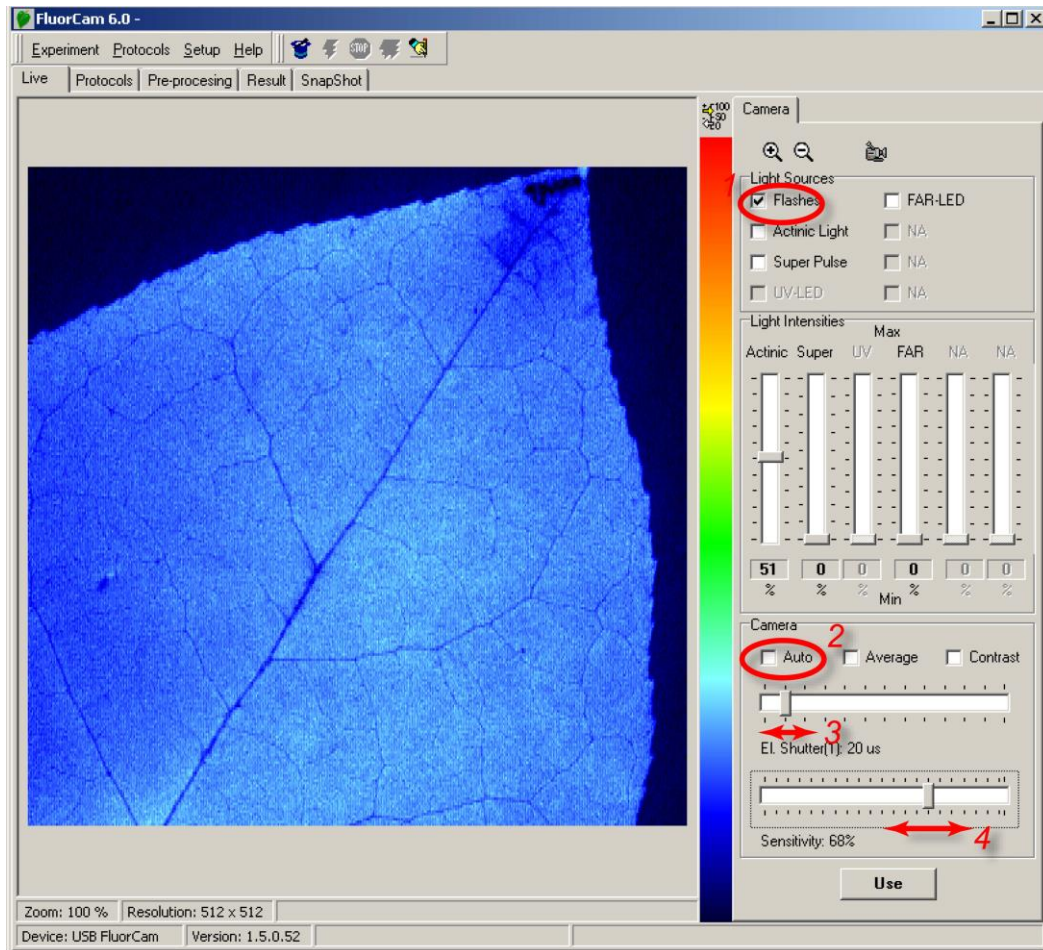
FIGURE 15. Live imaging in PAM mode with measuring flashes. The flashes are switched on by checking (1). The auto mode automatically adjusts the shutter and sensitivity to yield optimum signal. The auto mode can be switched off by (2). With the auto mode off, the shutter and sensitivity can be adjusted manually (3) and (4). Please, note that the shutter opening time controls also duration of the measuring flashes.

IV.B.4 Testing more features of the live window

- Switch on the PAM mode (flashes on). Set the camera sensitivity to ca. 60-80% and adjust the shutter opening time so that the signal of the dark-adapted leaf fluorescence is around 500 (out of maximal 4096).
- Set the actinic light intensity to ca. 50% and switch the light on (*1*). Observe the Kautsky effect on the computer screen. Test the effects of changing actinic light intensity. Switch the actinic light off.
- In the hardware versions containing super pulse feature, set the pulse intensity to ca. 50% and trigger the super pulse by (*2*). Observe the transition from F_0 to F_M and back on the computer screen. Repeat with changing super pulse intensity.
- In the hardware versions containing far-red light, set the far-red intensity to ca. 50% and switch the far-red irradiance on (*3*). The fluorescence signal slightly elevated by the actinic effects of the measuring light or by dim ambient irradiance in your lab will be reduced to F_0 level.

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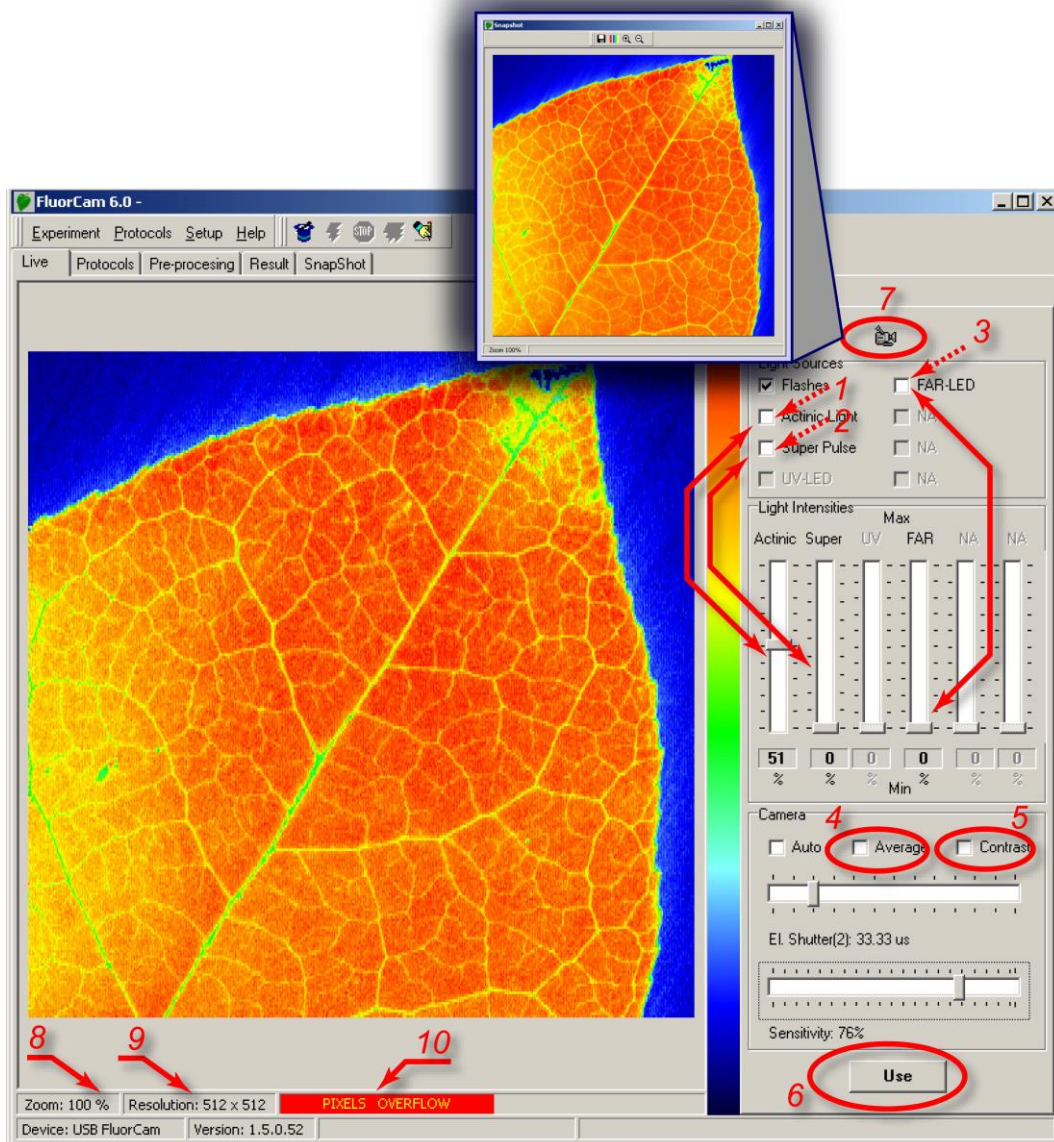
FIGURE 16. More features in live window. The actinic light is switched on and off and its intensity is set by (1). The super pulse is triggered and its intensity is set by (2). The far-red light is switched on and off and its intensity is set by (3). The average mode and the contrast modes are controlled by (4) and (5). The setting of the live window can be transmitted to protocol (see below) by (6). The image shown in the image window can be captured as a snapshot by (7). The FluorCam ID and Version are automatically recognized by instruments made in 2005 or later (8) and (9). Signal that is exceeding in any pixel the maximum CCD well capacity (4096 levels) is indicated by (10).

- Set the sensitivity to a high level and the shutter to the lowest level that still yields a leaf fluorescence signal that is clearly visible on the screen. With this setting, the images displayed on the computer screen will be relatively noisy. To reduce the noise, switch on the average mode by (*4*). Note the improvement of the signal-to-noise ratio. Averaging is also possible in the instrument protocols (see below).

- Decrease the camera sensitivity and the shutter to the lowest level that yields a leaf fluorescence signal that is low but visible on the screen. The contrast of the images will be low. To increase the contrast, switch on the contrast mode by (*5*). Note the improvement of the contrast that is achieved by re-scaling of the image window from 0-to-4096 to minimum-to-maximum.

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- The FluorCam is typically operated using a protocol. The protocol specifies intensity setting of an experiment. One can find optimum setting in the live window and transmit the thus found setting to protocol by (*6*).
- The fluorescence transients are recorded using protocol specification. As an alternative, a fluorescence image can be recorded also in live mode by clicking the snapshot icon (*7*).
- The FluorCam device ID and hardware version are automatically recognized by instruments made in 2005 or later (*8*) and (*9*).
- Signal that is exceeding, in any pixel, the maximum CCD well capacity (4096 levels) is indicated by the warning in (10). It is especially important in combination of measuring flashes with strong actinic light and strong super pulse intensity that can generate background signal comparable to fluorescence. Due to such a strong background, the CCD capacity can be exceeded even though the fluorescence signal excited by the measuring flashes does not approach the 4096 maximum. If this is the case, reduce the shutter opening time or the camera sensitivity to avoid the saturation.

IV.C.   Designing a protocol to capture fluorescence transients

Protocol is a list of instructions by which the user specifies what and when should the FluorCam instrument do. There are three modes of designing a protocol.

IV.C.1 Protocols à la carte.

The first choice is to use one of the pre-designed protocols that are available from the menu that opens upon clicking the wizard icon (1 in Fig.17). The advantage is not only the ease of use but also robustness of the offered protocols.

Upon clicking the selected name, the corresponding protocol is loaded into protocol window. There is no need to learn the code in which the protocol is written unless the user needs to design new protocols or to modify the existing protocols (see below). If you don't need any of these you can skip reading the next 3 sections (IV.C.2-4).
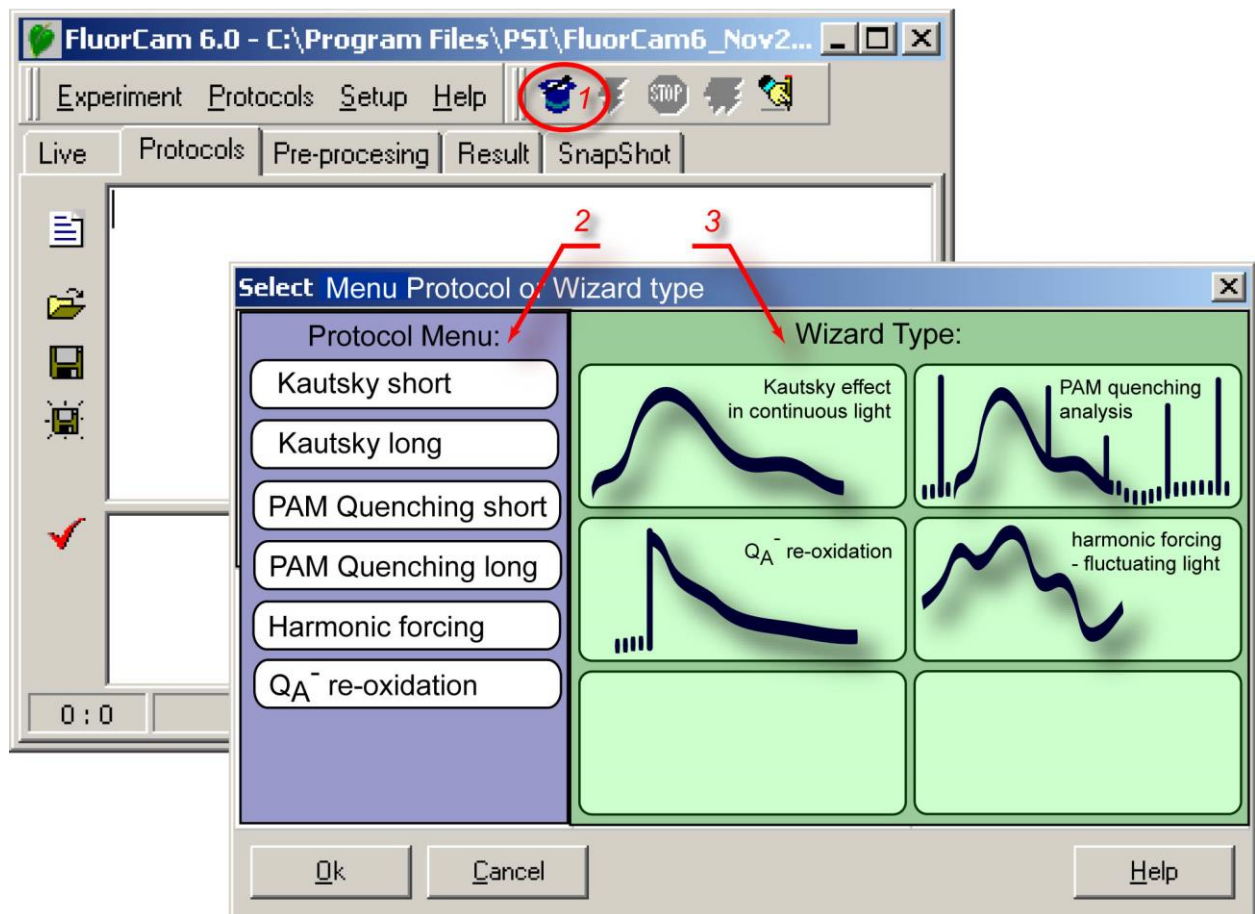
FIGURE 17. Protocol Menu and Wizard window.

IV.C.2 *Protocol template filled by wizard.*

Wizard offers flexibility in designing protocols without requiring from the user expertise in the protocol syntax. The flexibility may be required in many experimental situations. For example, the Kautsky effect transient largely depends on the actinic light intensity. A mutant or localized stress can be distinctly visible with a low irradiance and can be missed with a high one. The modification can be done either using wizard template or by modifying an existing protocol (see IV.C.3).

The wizard constructing protocols for Kautsky effect measurements in continuous light (Fig.18A) requires two parameters:
- 'Experiment duration' determines in seconds the period for which the plant is illuminated by the actinic light;
- 'Actinic light intensity' determines in percents the relative irradiance of the actinic light source.

The wizard for PAM quenching analysis (Fig.18B) requires the following parameters:
- 'Subtract background signals', if checked, leads to subtraction of background signals that are measured 40 ms before fluorescence is sampled during a measuring flash;
- 'F_0 duration' defines in seconds the period during which F_0 measurement is repeated once per second.
- F_M 'Pulse duration' defines in milliseconds duration of the flash that transiently reduces the plastoquinone pool and the Q_A acceptor.
- F_M 'Pulse intensity' sets relative power of the plastoquinone / Q_A reducing flash.

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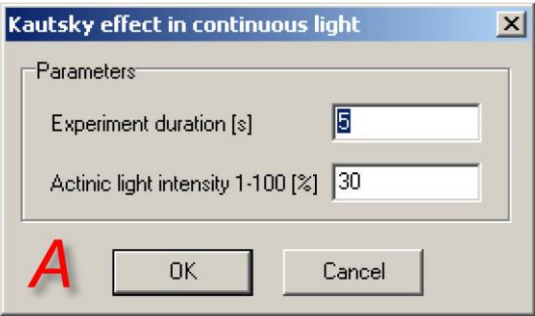
- 'Dark relaxation period after Fm' defines in seconds duration of the dark relaxation following the F_M measurement.
- 'Actinic light exposure' determines in seconds duration of the light on period.
- 'Actinic light intensity' determines in percents relative power of the actinic light.
- 'Number of pulses' defines the number of saturating flashes and of the Fm' measurements during the actinic light exposure.
- 'Dark relaxation period after Kautsky' determines in seconds duration of the dark relaxation following the Kautsky effect measurement.
- 'Number of pulses' defines the number of saturating flashes and of the Fm' measurements during the dark relaxation following the Kautsky effect measurement.

The wizard constructing protocols for Q_A^- re-oxidation kinetics (Fig.18C) requires the following parameters:
- 'Dark interval separating ST flashes' defines how long is the relaxation between two single-turnover (ST) flashes. This parameter defines also the period of the pump-and-probe measurement. One data point is collected per period.
- 'No. of data points / decade' defines the resolution of the measurement on logarithmic time scale. Keep in mind that the re-oxidation kinetics is measured in pump-and-probe mode and that number of data points / decade multiplied by the number of decades and by the dark interval between the measurements defines the total duration of the experiment. With the dark interval of 5 s separating the measurements, with 4 data points per decade and with 4 decades of time scale covered (100 µs, 1 ms, 10 ms, 100 ms), the total experiment time will be 80 s.
- 'First data points delay' defines how many microseconds pass between the single-turnover flash and the first measurement.
- 'ST flash duration' defines how long is the single-turnover flash. The flash should be short to prevent double-turnovers. At the same time, the flash should be saturating and the saturation requires high power and long duration. A compromise between the two requirements must be found.
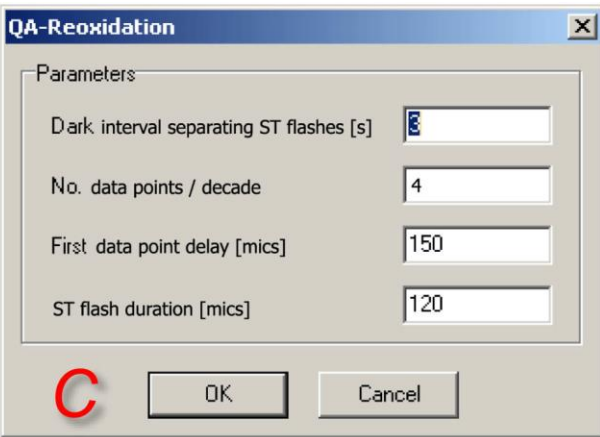
The wizard for harmonic forcing experiments (Fig.18D) requires the following parameters:
- 'F_0 duration' determines in seconds the period during which F_0 measurement is repeated once per second.
- F_M 'Pulse duration' determines in milliseconds duration of the flash that transiently reduces the plastoquinone pool and the Q_A acceptor.
- F_M 'Pulse intensity' sets relative power of the plastoquinone / Q_A reducing flash.
- Harmonic forcing 'Amplitude' defines in percents the relative change between the maximum irradiance and the mean irradiance of the oscillating light. For example, an amplitude of 40% with 0% offset means that the light will oscillate between 0 and 80% of the maximum actinic light irradiance. The mean irradiance during such oscillations will be 40% of the maximum.
- Harmonic forcing 'Offset' defines in percents the relative increase of the mid-irradiance. For example, an amplitude of 30% with 20% offset means that the light will oscillate between 20% and 2 x 30+20% = 80% of the maximum actinic light irradiance. The mean irradiance during such oscillations will be 20 + 30% = 50% of the maximum.
- Harmonic forcing periods define which oscillation periods are applied, for how long, and in what order. Typically, the first oscillation period is short and repeated longer to induce a stable periodic transient.
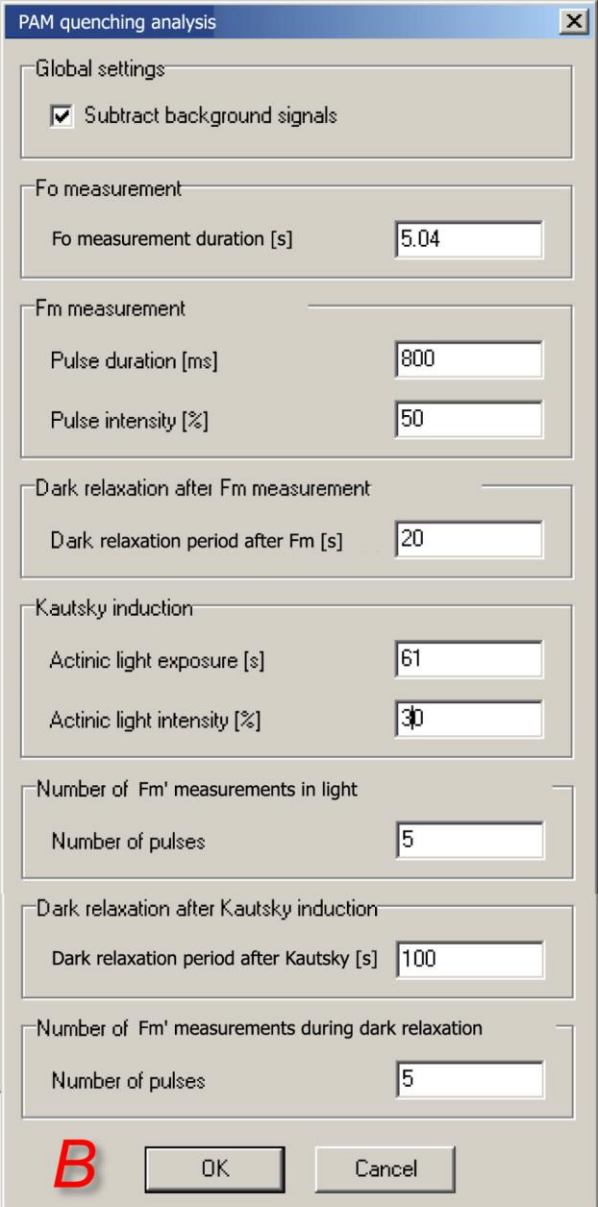
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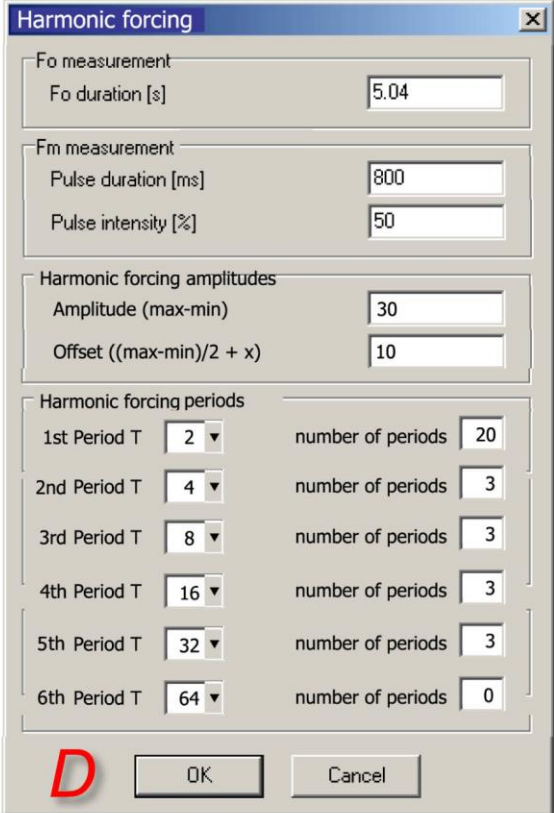
FIGURE 18. *Wizard windows. (A) The protocol for Kautsky effect measurement with continuous light excitation requires only to specify the duration of the actinic light exposure and the actinic light intensity. (B) The PAM quenching analysis consists of three phases: measurement of dark-adapted levels F_0 and F_M, measurement of Kautsky effect and of non-photochemical quenching in light. (C) The Q_A^- re-oxidation kinetics is measured in pump-and-probe with short saturating flashes repeated at a low frequency and measurements taking place with a pre-defined delay after the Q_A reducing flash. (D) The harmonic forcing protocol requires to specify F_0 and F_M levels, amplitude and offset of the actinic light modulation and up to six measurements with various forcing periods.*

IV.C.3 Modification of menu and wizard protocols

The pre-designed protocols offered in the menu or the protocols generated by wizard are displayed in the protocol window in form of a text file that can be edited (Fig.19). The protocols can be saved under their current name (3 in Fig.19) or saved under a new name (4 in Fig.19). The syntax of the modified protocol can be checked (9 in Fig.19). The FluorCam program displays potential warnings and errors revealed by syntax check in a separate text window (10 in Fig.19). Several lines of a protocol source code that are frequently and easily modified are shown in Fig.19 (5-8).
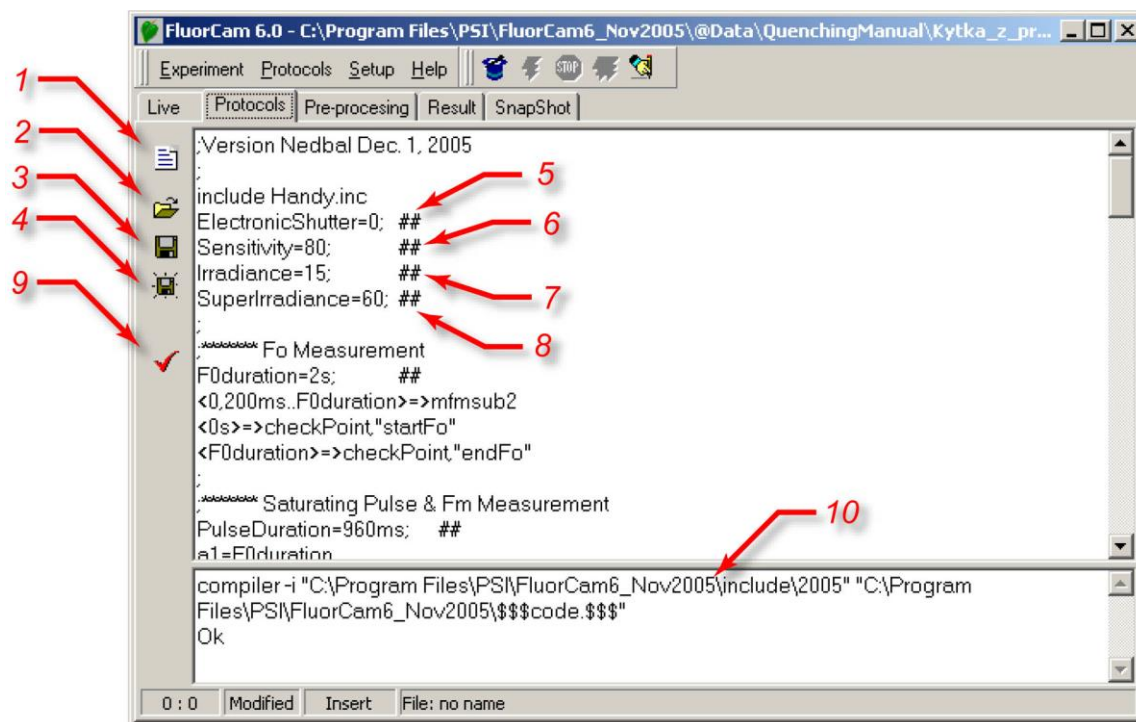
FIGURE 19. Protocol window. (1) New protocol icon erases the protocol memory. (2) Open an existing protocol. (3) Save the active protocol under its present name. (4) Save the active protocol under a new name.
(5) Line specifying electronic shutter opening time (0-12 for continuous light mode and 0-2 for PAM mode). (6) Line specifying the relative sensitivity of the detection (0-100%). (7) Line specifying the relative power of the actinic irradiance (0-100%). (8) Line specifying the relative sensitivity of the super pulse (0-100%). (9) Syntax check of the protocol source file and protocol compilation. (10) Syntax check report with identification of potential warnings and errors.

IV.C.4 Design of new protocols from scratch

The spectrum of protocols that are offered in the protocol menu and that can be designed with help of wizard templates is broad and covers all typical experimental designs required in plant science and in plant applied research. The users that do not need more specialized protocols can skip the present section and proceed to the Chapter V.

The spectrum of experiments that can be done with FluorCam hardware is broader than what can be covered by wizards. The full versatility of the hardware can be exploited by user-designed protocols.

The protocol syntax is explained here using the protocol for quenching analysis that is offered in the protocol menu. An example of measurement with this protocol is shown in Fig.3.

The protocol starts with the header section

```
;Quenchin analysis Dec. 1, 2005
include default.inc
include Handy.inc
ElectronicShutter=0;   ##
```

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```
       Sensitivity=80;          ##
       Irradiance=15;           ##
       SuperIrradiance=60;  ##
```

The protocol starts with the header section. The first line represents the comment format. The semicolon symbol (;) can start a comment section in any line. Comments are ignored by the protocol compiler.

The next two lines (include default.inc and include Handy.inc) specify the names of the files, which define the FluorCam compiler instructions and syntax of the FluorCam protocols. The default.inc defines those commands that are valid for all FluorCam versions. The Handy.inc file lists commands that are specific for a particular FluorCam version.

The line (ElectronicShutter=0) determines the duration of the interval for which is the electronic shutter opened during measurements. The electronic shutter settings are numbered and the actual duration of the shutter opening time is shown in Tab.1. The line ends by string (; ##) that is a comment ignored by the compiler that serves to mark a line which is easily modified. The modification is easy because it does not require modifications elsewhere in the protocol.

	PAM mode			continuous light mode									
Electronic shutter #	0	1	2	3	4	5	6	7	8	9	10	11	12
Shutter opening time	10 µs	20 µs	33 µs	100 µs	250 µs	500 µs	1 ms	2 ms	4 ms	6.3 ms	10 ms	16.67	20 ms

The sensitivity of the FluorCam detection is defined by the (Sensitivity=80) line. The sensitivity scale is linear and relative (0-100%): 0 meaning minimum sensitivity and 100% meaning maximum sensitivity. The maximum sensitivity range (80-100%) should be used only when necessary because it can be leading to a relatively low signal-to-noise ratio.

The actinic light power is determined by the (Irradiance=15) line. Again, the scale is linear and relative. The actual absolute irradiance at the sample plane must be measured by the user by any of the widely available PhaR meters. The absolute irradiance is determined by the actinic light setting and by the topology of the particular instrument and of the particular sample.

The power of the plastoquinone pool reducing flash, the SuperPulse, is defined by the (SuperIrradiance=60) line.

The header protocol section is followed by sections that primarily define new protocol variables, timing of individual trigger and measurement events.

```
;******* Fo Measurement
F0duration=2s;          ##
<0,200ms..F0duration>=>mfmsub2
<0s>=>checkPoint,"startFo"
<F0duration>=>checkPoint,"endFo"
```

The comment line (;******* Fo Measurement) is followed by introduction of a variable that defines duration of the F_0 measurement (F0duration=2s). The next line (<0,200ms..F0duration>=>mfmsub2) determines the F_0 measurement sequence. <0,200ms..F0duration> defines time series that is interpreted by the compiler using the following syntax elements: Parenthesis <> represent linear scale of the timing. The first event of <t_1, t_2,.. t_N> is at the time t_1, the second event is at t_2 ($t_2 = t_1 + \delta t$, where $\delta t = t_2 - t_1$), and the third event is at t_3 ($t_3 = t_1 + 2.\delta t$). The last event is determined by the last element of this series that is equal or shorter than t_N. The symbol => tells the compiler that at the instants specified by the time series the FluorCam must execute the command mfmsub2. The command is interpreted as m … measure, fm … flash & measure, and sub … subtract. First, an image is taken without the measuring flash to capture the background signal in every pixel. Second image is taken 40 ms later in parallel to a

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measuring flash. The signal in the second image consists of both, the background and fluorescence excited by the measuring flash. By subtracting the first image from the second one subtracts the background and obtains net fluorescence signal. The technique requires that the background is not changing on the time scale of tens of milliseconds (avoid measuring in a bright light of fluorescent tubes). Also, neither of the two measurements should reach saturation (4096).

The next two lines determine that all the images taken between <0s> and <F0duration> can be jointly addressed under the fluorescence feature name "Fo". The images can be, under this name, averaged, displayed and used for calculations in the image processing part of the software. The details of the prescribed processing formulae are defined in numerix.nmx and numerixavg.nmxa files.

The next protocol block determines timing of the F_M measurement:
```
;******* Saturating Pulse & Fm Measurement
PulseDuration=960ms;    ##
a1=F0duration
a2=a1+160ms
<a1+40ms>=>SatPulse(PulseDuration)
<a1+40ms>=>actinlightOn(PulseDuration)
<a2,a2+160ms..a1+PulseDuration>=>mfmsub2
<a2>=>checkPoint,"startFm"
<a1+PulseDuration>=>checkPoint,"endFm"
```
Following the comment line (;******* Saturating Pulse & Fm Measurement), three time variables are set: PulseDuration defines duration of the saturating Super Pulse, a1 renames the end of the previous series, and a2 defines the start of the F_M measurement.
The SuperPulse is triggered (=>SatPulse(PulseDuration)) 40ms after the end of the preceding series <a1+40ms>. The Pulse is on for an interval specified in PulseDuration. The actinic light is switched on in parallel (<a1+40ms>=>actinlightOn(PulseDuration)). The first measurement is taken 120 ms after the saturating flash is triggered (<a2,a2+160ms..a1+PulseDuration>=>mfmsub2). The measurement is delayed to allow full reduction of the plastoquinone pool by the Super Pulse. The last two command lines assign the images taken between a2 (= a1+160ms) and a1+PulseDuration a single collective name of a fluorescence feature - Fm.

The dark relaxation following the F_M measurement is defined in:
```
;******* Dark Relaxation Measurement
DarkRelaxation1=27s;  ##
b1=F0duration+40ms+PulseDuration
b2=80ms
b3=2s
<b1+b2, b1+2*b2, b1+4*b2, b1+8*b2, b1+16*b2>=>mfmsub2
<b1+b3, b1+2*b3..b1+DarkRelaxation1-b3>=>mfmsub2
<b1+DarkRelaxation1-40ms>=>mfmsub2
```
Dark relaxation period is set to 27s (DarkRelaxation1=27s). The variable b1 defines start of the measurement of the relaxation kinetics (b1=F0duration+40ms+PulseDuration). The variable b2 defines (b2=80ms) the step of the initial logarithmic measurement time series following the Super Pulse <b1+b2, b1+2*b2, b1+4*b2, b1+8*b2, b1+16*b2>=>mfmsub2. The second time series resets the measurement for the time scale of seconds (b3=2s, <b1+b3, b1+2*b3..b1+DarkRelaxation1-b3>=>mfmsub2). The last line of the protocol block defines the last measurement point before the actinic light exposure. The data point is set to the last FluorCam period that is still taken in dark.

The actinic light is switched on for 71s (ALPeriod=71s) at time c1:
```
;******* Actinic light Exposure
ALPeriod=71s;      ##
c1=F0duration+PulseDuration+40ms+DarkRelaxation1
<c1>=>actinlightOn(ALPeriod)
```

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The Kautsky effect is measured during the actinic light period:

```
;******* Kautsky Effect Measurement
c2=40ms
c3=2s
<c1+c2, c1+2*c2, c1+4*c2, c1+8*c2, c1+16*c2, c1+32*c2>=>mfmsub2
<c1+c3, c1+2*c3..c1+ALPeriod-80ms>=>mfmsub2
<c1+c2+1s>=>checkPoint,"startFp"
<c1+c2+2s>=>checkPoint,"endFp"
```

The first data point is taken 40ms after the actinic light is switched on (c1+c2). The initial measurement series (<c1+c2, c1+2*c2, c1+4*c2, c1+8*c2, c1+16*c2, c1+32*c2>=>mfmsub2) defines the data points 40ms, 80ms, 160ms, 320ms, 640ms, and 1.28s after the actinic light is switched on (c1). The subsequent measurement series (<c1+c3, c1+2*c3..c1+ALPeriod-80ms>=>mfmsub2) continues with 2s, 4s, 8s, and so until the end of the actinic light exposure. The last two lines define new fluorescence feature name Fp. This feature is defined in the numerix.nmx and numerixavg.nmxa files as the image in which the averaged signal reaches a local maximum.

The following block defines the first SuperPulse reducing transiently the plastoquinone pool during the actinic light exposure:

```
;******* Saturating Pulses - Fm_L Quenching Analysis
f1=c1+<9s>;                    ##
f11=f1#<160ms,320ms..960ms>
f11=>mfmsub2
f1=>checkPoint,"startFt_L1"
f1+960ms=>checkPoint,"endFt_L1"
;--------
f2=f1+1s
f2+40ms=>SatPulse(PulseDuration)
f21=f2#<160ms,320ms..PulseDuration>
f21=>mfmsub2
f2=>checkPoint,"startFm_L1"
f2+PulseDuration=>checkPoint,"endFm_L1"
```

First, the actual fluorescence before the saturating Super Pulse is measured starting at 9s after the actinic light is switched on (f1=c1+<9s>). The measurements are defined by the time series (f11=f1#<160ms,320ms..960ms>) and executed by (f11=>mfmsub2). The fluorescence feature Ft_L1 is defined thereafter (f1=>checkPoint,"startFt_L1", f1+960ms=>checkPoint,"endFt_L1").

The origin of the Super Pulse flash is set to 10.04s after the actinic light is switched on (f2=f1+1s, f2+40ms=>SatPulse(PulseDuration)). The measurements are defined by the time series (f21=f2#<160ms,320ms..PulseDuration>) and executed by (f21=>mfmsub2). The fluorescence feature Fm_L1, the maximum fluorescence taken in the first flash during the actinic light period, is defined thereafter (f1=>checkPoint,"startFt_L1", f1+960ms=>checkPoint,"endFt_L1").

The Ft_Ln and Fm_Ln measurements are repeated at 19 / 20s, at 29 / 30s, and at 49 / 50s:

```
f3=c1+<19s>;                   ##
f31=f3#<160ms,320ms..960ms>
f31=>mfmsub2
f3=>checkPoint,"startFt_L2"
f3+960ms=>checkPoint,"endFt_L2"
;--------
f4=f3+1s
f4+40ms=>SatPulse(PulseDuration)
f41=f4#<160ms,320ms..PulseDuration>
f41=>mfmsub2
f4=>checkPoint,"startFm_L2"
f4+PulseDuration=>checkPoint,"endFm_L2"
;^^^^^^^^^^^^^^^^^
f5=c1+<29s>;                   ##
```

```
f51=f5#<160ms,320ms..960ms>
f51=>mfmsub2
f5=>checkPoint,"startFt_L3"
f5+960ms=>checkPoint,"endFt_L3"
;--------
f6=f5+1s
f6+40ms=>SatPulse(PulseDuration)
f61=f6#<160ms,320ms..PulseDuration>
f61=>mfmsub2
f6=>checkPoint,"startFm_L3"
f6+PulseDuration=>checkPoint,"endFm_L3"
;^^^^^^^^^^^^^^^
f7=c1+<49s>;                        ##
f71=f7#<160ms,320ms..960ms>
f71=>mfmsub2
f7=>checkPoint,"startFt_L4"
f7+960ms=>checkPoint,"endFt_L4"
;--------
f8=f7+1s
f8+40ms=>SatPulse(PulseDuration)
f81=f8#<160ms,320ms..PulseDuration>
f81=>mfmsub2
f8=>checkPoint,"startFm_L4"
f8+PulseDuration=>checkPoint,"endFm_L4"
;^^^^^^^^^^^^^^^
```

The steady state fluorescence features Ft_Lss and Fm_Lss are measured at 69 / 70s:
```
f9=c1+<69s>;                        ##
f91=f9#<160ms,320ms..960ms>
f91=>mfmsub2
f9=>checkPoint,"startFt_Lss"
f9+960ms=>checkPoint,"endFt_Lss"
;--------
f10=f9+1s
f10+40ms=>SatPulse(PulseDuration)
f101=f10#<160ms,320ms..PulseDuration>
f101=>mfmsub2
f10=>checkPoint,"startFm_Lss"
f10+PulseDuration=>checkPoint,"endFm_Lss"
```

The actinic light period can be followed by a short exposure to far-red irradiance that selectively excites Photosystem I and, thus, oxidizes rapidly the plastoquinone pool (Fig.2). In this way, the minimum fluorescence of a light adapted plant, Fo_Lss, can be measured. The far-red irradiance is switched off during the measurement because it generates a background signal that would saturate the CCD camera chip:
```
;******* Far-Red Exposure
FRPeriod=2s;            ##
d1=F0duration+PulseDuration+40ms+DarkRelaxation1+ALPeriod
<d1>=>IR(520ms)
<d1+640ms>=>IR(120ms)
<d1+840ms>=>IR(120ms)
<d1+1040ms>=>IR(120ms)
<d1+1240ms>=>IR(120ms)
<d1+1440ms>=>IR(120ms)
<d1+1640ms>=>IR(120ms)
<d1+1840ms>=>IR(120ms)
<d1+600ms,d1+800ms..d1+FRPeriod>=>mfmsub2
<d1+1200ms>=>checkPoint,"startFo_Lss"
<d1+FRPeriod>=>checkPoint,"endFo_Lss"
```

The dark relaxation following the actinic light period is monitored for 100s:

```
;******* Dark relaxation after actinic light period
DarkRelaxation2=100s
h1=F0duration+PulseDuration+40ms+DarkRelaxation1+ALPeriod+FRPeriod
;******* Relaxation Measurement
h2=40ms
h3=2s
<h1+h2, h1+2*h2, h1+4*h2, h1+8*h2, h1+16*h2, h1+32*h2>=>mfmsub2
<h1+h3, h1+2*h3..h1+DarkRelaxation2-80ms>=>mfmsub2
```

The dark relaxation of the non-photochemical quenching is measured during 3 flashes at 29/30, 59/60 and 89/90 seconds after the actinic light is switched off. Each flash is followed by a short far-red irradiance to reveal the instantaneous $F0$ level:

```
;******* Saturating Pulse - Fm_D1 Quenching Analysis
g1=h1+<29s>;                    ##
g11=g1#<160ms,320ms..960ms>
g11=>mfmsub2
g1=>checkPoint,"startFt_D1"
g1+960ms=>checkPoint,"endFt_D1"
;--------
g2=g1+1s
g2+40ms=>SatPulse(PulseDuration)
g21=g2#<160ms,320ms..PulseDuration>
g21=>mfmsub2
g2=>checkPoint,"startFm_D1"
g2+PulseDuration=>checkPoint,"endFm_D1"
;^^^^^^^^^^^^^^^^
;******* Far-Red Exposure D1
gg1=g2+40ms+PulseDuration
gg1=>IR(520ms)
gg2=gg1#<600ms,800ms,1200ms,1400ms,1600ms,1800ms>
gg2=>mfmsub2
gg3=gg1#<640ms,840ms..FRPeriod>
gg3=>IR(120ms)
gg1+1200ms=>checkPoint,"startFo_D1"
gg1+FRPeriod=>checkPoint,"endFo_D1"
;^^^^^^^^^^^^^^^^
;******* Saturating Pulse - Fm_D2 Quenching Analysis
g3=h1+<59s>;                  ##
g31=g3#<160ms,320ms..960ms>
g31=>mfmsub2
g3=>checkPoint,"startFt_D2"
g3+960ms=>checkPoint,"endFt_D2"
;--------
g4=g3+1s
g4+40ms=>SatPulse(PulseDuration)
g41=g4#<160ms,320ms..PulseDuration>
g41=>mfmsub2
g4=>checkPoint,"startFm_D2"
g4+PulseDuration=>checkPoint,"endFm_D2"
;^^^^^^^^^^^^^^^^
;******* Far-Red Exposure D2
gg3=g4+40ms+PulseDuration
gg3=>IR(520ms)
gg4=gg3#<600ms,800ms,1200ms,1400ms,1600ms,1800ms>
gg4=>mfmsub2
gg5=gg3#<640ms,840ms..FRPeriod>
gg5=>IR(120ms)
gg3+1200ms=>checkPoint,"startFo_D2"
gg3+FRPeriod=>checkPoint,"endFo_D2"
;^^^^^^^^^^^^^^^^
```

```
;******* Saturating Pulse - Fm_D3 Quenching Analysis
g5=h1+<89s>;                    ##
g51=g5#<160ms,320ms..960ms>
g51=>mfmsub2
g5=>checkPoint,"startFt_D3"
g5+960ms=>checkPoint,"endFt_D3"
;--------
g6=g5+1s
g6+40ms=>SatPulse(PulseDuration)
g61=g6#<160ms,320ms..PulseDuration>
g61=>mfmsub2
g6=>checkPoint,"startFm_D3"
g6+PulseDuration=>checkPoint,"endFm_D3"
;^^^^^^^^^^^^^^^^^^
;******* Far-Red Exposure D1
gg1=g6+40ms+PulseDuration
gg1=>IR(520ms)
gg2=gg1#<600ms,800ms,1200ms,1400ms,1600ms,1800ms>
gg2=>mfmsub2
gg3=gg1#<640ms,840ms..FRPeriod>
gg3=>IR(120ms)
gg1+1200ms=>checkPoint,"startFo_D3"
gg1+FRPeriod=>checkPoint,"endFo_D3"

;END ****************************************************************
```

IV.D. Experiment and data pre-processing

The experiment encoded in the protocol is executed upon clicking the trigger (1 in Fig.20). First, the protocol is compiled in the computer and uploaded to the FluorCam *via* the USB-2 cable. The experiment proceeds according to the protocol instructions. The experiment can be interrupted by clicking the stop sign (2 in Fig.20)[2].
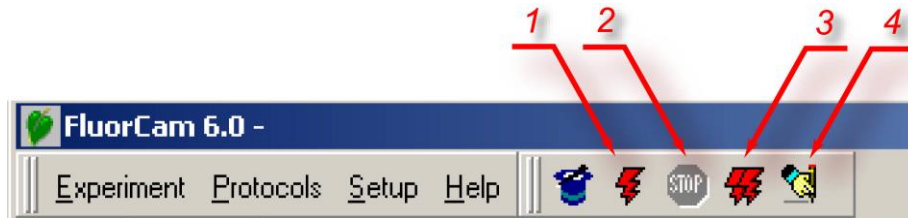
FIGURE 20. Top icon bar of the FluorCam software. (1) Trigger starting compilation of the protocol (if not done in the protocol window) and initiating execution of the experiment. (2) Stop interrupts execution of the experiment. The experiment can be re-started but not resumed after the stop is used. (3) Multiple trigger with automatic bar code reading and file assignment. (4) Manually activated bar code reader.

The sample and the experiment can be assigned a database code from an optional bar code reader: either automatically, in serial experiments in the multiple trigger mode (3 in Fig.20), or manually by the bar code reader activation (4 in Fig.20).

IV.D.1 Experiment

Upon triggering the experiment, the protocol is compiled and uploaded from computer to FluorCam. The progress of compilation and upload is shown in LogView window that

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[2] Attention: The experiment interrupted in this way cannot be resumed and the data are lost.

transiently appears on the computer screen. The entire progress of the experiment is monitored and recorded in 'psiserver.log' and in 'logfile.log' files in the FluorCam directory.
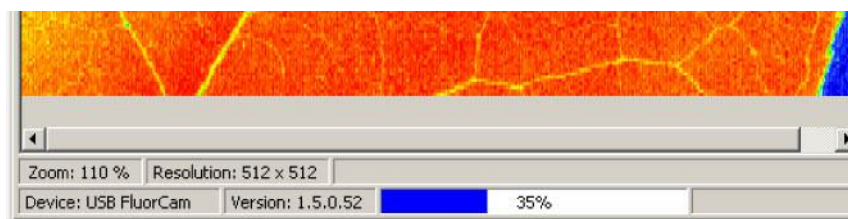
FIGURE 21. The bottom bar shows in percents approximately the progress of the experiment.
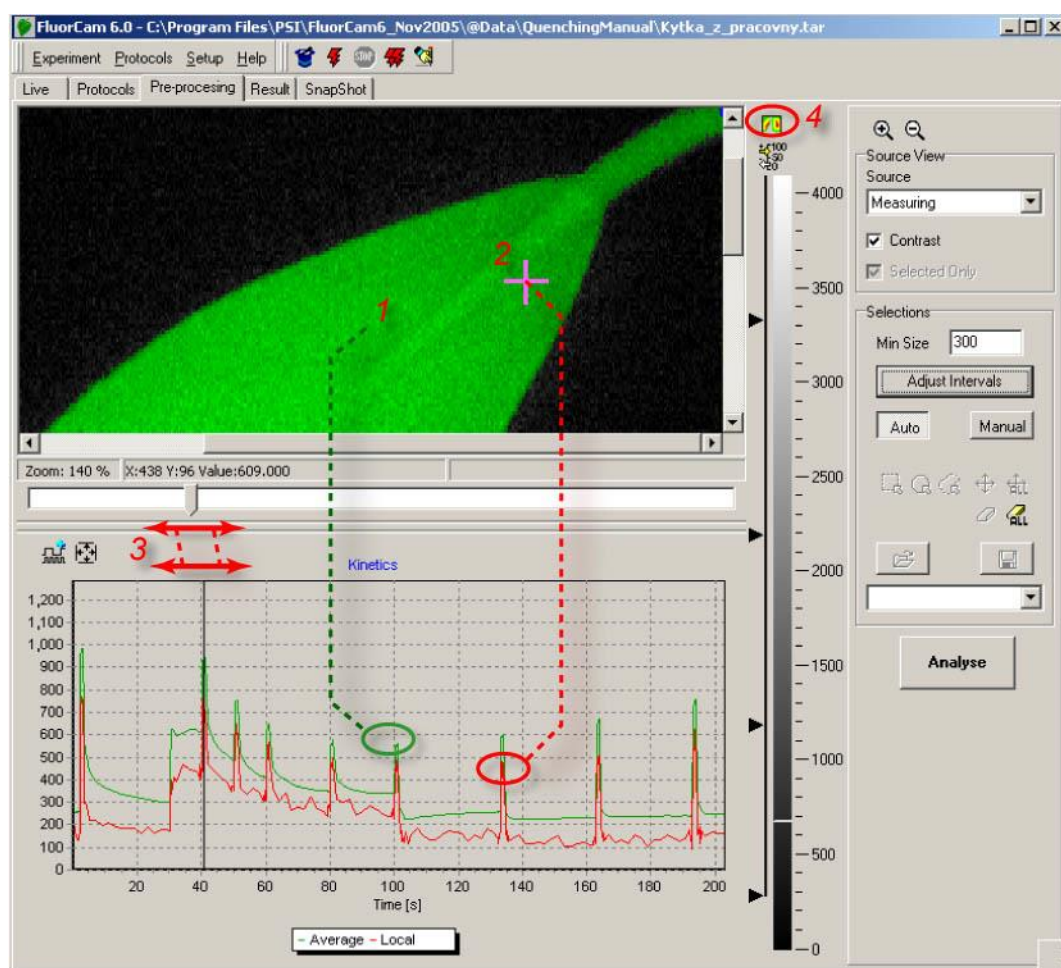
FIGURE 22. The pre-processing window shows a tentatively image segmented fluorescence image in the top panel. The green shading shows the region-of-interest that most likely represent fluorescing object. The surrounding area is tentatively identified as background. The signal in the region-of-interest is integrated (1) and kinetics of the integrated signal is shown in the bottom kinetic panel. The cursor (2) can select a pixel anywhere in the image and the transient in the selected pixel is be shown by the red kinetic trace. The displayed image can be changed by moving the sliding cursor (3). The display can toggle between 'show-all', 'show-image', and 'show-kinetics' modes by clicking (4).

IV.D.2 Data upload, pre-processing and selection of regions-of-interest

The data are uploaded to the computer RandomAccess Memory (RAM) during the experiment. As soon as the experiment is over, the computer transfers the data from RAM to its hard drive forming a temporary experiment file in 'psitemp' folder. Simple automatic image segmentation is proposed by the FluorCam software to separate the image regions with fluorescence signal

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from dark background. This segmentation is only tentative and can fail, for example, when there is no background or when the signal is too low.

Fig.22 shows the FluorCam window offering more pre-processing features. The green area in the image (1 in Fig.22) indicates the region-of-interest that is distinct from the background. The signal integrated over all pixels of this region is represented in the lower panel by the green kinetic trace[3]. Using computer mouse, one can place a cross cursor (2 in Fig.22) anywhere in the image window and the fluorescence transient corresponding to the pixel selected by the cursor is shown by the red kinetic trace.

The displayed image can be changed by sliding cursor (3 in Fig.22) along the time axis. By clicking the view icon (4 in Fig.22) one can toggle between the 'show-all', 'show-image', and 'show-kinetics' display modes.
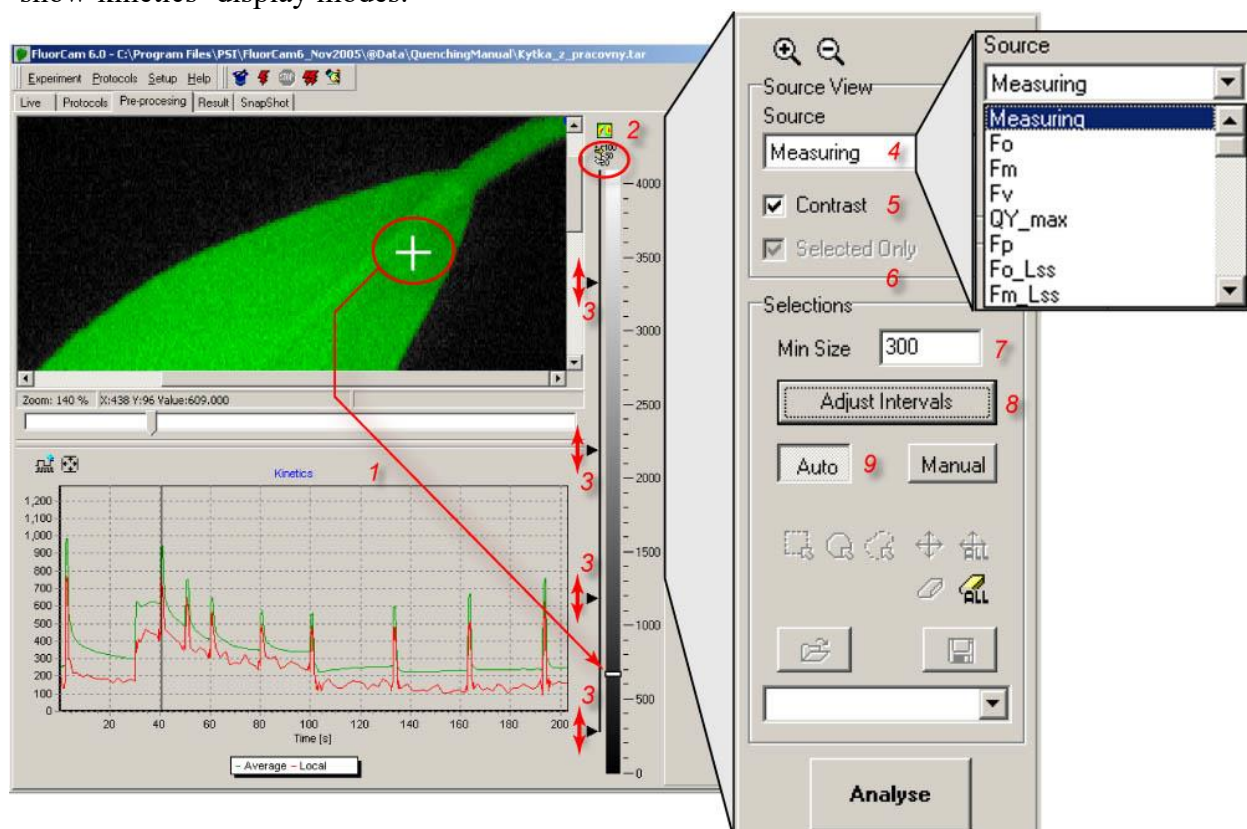
FIGURE 23. More pre-processing tools. The signal at the position of the cross cursor (1) is shown by the indicator on the scale bar (1 arrow). The scale bar can be displayed with or without numeric scale (2). A finer segmentation of the image is possible by using and moving the scale dividers (3, see also Fig.24). By default, the image shown in this window corresponds to the frame that is selected by the sliding cursor (Fig.22). However, one can also display images that are generated by frame combinations defined in the protocol and in the numerix.nmx and in the numerixavg.nmx files. The list of available images is available in the 'Source' field(4). Rescaling between minimum and maximum signal levels is possible by selecting the 'Contrast' field (5). The 'Selected only' field (6) is relevant only in a second iteration of the image segmentation and, if checked, limits the segmentation routines to the region-of-interest that was selected and analyzed in the primary segmentation step. The 'Min Size' field (7) defines a minimum number of pixels that are considered to be the user defined region-of-interest. The'Adjust Intervals' switch (8) triggers the tentative image segmentation that roughly separates the background from potential region-of-interest. The 'Auto' mode (9) limits the segmentation to 'Adjust Intervals' pre-segmentation (8) and to manual movement of scale dividers (3).

Fig.23 shows more features of the pre-processing window. The signal level in the pixel selected by the cross cursor is indicated in the scale bar (1 in Fig.23). The scale bar can be shown with or without the numeric scale (2 in Fig.23). The segmentation of the image can be assisted by the

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[3] It can take several seconds to calculate this integrated transient.

four scale dividers (3 in Fig.23) that separate up to five regions-of-interest consisting of pixels within the given signal range. The regions-of-interest can be selected and de-selected by clicking the black vertical line along the corresponding scale bar segment. The image segment with pixels in the lowest signal range 0-300 is de-selected in Fig.23. It is the black segment of the image window showing the leaf background. By moving the scale divider from 300 to lower values, pixels of the background will start to be included in the image processing and averaging. The image that is used for segmentation can be either any frame that is selected by the cursor from the measured sequence (3 in Fig.22) or any frame or combination of frames that is pre-labeled and defined in numerix.nmx files. The list of the images that are pre-labeled and available for segmentation is provided in the 'Source' field (4 in Fig.23). In this way, one can separate for example regions-of-interest that differ in quantum yields or in non-photochemical quenching rather than in directly captured fluorescence signals (e.g., F_0 or F_M).

Sometimes, e.g. with F_0, the measured image can be too dim for segmentation. Then, one can use Contrast (5 in Fig.23) to re-scale the signal between the maximum and the minimum signal levels.

The 'Selected Only' field (6 in Fig.23) is active for secondary image segmentation. One can, for example, use one fluorescence parameter to define the primary region-of-interest and perform a secondary image segmentation only within this primary region-of-interest using another fluorescence parameter.

The 'Minimum Size' field (7 in Fig.23) limits the size of the regions-of-interest that are included in the image processing. By setting this value low one can include small objects but can, unintentionally, also include small groups of pixels that are separated by an artifact of a noisy signal.

The 'Adjust Intervals' trigger (8 in Fig.23) re-sets the lowest scale divider (3 in Fig.23) to a minimum of the image histogram so that the background can be tentatively de-selected.

The 'Auto' mode (9 in Fig.23) relies on image segmentation by the scale dividers (3 in Fig.23) and by the 'Adjust Intervals' routine.

The 'Manual' mode of image segmentation is selected by (1 in Fig.24). The regions-of-interest can be square (2 in Fig.24), eliptic (3 in Fig.24), or polygonal (4 in Fig.24). Individual region of interest can be moved by 5 in Fig.24. The multiple regions-of-interest can be moved as a group by 6 in Fig.24. Individual or all regions-of-interest can be erased by 7 and 8 in Fig.24. The regions-of-interest can be saved and reloaded by 10 and 9 in Fig.24. This is a particularly handy feature in those situations when a reproducible pattern, e.g., of multi-well plates, is repeatedly imaged (11 in Fig.24).
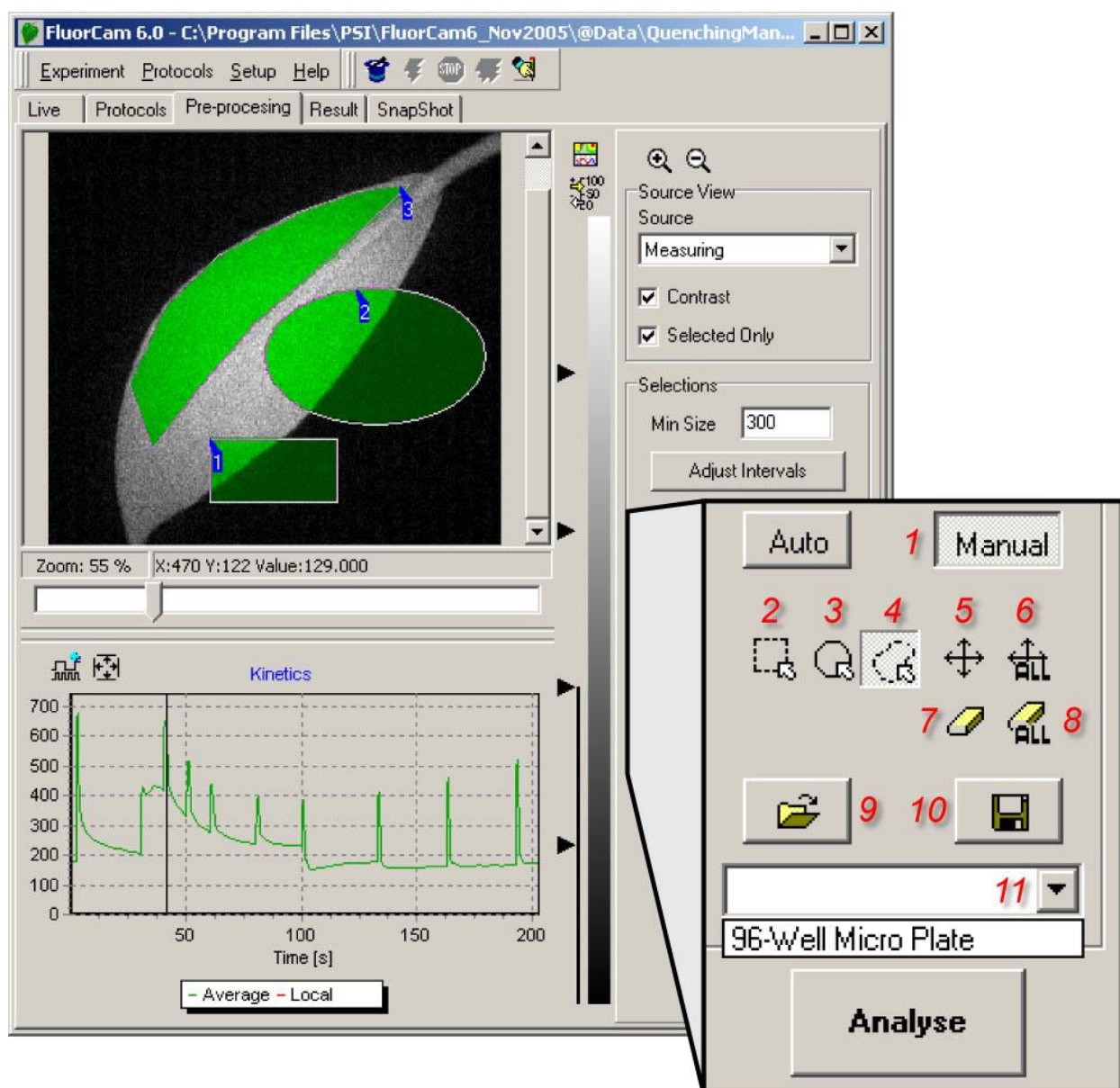
FIGURE 24. The manual image segmentation is selected by (1). The segments are rectangular (2), elliptic (3) or polygonal (4). The segments can be moved individually (5) or in group (6). The segments can be erased by (7) and (8). The segments can be saved (10) and loaded from memory (9 and 11). The signal in the selected regions-of-interest can be integrated by 'Analyze'.

The segmentation of the image by scale dividers is explained in detail in Fig.25.
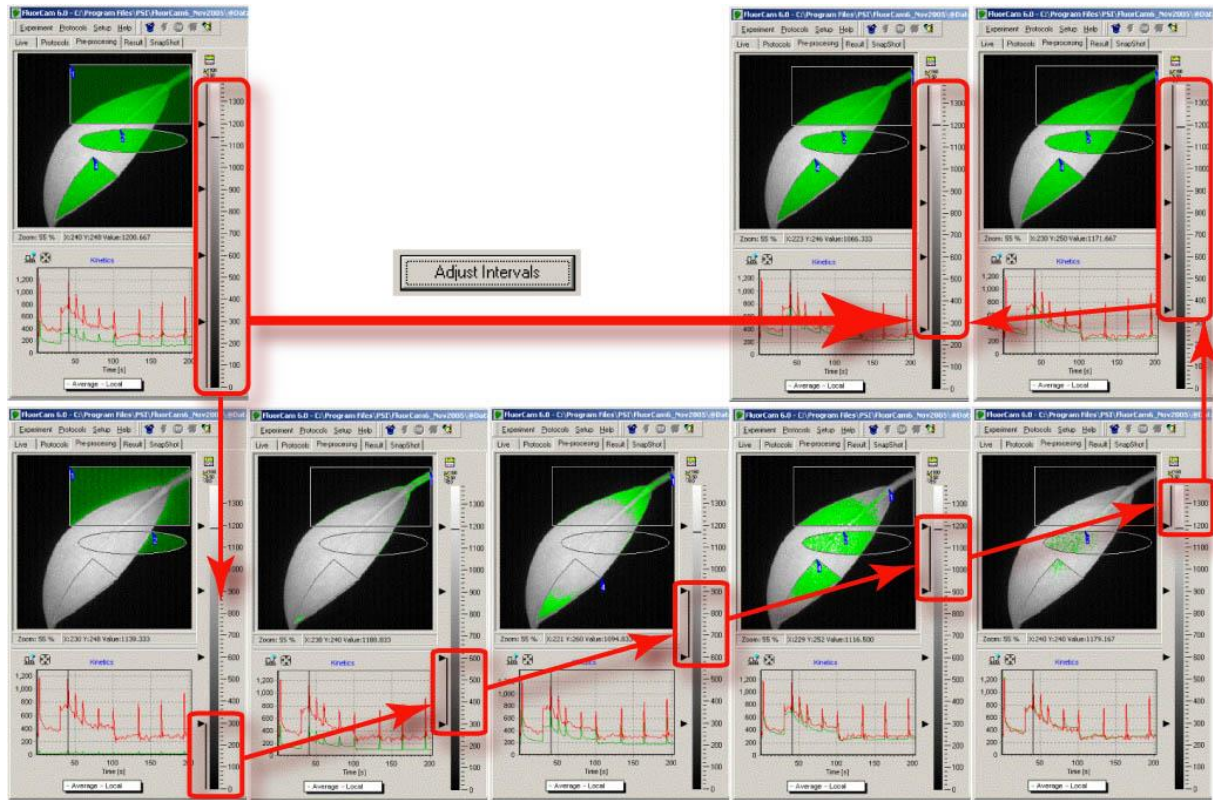
FIGURE 25. *The image segmentation by scale dividers. In the top left panel, the space dividers are distributed roughly uniformly from 0 to the maximal signal level and all five scale ranges are selected as indicated by the vertical line along the scale bar. With the entire signal range selected, the square, elliptic as well as polygonal shapes (1-3) are fully selected including the segments that show the background. Upon pressing 'Adjust Intervals', the lowest scale divider is moved to the histogram minimum that typically separates background from useful signal range (thick red arrow). The function of the scale dividers can be demonstrated by sequentionally selecting the corresponding signal range. First, select the lowest range 0-300 as in the lower leftmost panel. The selected range corresponds to background only segment shown in the panel image window. The green kinetic trace is close to zero level confirming that the selected segment is indeed background. The red kinetic trace shows for comparison kinetics in one of leaf pixels selected by the cursor. By selecting only the 300-600 signal range (lower row, second panel from the left), one excludes the background and selects only the stem pixels that, being in image periphery, exhibit low signal levels. The green kinetic trace already shows a typical pattern of the Kautsky effect that is shifted to lower signal levels compared to the red kinetic trace in the pixel selected by the cursor. One can scan through the other scale ranges selected by the other dividers.*

IV.E. Results analysis
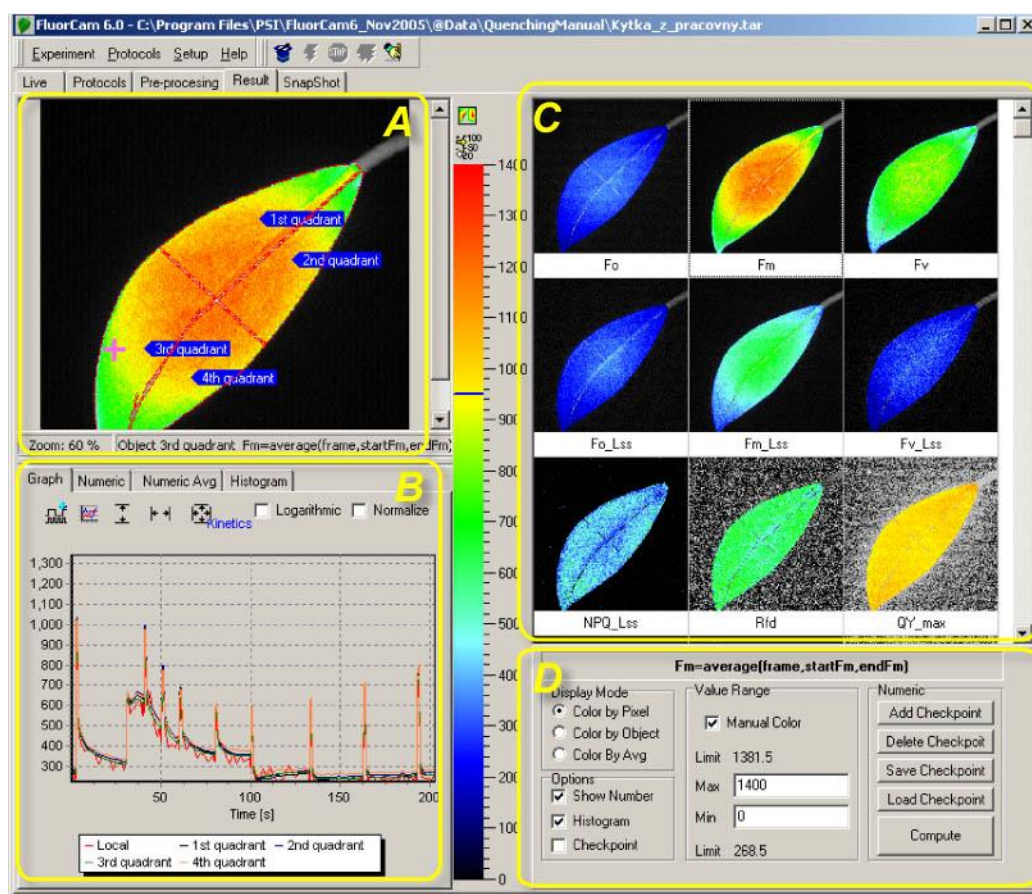
FIGURE 26. *The Results window consists of four panels (marked by yellow color). The panel A represents spatial dimensions by one image of the fluorescence transient sequence. The Panel B represents time dimension by showing kinetics of the fluorescence signals averaged over the segments that were selected prior analysis in the Pre-processing window and that, if selected, are shown in Panel A. Panel C shows all images defined and labeled in the numerix.nmx and numerixavg.nmx files. By clicking any of the frames in the Panel C, one transfers the selected image to the Panel A window. The Panel D offers controls to customize the Results panel.*
Panel A can be zoomed in and out (1). The field (2) informs about the segment that is currently pointed by the cursor (if any) and about the image that is selected by clicking any of the frames in the panel C (here 3). By pointing the cursor to a particular segment, one can rename it (4). By pointing anywhere in the image, one can save its bitmap. By clicking the top icon in 5, one can toggle between two panels A (space) + B(time), show A (space) and show B (time). The bottom icon in 5 toggles between showing and hiding the numeric scale.

IV.E.1 Spatial heterogeneity of the measured and calculated parameters

The Results window (Fig.26) shows, in the panel C, all images that are calculated in analysis of the pre-processing window (Fig.24). These images are either directly captured and averaged frames (e.g., F_0, F_M, F_t) or images calculated from the captured frames (e.g., $F_V = F_M - F_0$, $NPQ = (F_M - F_M')/F_M'$). Any of the images in the panel C can be selected by mouse clicking. The selected image is shown in an expanded form in the panel A. The expanded image is segmented according to the definition in the pre-processing window (Fig.24). The signal in all pixels of each segment is integrated and the averaged value is representing the segment fluorescence at given time point. Time sequences of these fluorescence levels constitute fluorescence transients that are shown in the panel B. These segment-averaged transients are supplemented by a transient that represents a pixel pointed by the mouse cursor (the noisy red line in the panel B). The panel D serves to customize the visualization of the results.
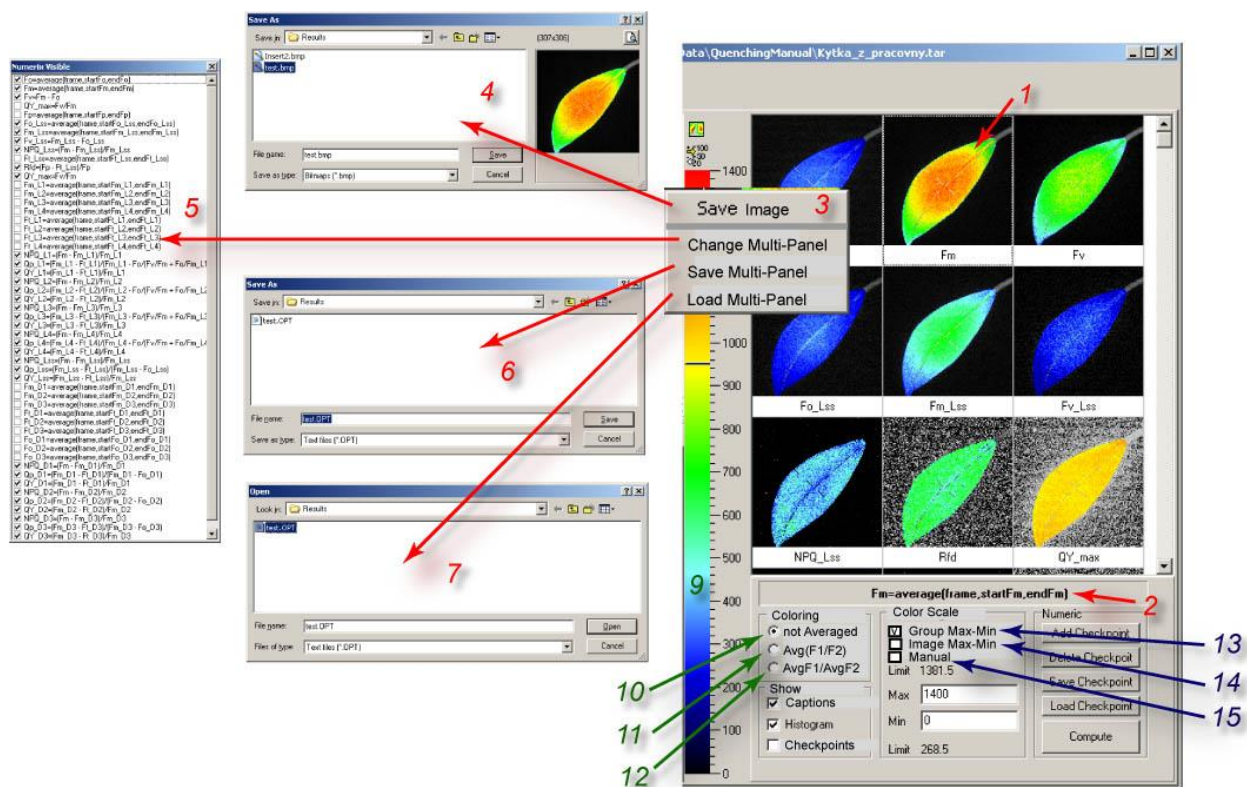
FIGURE 27. Visualization of the spatial heterogeneity of the fluorescence emission. Any image from the multi panel window can be selected for a closer inspection by left-clicking it by mouse (1). The formula by which is the selected image calculated is shown in the field (2). The right click of the mouse opens a sub-menu (3). By selecting 'Save Image', one can save the corresponding image in the bitmap format (4) for later image presentation or manipulation. By selecting 'Change Multi-Panel', one can show or hide the images in this window (5). The reason for this option comes from the fact that, typically, only a sub-set of the images that are calculated in the analysis is shown by default. By selecting 'Save Multi-Panel' (6) or 'Load Multi-Panel' (7), one can save or load the configuration of the window.

The false color scheme of the images can be changed by clicking the color scheme bar (9). With (10) selected, the images are colored pixel-by-pixel without averaging. By selecting (11), one switches to the mode in which the values are calculated in each pixel and averaging is done after the calculation, e.g., $Average(F_V/F_M)$. By selecting (12), one switches to the mode in which values are first averaged and then used for a calculation, e.g., $Average(F_V)/Average(F_M)$.

The visual perception is largely controlled by relative scaling of the false colors. By selecting (13), one chooses a default grouping of the fluorescence images with identical color scales. Typically, all directly measured signals (e.g., F_0, F_M) and their linear combinations (e.g., F_V, F_P) are shown using one scale so that, e.g., F_0 is blue, F_M is red. Ratio images (e.g., NPQ, QY) are shown with another scale.

Alternatively, one can select (14) to see the heterogeneity of the image always with the maximum resolution between maximum and minimum of the signal in a particular image. Sometimes it can be of an advantage to define the scale limits manually that is achieved by clicking (15) and by typing the chosen minimum and maximum limits.

Saving of a particular image as well as changing, saving and loading customized configuration of the multi panel window of Results is described in Fig.27 (red numbers and arrows).

The mode of the visual presentation can be pixel-by-pixel without any averaging (10).

Alternatively, one can be interested only in the averaged values of measured or calculated signals. With (11) selected, the software first calculates the requested parameter in each pixel and averages such calculated parameters over selected segment of the image. For example, assuming that the fluorescence signal in a hypothetical 2-pixel segment is: $F_{01}=1.5$ and $F_{02}=0.5$. At another time, the fluorescence signals are: $F_{M1}=2$ and $F_{M2}=8$. The parameter defined as $F_{ratio}=(F_M-F_0)/F_M$ is first calculated for each pixel: $F1_{ratio} = (2-1.5)/2 = 0.25$ and $F2_{ratio} = (8-0.5)/8 \approx 0.94$. The average value of F_{ratio} in the 2-pixel segment is 0.59. All three pixels are assigned a color that corresponds to 0.59.

The mode selected by (11) yields mathematically correct averages but has one severe limitation in case that the signal-to-noise ratio is low. In that case, fluorescence signals or calculated differences of fluorescence signals can approach 0. If these signals are used in denominator of a ratio, such pixels can contribute to the averages by disproportionally large parameter values. This noise artifact is limited in the mode (11) by eliminating from the image the pixels that contribute to the averaging by a value that is outside of the pre-set limits.

Alternatively, the user can avoid this noise artifact by choosing the mode (12). Then, the software first calculates the average values and makes the ratios of the averaged parameters. In the example described above, the averaged signal F_0 is 1 and the averaged signal F_M is 5. The ratio of the averages is $F_{ratio} = (F_M-F_0)/ F_M = (5-1)/5 = 0.8$. This value is not the numerically correct average of the parameter F_{ratio} in the two pixels ($F1_{ratio} = 0.25$, $F2_{ratio} \approx 0.94$) and its interpretation can be difficult. On the other hand, the averaged values of F_M and F_0 are much less susceptible to noise artifacts and typically the numerical error in using the ratio $[Average(F_M) – Average(F_0)] / Average(F_0)$ instead of the correct one $Average [(F_M - F_0)/ F_0)]$ is very low. It is up to the user to choose a better option for the particular experimental situation. With high signal-to-noise ratio it is typically possible to use the mathematically correct mode (11) whereas in experiments with low signal-to-noise it is certainly better to use the mode (12). These two modes are available for the tabulated numerical values of the fluorescence parameters as described in Fig.29.
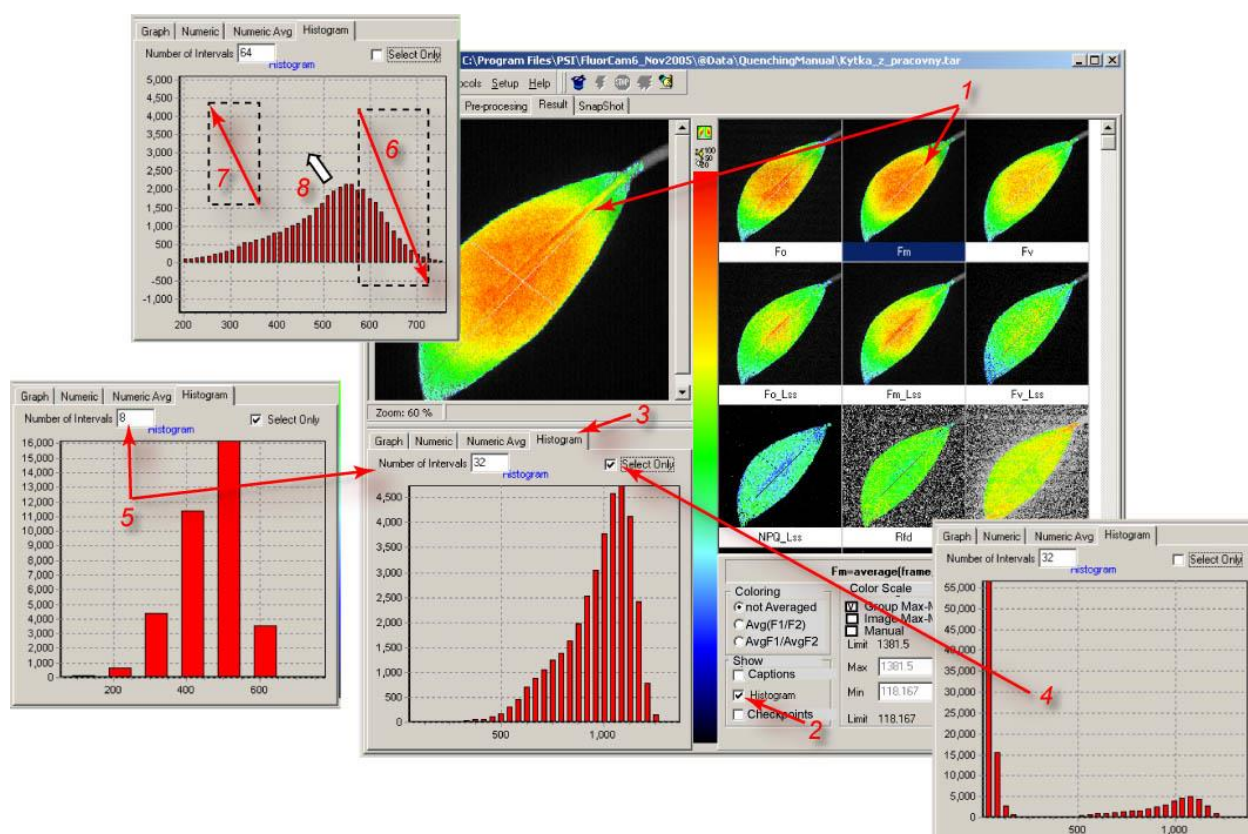
FIGURE 28. Histogram. The histogram of any selected image (1) can be enabled / disabled by (2). If enabled, the window (3) can be opened to see number of pixels corresponding to given interval of signal levels within the selected segment or within the entire image (if 4 is not checked. The number of signal levels that are included in the histogram can be modified by (5). The details of the histogram can be inspected by pressing the left mouse button and by dragging the other extreme of a rectangular zoom area in the direction indicated by the arrow 6 (top-left to bottom-right). The show-all zoom can be re-established by dragging in the opposite direction (7). By pressing the right mouse button with the cursor in the histogram plot area (8), one can move around the detailed view of the histogram

IV.E.2 Kinetics of the measured and calculated parameters

The kinetics of the fluorescence parameters can be qualitatively inspected in the multi-panel window described in Figs.26 and 27. The quantitative assessment of the kinetics can be obtained using the Graph window in Results (Fig.29).
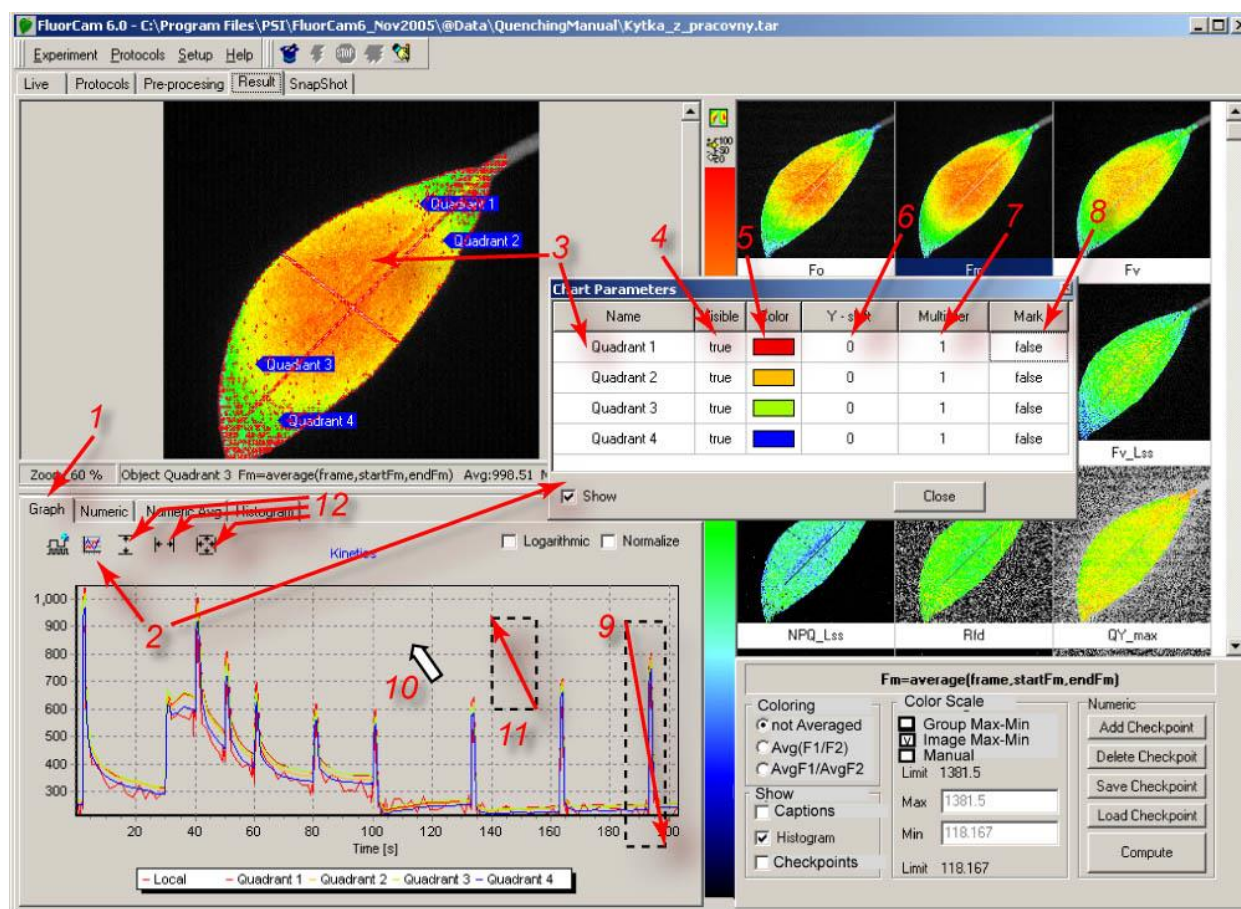
FIGURE 29. Kinetics of the fluorescence transient is displayed by selecting (1). The transients of the segments defined in the Pre-Processing window are shown in format defined in the Chart Parameters window (2). The caption of individual segments can be modified either by clicking the segment in the main image window (3 left arrow) or by changing the name directly in (3 right arrow). Any particular segment can be shown or hidden (4). The color of the trace as well as the color of the segment in the Averaged modes (11 and 12 in Fig.27) can be changed by (6) – Sometimes the kinetic traces strongly overlap and it is better to use an offset (7). Also, one may need to re-normalize the trace using a multiplication factor (7). Details of the kinetic trace can be inspected also noting the numerical values of individual data points (8, and in Fig.30). The details of the kinetic traces can be zoomed in by pressing and dragging the left mouse button in the direction (9). The zoomed-in graph can be moved around by pointer dragging with the right mouse button pressed (10). The graph can be zoomed out by dragging cursor by the left mouse button in the direction (11). Alternatively, the zooming out can be performed by clicking the icons group 12: left icon for signal dimension, middle icon for the time dimension, and right icon for both dimensions.

The kinetic traces can be zoomed in and out using the same convention as described in Fig.28 for the histogram. The zoom-in area can be defined by pressing the left mouse button and dragging it in the direction top-left to bottom-right (9 in Fig.29). The zoomed-in graph can be moved around by dragging with the right mouse button pressed (10). The original show-all zoom can be re-established by left button dragging in the bottom-right to top-left direction.
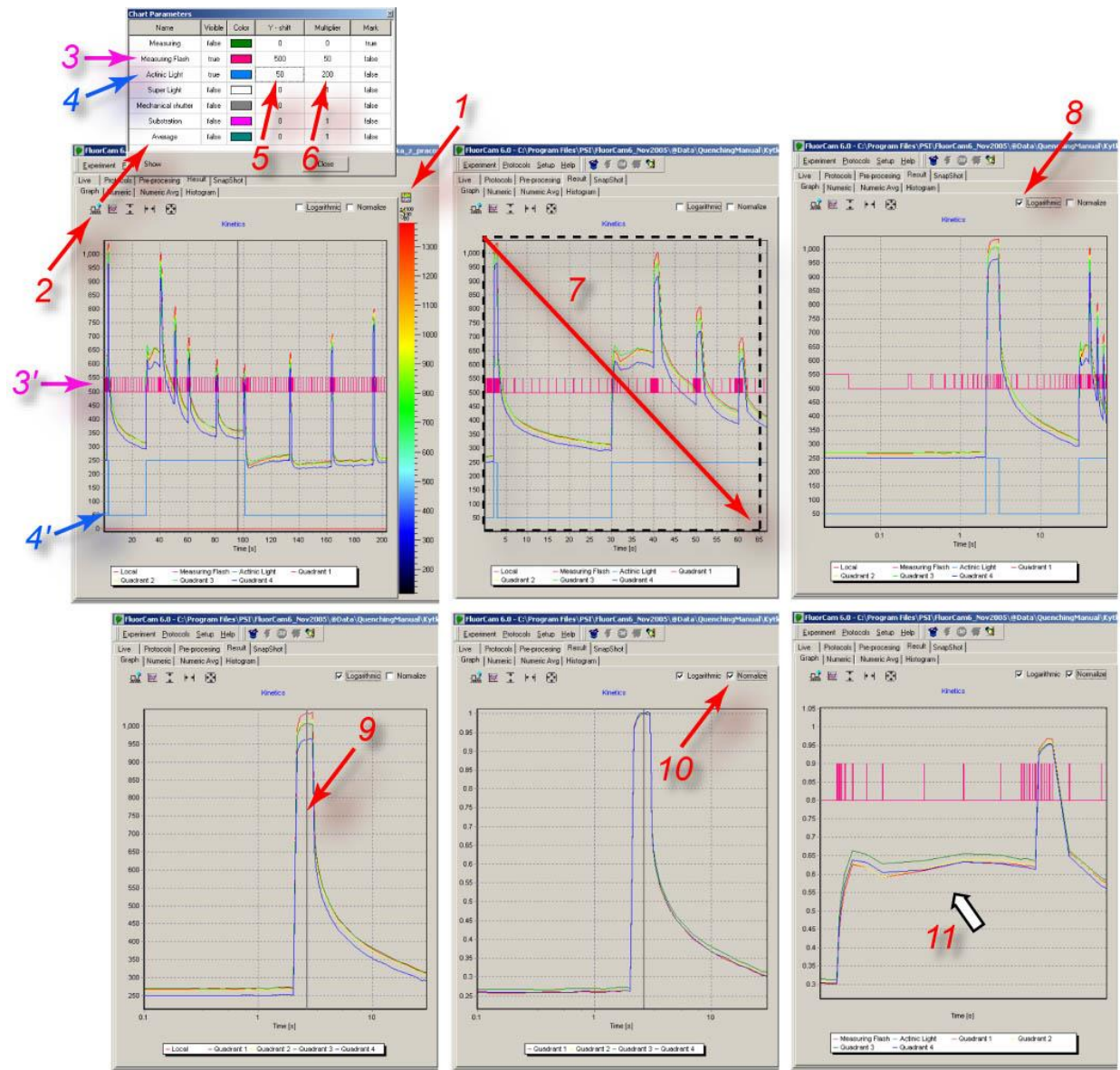
FIGURE 30. Additional visualization features available for kinetic analysis. The panel showing the fluorescence transient can be expanded by (1). By clicking (2), one can open the window that can visualize time course of controlling signals defined in the protocol. The magenta arrow (3) shows the line that, by clicking true in 'Visible' column includes the measuring flashes in the kinetic window (3' below). The blue arrow (4) shows the corresponding line for the actinic light on/off status. The blue line (4' below) shows the actinic light signal in the kinetic window. The 'Y-shift' (5) and the 'Multiplier' (6) columns offset and re-scale the control signals. Details of the transients can be inspected by zooming-in (7) by the left mouse button dragging. The show-all zoom level can be restored by dragging in the opposite direction (see Fig.29). Details of the initial phase of the fluorescence transient can be effectively visualized using logarithmic time scale (8). Double-clicking of the left mouse button positions a cursor (9) that defines timing for the normalization (10). The graph can be moved within the window by cursor dragging with pressed right mouse button.

Fig.30 demonstrates additional features that are available for the kinetic analysis of the fluorescence transients.

Fig. 31 shows a zoomed-in detail of the kinetic trace showing the measurement of F_M.
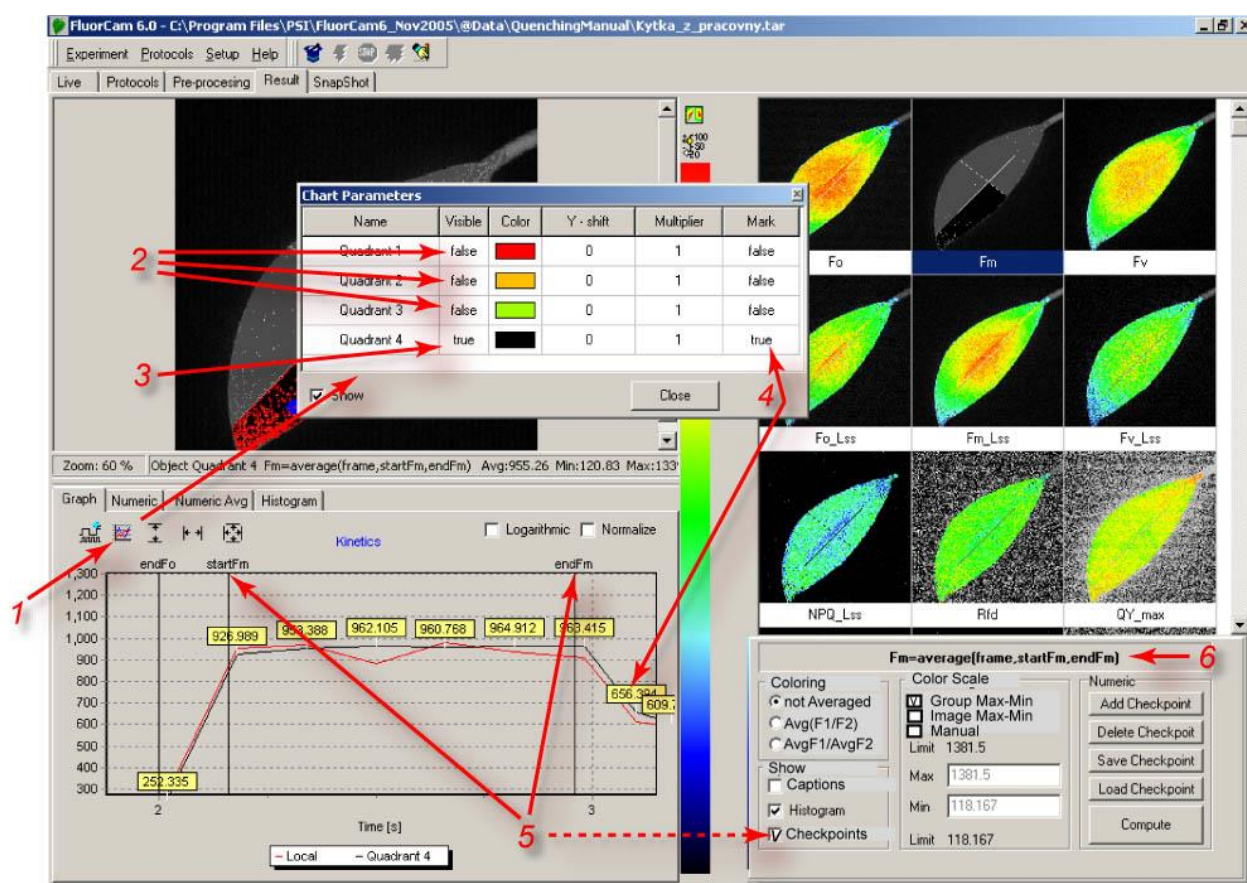
FIGURE 31. The attributes of the zoomed fluorescence transient can be set in the 'Chart Parameters' window (1). The signals of the first 3 segments are hidden (2) and only the fourth transient shown (3). The color line is black. The red noisy line corresponds to a pixel that is pointed by the cursor. The black kinetic trace of the fourth segment is shown with marks (4) that show the numeric value of the signal. One can also see timing for individual checkpoints (5, dotted line) that are used to calculate fluorescence signals for the images in the multi-panel window. Here, for example, the checkpoints StartFm and EndFm are shown (5) that are used to calculate the averaged F_M signal between them. The definition of F_M printed in (6) is stored in the numerix.nmx and numericsavg.nmx files.

IV.E.3 More on checkpoints

The checkpoints are marks separating and naming different phases of the fluorescence transient. The paragraph IV.C.4 describes how can be the checkpoints defined in the experimental protocol. For example, by writing

<0s>=>checkPoint,"startFo"
<F0duration>=>checkPoint,"endFo"

and

<a2>=>checkPoint,"startFm"
<a1+PulseDuration>=>checkPoint,"endFm"

one defines 4 checkpoints named:

- startFo as the first measurement after 0s
- endFo as the first measurement after the time defined in the variable F0duration
- startFm as the first measurement after the time defined in the variable a2
- endFm as the first measurement after the time defined in the variable a1+PulseDuration

Clearly, one can use these 4 checkpoints to operate with 4 different frames or, more typical, to operate with 2 sets of frames, each limited by start and end timing.

The use of the checkpoints and the potential arithmetic operations with the frames defined by checkpoints is defined in the numerix.nmx and numerixavg.nmx files. For example, one can find there the following lines:

a: <EXIST name="startFo"/>

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b: <NOEXIST name="endFo"/>

c: <FORMULA var="Fo" term="frame{startFo}" accuracy="2" visible="true" export="true"/>

that can be de-coded in the following way: if a checkpoint named "startFo" is defined in the protocol and if there is no definition of any checkpoint named "endFo", the frame labeled "startFo" will be used as "Fo" frame variable using the prescribed numeric accuracy (two digits after the decimal point. The "Fo" frame variable will be represented in the multi-panel results window (visible="true") and will be included in the export files (export="true").

The piece of protocol described above contains the definition of the checkpoint "endFo" and, thus, block of numerix file applies:

<EXIST name="startFo"/>
<EXIST name="endFo"/>
<FORMULA var="Fo" term="average(frame,startFo,endFo)" accuracy="2" visible="true" export="true"/>

defining the "Fo" frame variable as an average of all frames that are between "startFo" and "endFo" checkpoints.

Similarly, the "Fm" frame variable is defined by

<EXIST name="startFm"/>
<EXIST name="endFm"/>
<FORMULA var="Fm" term="average(frame,startFm,endFm)" accuracy="2" visible="true" export="true"/>

One can also define arithmetic operations with frames, e.g., by:

<FORMULA var="Fv" term="Fm - Fo" accuracy="2" visible="true"/>
<FORMULA var="QY_max" term="Fv/Fm" accuracy="2" visible="false"/>

This is the way in which the checkpoints define the images in the multi-panel results window and the parameters calculated in the Numeric and Nimeric Avg tables.

The checkpoints can be moved by deleting and adding. In any case, only the checkpoint names that are properly defined in the numerix and numerixavg.nmx files can be used to define images in the multi-panel window and the numeric tables.

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VII. References

Govindjee (1995) Sixty-three years since Kautsky: Chlorophyll a fluorescence. Australian Journal of Plant Physiology 22: 131-160

Govindjee and Papageorgiou G (2004) Chlorophyll fluorescence: A signature of photosynthesis. Govindjee (ed.). Springer Verlag, Dordrecht

Kautsky H and Hirsch A (1931) Neue Versuche zur Kohlensäureassimilation. Naturwiss. 19: 964

Lichtenthaler H and Miehé J (1997) Fluorescence imaging as a diagnostic tool for plant stress. Trends in Plant Science 2: 316-320

Nedbal L, Soukupova J, Kaftan D, Whitmarsh J and Trtilek M (2000) Kinetic imaging of chlorophyll fluorescence using modulated light. Photosynthesis Research 66: 3-12

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

Kautsky H and Hirsch A (1931) Neue Versuche zur Kohlensäureassimilation. Naturwiss. 19: 964