

Instruction Manual and User's Guide



FluorCam



**Photon
Systems
Instruments**

Professional Instruments
for Plant Science, Biotechnology
and Agriculture

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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)
$F_{0_Ln}^1$	$\approx F_0 / ((F_M - F_0) / F_M + F_0 / F_{M_Ln})$	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^2	$(F_M - F_{M_Dn}) / F_{M_Dn}$	instantaneous non-photochemical quenching during dark relaxation	non-photochemical quenching relaxing in dark
NPQ_Ln^2	$(F_M - F_{M_Ln}) / F_{M_Ln}$	instantaneous non-photochemical quenching during light adaptation	non-photochemical quenching induced in light
NPQ_Lss^2	$(F_M - F_{M_Lss}) / F_{M_Lss}$	steady-state non-photochemical quenching	steady-state non-photochemical quenching in light

qP_Dn ³	$\frac{(F_{M_Dn} - F_{t_Dn})}{(F_{M_Dn} - F_{0_Dn})}$	coefficient of photochemical quenching during dark relaxation	estimate of the fraction of open PSII reaction centers $PSII_{open} / (PSII_{open} + PSII_{closed})$
qP_Ln ³	$\frac{(F_{M_Lss} - F_{t_Lss})}{(F_{M_Lss} - F_{0_Lss})}$	coefficient of photochemical quenching during light adaptation	estimate of the fraction of open PSII reaction centers $PSII_{open} / (PSII_{open} + PSII_{closed})$
qP_Lss ³	$\frac{(F_{M_Lss} - F_{t_Lss})}{(F_{M_Lss} - F_{0_Lss})}$	coefficient of photochemical quenching in steady-state	estimate of the fraction of open PSII reaction centers $PSII_{open} / (PSII_{open} + PSII_{closed})$
QY_Dn ⁴	$\frac{(F_{M_Dn} - F_{t_Dn})}{F_{M_Dn}}$	instantaneous PSII quantum yield during dark relaxation	PSII quantum yield relaxing in dark
QY_Ln ⁴	$\frac{(F_{M_Ln} - F_{t_Ln})}{F_{M_Ln}}$	instantaneous PSII quantum yield during light adaptation	PSII quantum yield induced in light
QY_Lss ⁴	$\frac{(F_{M_Lss} - F_{t_Lss})}{F_{M_Lss}}$	steady-state PSII quantum yield	steady-state PSII quantum yield in light
$Fv/Fm (QY_max)$ ⁴	F_v / F_m	maximum PSII quantum yield	maximum PSII quantum yield in dark-adapted state
Fv/Fm_Ln ⁵	$\frac{(F_{m_Ln} - F_{0_Lss})}{F_{m_Ln}}$	PSII quantum yield of light adapted sample	PSII quantum yield in light-adapted state
Fv/Fm_Lss ⁵	$\frac{(F_{m_Lss} - F_{0_Lss})}{F_{m_Lss}}$	PSII quantum yield of light adapted sample at steady-state	PSII quantum yield in light-adapted steady-state
R_{fd_Ln} ⁶	$\frac{(F_p - F_{t_Ln})}{F_{t_Ln}}$	instantaneous fluorescence decline ratio in light	empiric parameter used to assess plant vitality
R_{fd_Lss} ⁶	$\frac{(F_p - F_{t_Lss})}{F_{t_Lss}}$	fluorescence decline ratio in steady-state	empiric parameter used to assess plant vitality

¹ **Oxborough K, Baker NR** (1997) Resolving chlorophyll *a* fluorescence images of photosynthetic efficiency into photochemical and non-photochemical components: calculation of qP and F_v'/F_m' without measuring F_0' . *Photosynthesis Research* 54: 135-142

² **Horton P, Ruban AV** (1992) Regulation of photosystem-II. *Photosynthesis Research* 34: 375-385

³ **Horton P, Ruban AV, Walters RG** (1996) Regulation of light harvesting in green plants. *Annual Review of Plant Physiology and Plant Molecular Biology* 47: 655-684

⁴ **Genty B, Briantais JM, Baker NR** (1989) The relationship between quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochimica et Biophysica Acta* 990:87-92

- ⁵ **Oxborough K** (2004) Imaging of chlorophyll a: theoretical and practical aspects of an emerging technique for the monitoring of photosynthetic performance. *Journal of Experimental Botany* 55: 1195-1205
- ⁶ **Lichtenthaler HK, Mische JA** (1997) Fluorescence imaging as a diagnostic tool for plant stress. *Trends in Plant Science* 2: 316-320

II. Introduction to Chlorophyll Fluorescence Techniques

II.A. Chlorophyll Fluorescence

Measurement of chlorophyll fluorescence kinetics is an extremely important technique for the non-invasive study of photosynthetic dynamics in intact plants, algae, and in cyanobacteria.

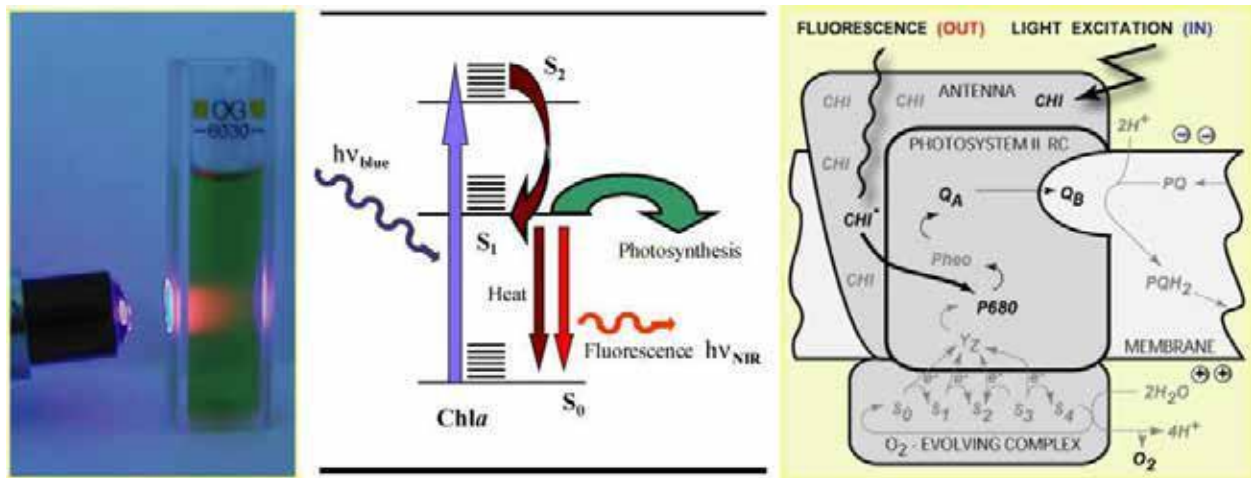


FIGURE 1. The left panel shows a cuvette containing a chlorophyll solution that is illuminated by a beam of blue light. The red light emanating from the solution results from chlorophyll fluorescence emission. The change from the absorbed blue light to the emitted red fluorescence is explained by the 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 excitation energy (FIGURE 1). The more effective is photosynthetic energy conversion, the lower the chlorophyll fluorescence yield, and *vice versa*. In a healthy, dark-adapted plant, photosynthetic capacity is available at its maximum and, thus, chlorophyll fluorescence yield is minimal (F_0). The photosynthetic capacity can be reduced to zero by herbicide or by a pulse of strong light that transiently congests the photosynthetic electron transport pathway. With photosynthetic yield at zero, fluorescence emission reaches a maximum (F_M). An exhaustive review of all aspects of chlorophyll fluorescence emission is provided by 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 (FIGURE 2). The antenna complexes deliver captured energy to Photosystem II and Photosystem I reaction centers. The effectiveness of the energy capture, and of its transfer to the reaction

centers, is determined 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 photosynthesis is blocked, e.g.: by congestion of the plastoquinone pool that mediates electron transport from Photosystem II to the Cyt b/f complex (FIGURE 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.

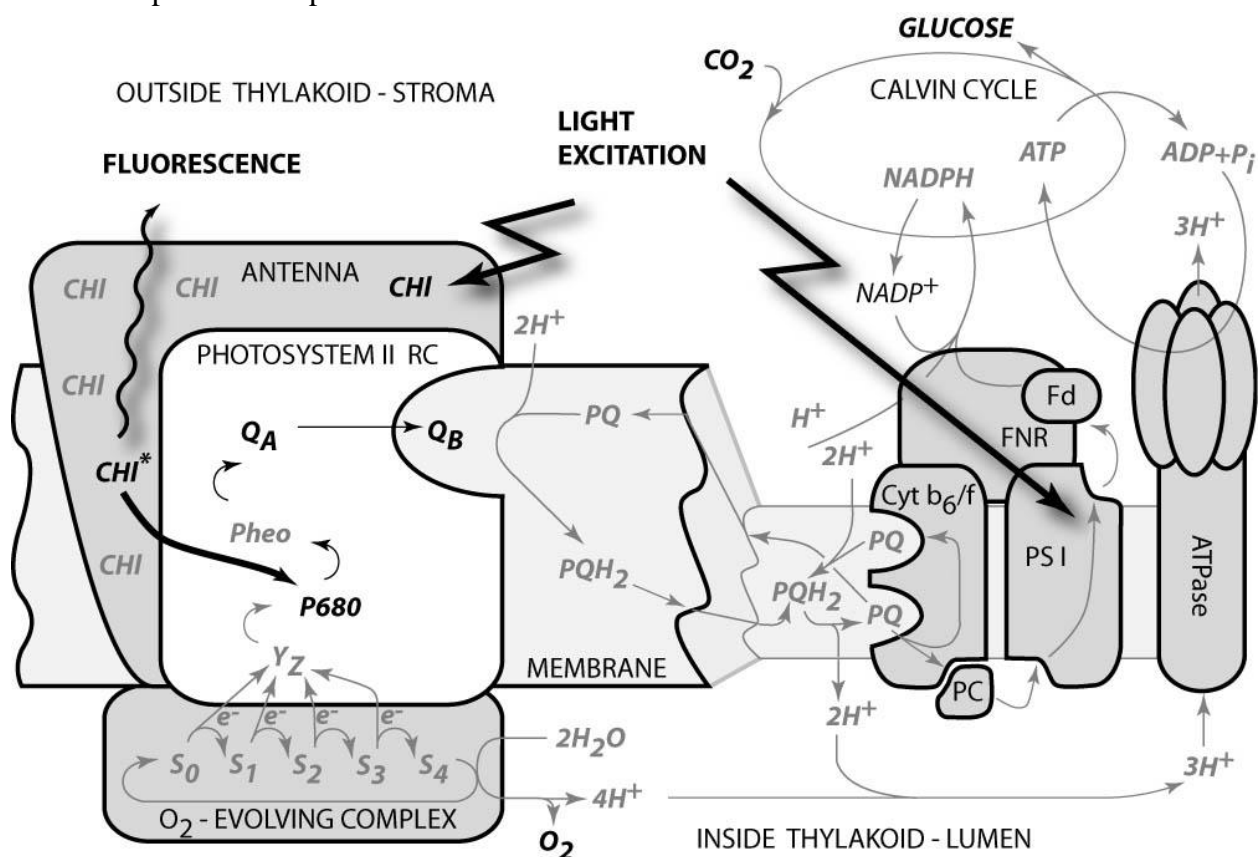


FIGURE 2. Schematic presentation of the principal photosynthetic modules in plants and green algae. Light is absorbed by antenna pigments of Photosystem II (in front) and of Photosystem I (in back). The excitons generated in the antennae are rapidly transferred to the reaction centers where their energy 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 O_2 -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 Q_A . A mobile plastoquinone pool shuttles two electrons sequentially taken from Q_A - and two protons taken from the stromal side of the membrane to the luminal side of cytochrome b_6/f complex where the protons are released and electrons are sent to PSI. PSI uses the excitonic energy to generate reducing $NADPH.H^+$. The charge transfer reactions in the thylakoid membrane result also in accumulation of protons on the luminal 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 the Calvin-Benson cycle to assimilate inorganic CO_2 into sugars.

¹ Irradiance of $2000 \mu\text{mol}(\text{photons}).\text{m}^{-2}.\text{s}^{-1}$, typical of a sunny summer day at noon in Central Europe, results in ca. 1200 excitons arriving to the reaction center per second when the effective antenna cross section of 100 \AA^2 .

II.C. Essentials of Chlorophyll Fluorescence Technique

II.C.1 Kautsky Effect Measured in Continuous Light Mode

The Kautsky effect represents the complex dynamics of chlorophyll fluorescence emission and plant photochemical yields during transition from a dark-adapted to a light-adapted state (Kautsky and Hirsch 1931, Govindjee 1995).

The 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 $\mu\text{mol (photons).m}^{-2}.\text{s}^{-1}$). 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 irradiance means that the dynamics of the fluorescence transients will be faster and that the signal will proportionally rise. Another aspect of continuous light measurement is that the fluorescence signal cannot be discriminated from background light. Also, 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) obscures 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).

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

- Upper estimate of F_0 fluorescence emission of a dark-adapted plant;
- F_P peak fluorescence attained during the first second(s) of the transient when the plastoquinone pool is largely congested by the light-driven electron flux (*FIGURE 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. This parameter 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 and Miehé (1997) to propose the R_{fd} fluorescence ratio ($R_{fd} = (F_P - F_t_{LSS})/F_P$) as an empirical parameter to quantify plant fitness. Measurement of the R_{fd} parameter requires only very simple imaging instrumentation and can be measured with low noise. The measurement is relatively time consuming as it requires light adaptation that can take tens of seconds, or up to a few minutes, depending on the measured system and on incident irradiance.

II.C.2 Kautsky Effect Measured in Pulse-Amplitude-Modulated Mode (PAM)

The transitions from the dark-adapted state to the light-adapted state and back can be investigated in great detail by the 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]. Similar 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 the fluorescence yield $\Phi(t)$ that is multiplied by the effective antenna size $\sigma_{PSII}(t)$.

- The measurement starts with a dark-adapted plant that is characterized by a low, minimum fluorescence emission signal F_0 (FIGURE 3). F_0 is interpreted as the fluorescence signal from open PSII reaction centers with their primary quinone acceptor Q_A fully oxidized (FIGURE 2). The open reaction centers have maximum q_P photochemical quenching and minimum Φ_0 fluorescence yield. At the same time, NPQ non-photochemical quenching is fully relaxed with the effective antenna size at its maximum $\sigma_{PSII}(\text{dark})$.

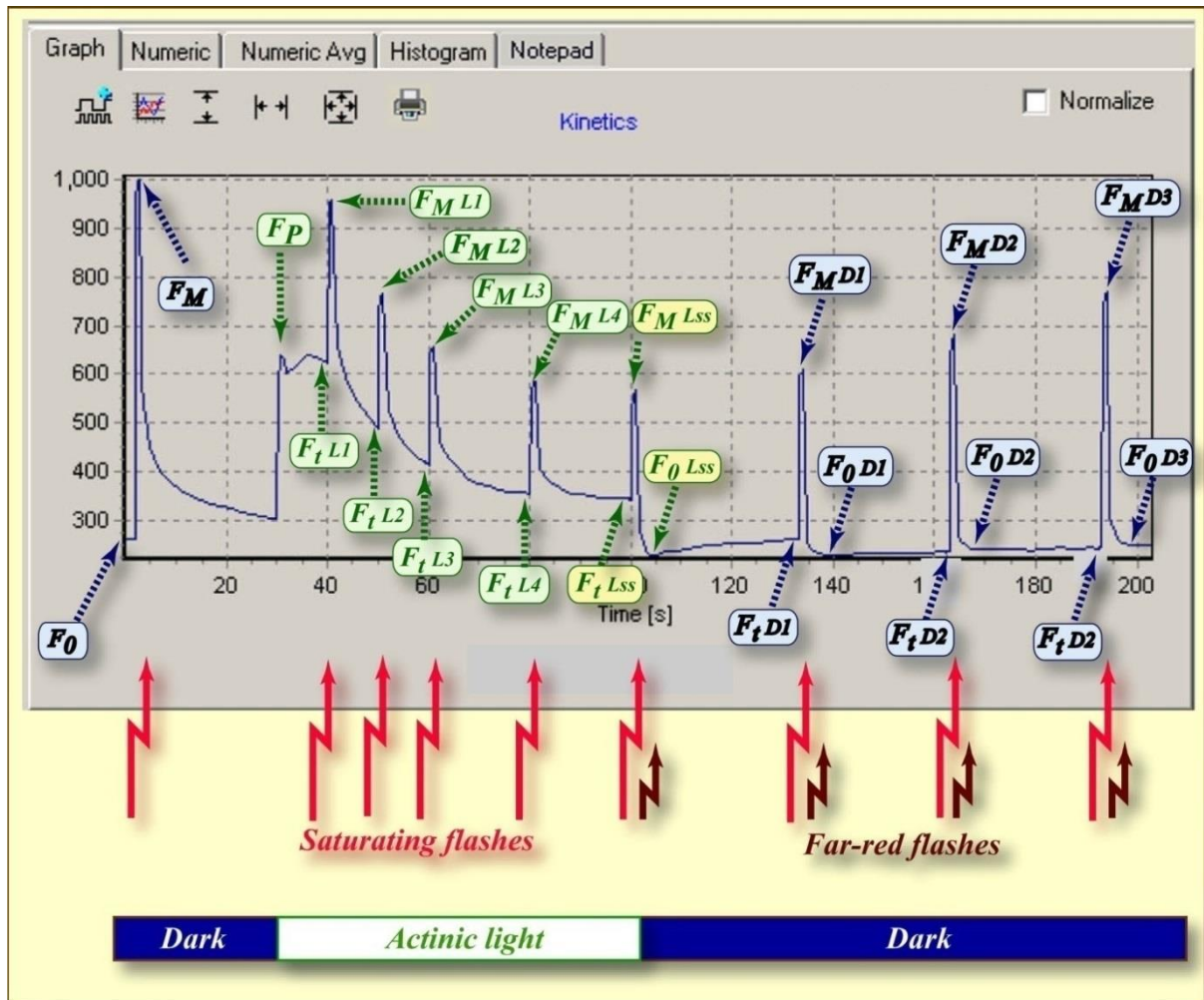


FIGURE 3. FluorCam window showing the result of an induction experiment measured in PAM -mode. The blue labels mark the fluorescence levels measured either in the dark (F_0 , F_M) or during dark relaxation (F_{t_Dn} , F_{M_Dn} , F_{0_Dn}). The green labels mark the fluorescence levels measured during light adaptation (F_{t_Ln} , F_{M_Ln}). The yellow labels show the steady-state fluorescence levels attained in continuous light (F_{t_Lss} , F_{M_Lss} , F_{0_Lss}). The bright-red arrows indicate timing of flashes that transiently saturate the electron transport chain, reducing the plastoquinone pool and Q_A acceptor. The closed reaction centers do not quench fluorescence photochemically, and the fluorescence signal reaches a local maximum that is modulated only by 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 results in maximum quenching by photochemical charge separation, and a minimum fluorescence signal F_{0_Dn} that is modulated only by residual non-photochemical quenching.

- The measurement continues with a dark-adapted plant that is exposed to a strong flash of light (marked by the red arrow in *FIGURE 3*) that transiently reduces the plastoquinone pool and the primary quinone acceptor QA (*FIGURE 2*). The quenching by photochemistry of the reaction centers (qP) is eliminated and the fluorescence yield reaches its maximum (ΦM). 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 FM ($\approx \sigma PSII \cdot \Phi M$) is at its maximum. This signal is often called FM maximum fluorescence (yield).
- The variable fluorescence FV signal is defined as the difference $FM - F0$. FV and the FM maximum fluorescence value are used to calculate the maximum quantum yield of Photosystem II photochemistry $QY_{max} = FV / FM$.
- Dark relaxation following the saturating flash leads to re-oxidation of the plastoquinone pool and of the primary quinone acceptor QA⁻. The decline of fluorescence signal from the FM level back to the $F0$ dark-adapted level (*FIGURE 3*) is dependent on the kinetics of reaction centers re-opening.
- The Kautsky effect is induced by actinic light (marked by the green-white horizontal bar in *FIGURE 3*) that drives primary charge separation in the reaction centers of both photosystems (*FIGURE 2*). First, the signal rises to the FP peak fluorescence level as the plastoquinone pool becomes largely reduced by Photosystem II activity (*FIGURE 2*). Later, the signal declines to the Ft_Lss steady-state fluorescence level (*FIGURE 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 electron transport processes that relieve the congestion of charge transport intermediates, fluorescence decline is further enhanced by photoprotective non-photochemical quenching that diminishes maximum fluorescence from FM in the dark to the much lower level FM_Lss in the light adapted steady-state. Steady-state non-photochemical quenching is quantified by $NPQ_Lss = (FM - FM_Lss) / FM$. Another parameter introduced to characterize the Kautsky effect is the Rfd fluorescence decline ratio ($Rfd = (FP - Ft_Lss) / FP$) that is used to assess plant vitality [Lichtenthaler and Miehe 1997].
- Saturating flashes (bright-red arrows in *FIGURE 3*) eliminate photochemical quenching transiently by reducing the plastoquinone pool and the QA acceptor (*FIGURE 2*). The fluorescence signal reaches local maxima that reveal the dynamics of non-photochemical quenching during light adaptation (FM_Ln). Non-photochemical quenching is quantified by $NPQ_Ln = (FM - FM_Ln) / FM$.
- The 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 $F0_Ln \approx F0 / ((FM - F0) / FM + F0 / FM_Ln)$. This estimate is needed to assess the fraction of PSII reaction centers that are open at a given time. This fraction is characterized by photochemical quenching $qP_Ln (\equiv PSII_{open} / (PSII_{open} + PSII_{closed})) = (FM_Ln - Ft_Ln) / (FM_Ln - F0_Ln) \approx (FM_Ln - Ft_Ln) / (FM_Ln - F0 / ((FM - F0) / FM + F0 / FM_Ln))$.
- Finally, saturating flashes serve to estimate the instantaneous quantum yield of PSII photochemistry $QY_Ln = (FM_Ln - Ft_Ln) / FM_Ln$.
- The instantaneous signal Ft_Lss , and the maximum signal FM_Lss in the light-adapted steady-state, are measured before and during the saturating flash at the end of the actinic light period (yellow labels in *FIGURE 3*). This measurement is used to assess steady state non-photochemical quenching ($NPQ_Lss = (FM - FM_Lss) / FM$), the fluorescence decline ratio ($Rfd = (FP - Ft_Lss) / FP$), and the steady-state quantum yield of PSII photochemistry ($QY_Lss = (FM_Lss - Ft_Lss) / FM_Lss$).

- The minimum fluorescence signal in the light-adapted steady-state $F0_Lss$ is measured immediately after the actinic light is switched off and the plastoquinone pool and QA acceptor are re-oxidized by a far-red flash (dark-red arrow in *FIGURE 3*). The measured $F0_Lss$ signal is used to calculate the steady-state fraction of open reaction centers estimated from the level photochemical quenching qP_Lss ($\equiv PSII_{open} / (PSII_{open} + PSII_{closed}) = (FM_Lss - Ft_Lss) / (FM_Lss - F0_Lss)$).
- Dark relaxation (dark-blue horizontal bar in *FIGURE 3*) following the actinic light period is monitored by measuring the fluorescence signal before the saturating flashes (Ft_Dn), during the saturating flashes (FM_Dn) and during the far-red flashes ($F0_Dn$).
- Fluorescence signals measured during the dark period are used to calculate the relaxation of non-photochemical quenching $NPQ_Dn = (FM - FM_Dn) / FM$, the re-opening of PSII reaction centers qP_Dn ($\equiv PSII_{open} / (PSII_{open} + PSII_{closed}) = (FM_Dn - Ft_Dn) / (FM_Dn - F0_Dn)$), and the relaxation of PSII quantum yield $QY_Dn = (FM_Dn - Ft_Dn) / FM_Dn$.

III. FluorCam Instrument

FluorCam is the name for a family of kinetic imaging fluorimeters developed by Photon Systems Instruments, spol. s r.o. (*FIGURE 5*). Every FluorCam version contains a CCD camera, with standard modules to capture and grab images, and modules to control the measuring light flashes, actinic light and saturating flashes that are generated by version -dependent light sources. Both the camera and the light sources use the same high-capacity power supply. The operation of the camera and light sources is controlled by a protocol in FluorCam software (see section V) that is transmitted to the camera *via* a Ethernet cable. The acquired data are transmitted from the camera to the computer by the same cable.

FIGURE 4 shows a scheme of the FluorCam macroscopic systems (Handy FluorCam, Closed FluorCam, Open FluorCam and custom-made large-scale systems). In some versions, light sources can be limited to measuring flashes and actinic light source. The control unit can be integrated in the camera in some versions or can be in form of a separate hub.

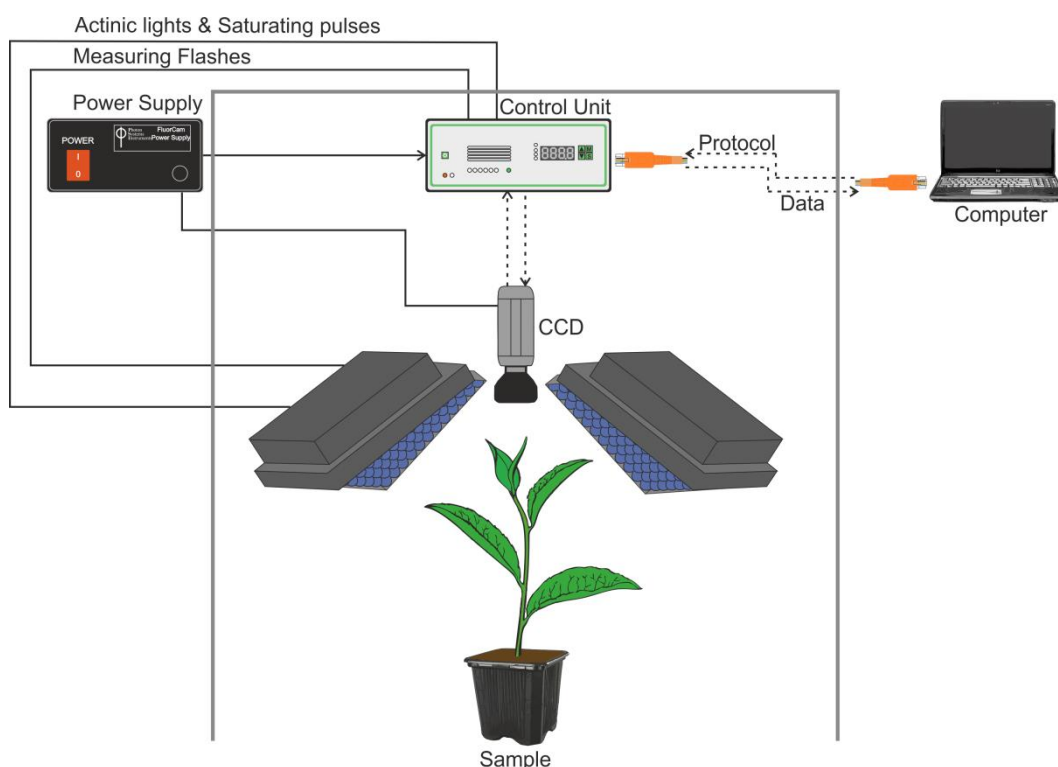


FIGURE 4. Scheme of macroscopic FluorCam versions

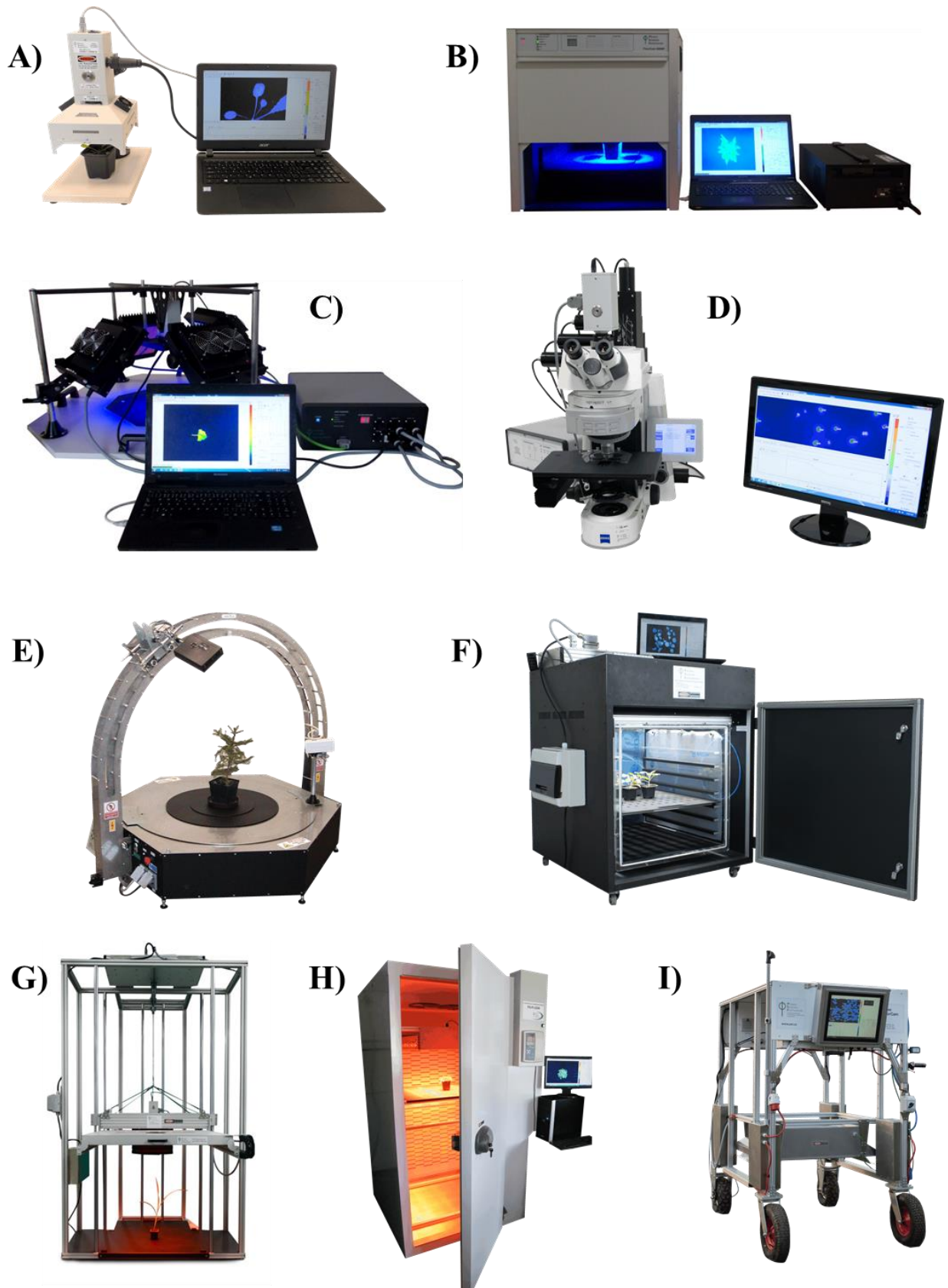


FIGURE 5. FluorCam hardware versions: (A) Portable **Handy FluorCam** for single leaf/Petri dish measurements. (B) **Closed FluorCam** with fixed working distance for small plant/Petri dish measurements. (C) **Open FluorCam** with variable working distance and angles for small plant/Petri dish measurements. (D) **Fluorescence Kinetic Microscope** with Zeiss Axio Imager. (E) Customized **Arch FluorCam** for multi-angle fluorescence imaging of small and medium-size plant. (F) Customized large **Open FluorCam** installed in light isolated and gass proof chamber. (G) Customized large **Open FluorCam** for indoor fluorescence imaging of 1 m high plants. (H) Customized **Open FluorCam** installed in step-in growing chamber FytoScope. (I) **Rover FluorCam** is easy-moving model for large-scale scanning in the field.

III.A. Closed FluorCam

Closed FluorCam is a durable laboratory version of the FluorCam instrument for measurements on small plants, attached or detached leaves, algal cultures etc. (*FIGURE 6*). The camera field of view can cover the samples up to 13×13 cm. Selectable shelving system allows precise measurement of samples of diverse sizes. The cabinet may be closed for dark-adaptation. The position of the LED panels and the camera objective are fixed and do not need any adjustment. The only manual controls is focusing of the lens. A scheme of a typical configuration is shown in *FIGURE 6*.

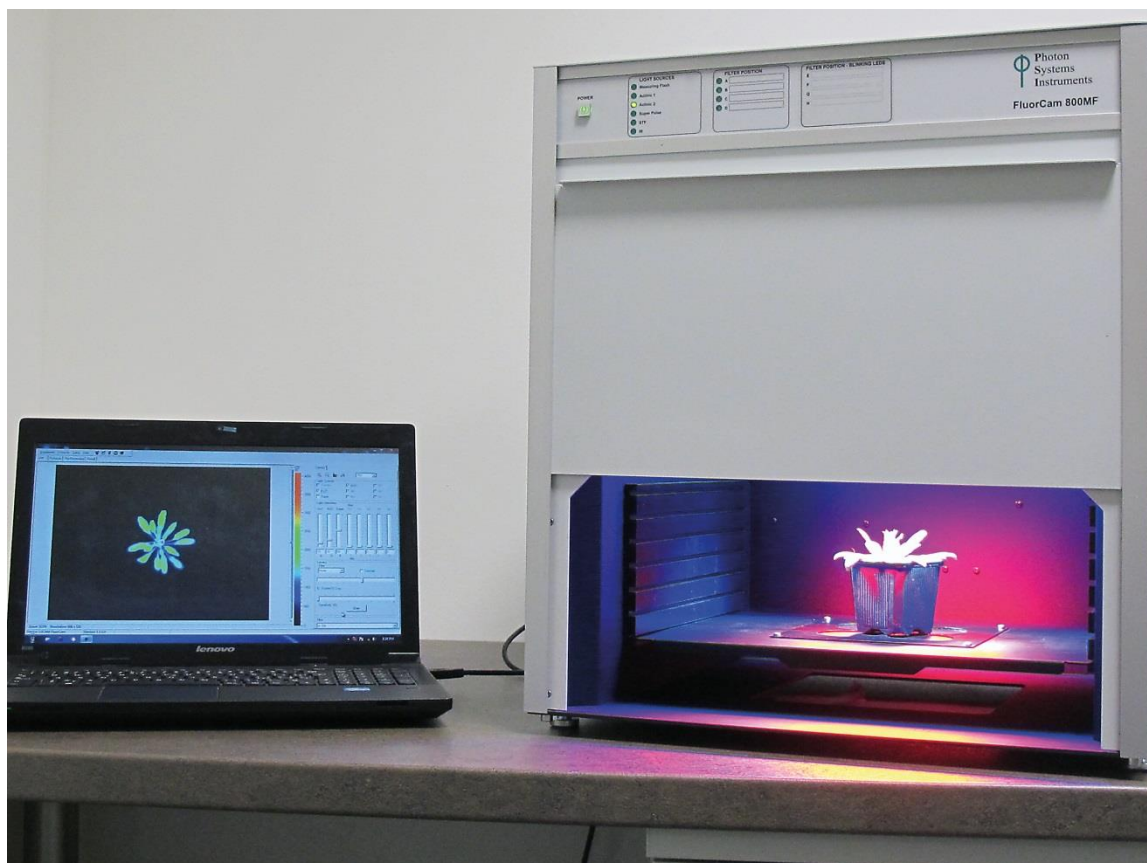


FIGURE 6. Closed FluorCam

III.B. Handy FluorCam

Handy FluorCam is a compact portable version of the FluorCam instrument for measurements on a single leaf (*FIGURE 7*) or on samples in a Petri dish. The position of the LED panels and the camera objective are fixed and do not need any adjustment. The only manual controls are focusing and zoom of the objective. The whole instrument can be carried in a convenient bag over the shoulder. It can be attached to tripod and can also run on batteries.

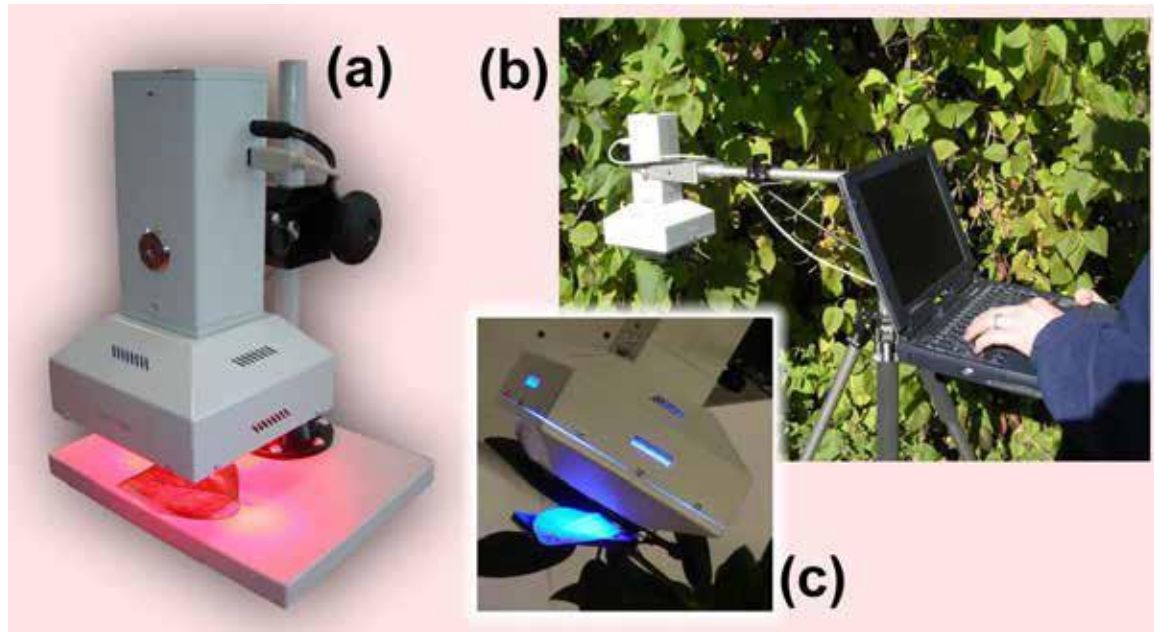


FIGURE 7. Handy FluorCam. (a) Portable device version for single leaf/Petri dish measurements in laboratory. (b) Handy FluorCam field operation. (c) Blue-color device version with its leaf clip open.

III.C. Open FluorCam

The Open FluorCam (*FIGURE 8*) is a particularly versatile version of the FluorCam instrument for measurements on a single leaf or on a small plant. The LED panels and the light source generating saturating flashes can be arranged at various angles and distances from the sample. The position of the camera may also be adjusted for added precision. This allows optimization of the imaging configuration for samples of different morphology. The maximum area for imaging with standard hardware is 13×13 cm, respectively 20×20 cm, depending on the size of selected light sources. The FluorCam hub can support up to four LED panels.

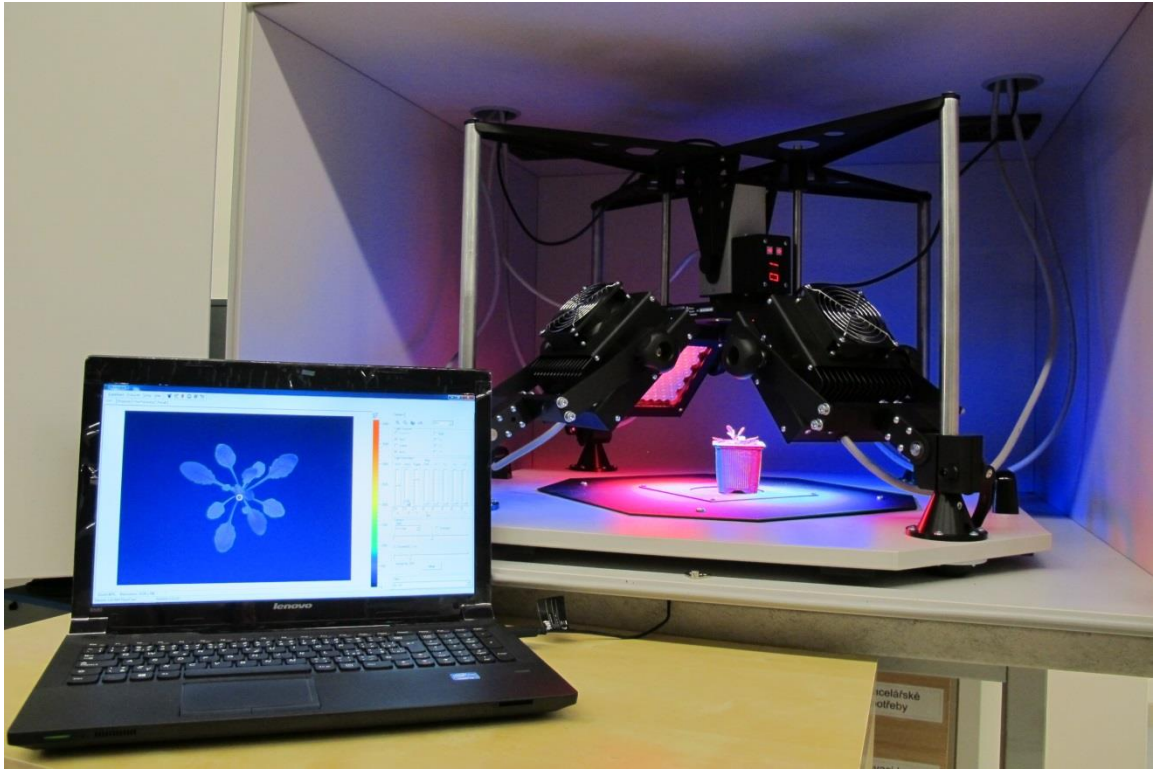


FIGURE 8. Open FluorCam. The positions and angles of light sources, and of the camera, can be adjusted to provide the optimum configuration for irradiance homogeneity with samples that have different morphology, making thus the instrument very flexible.

III.E. Fluorescence Kinetic Microscope

Fluorescence Kinetic Microscope extends the complete capacity of kinetic Chl-fluorescence imaging to the realm of individual cells and sub-cellular structures. It 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 type of the light sources. Up to three LED systems with two dichroic mirrors can be integrated into the module that fits the microscope condenser port. Measuring flashes, actinic light, saturating flashes as well as a source for 'fluorescence protein' excitation are typically supplied in this module. A far-red light source is mounted separately in the transmission direction. A typical configuration of the Micro FluorCam is shown in *FIGURE 9*.



FIGURE 9. Scheme of Micro FluorCam

III.F. Custom-Made FluorCam Versions

The large Open FluorCams can be mounted to various customized systems, eventually with motorized or manual positioning of the light sources and camera.

The **Rover FluorCam** (*FIGURE 10*) is an extremely stable structure with large and solid wheels that allow easy movement in field due to its motorized wheels. From small to large plants can be studied *in situ* without detaching or destroying them. The system scans area of 35×35 cm and its height can be adjusted from 20 cm to 150 cm. If required, an additional camera can be used for true-color analysis.



FIGURE 10. Rover FluorCam is a remarkably stable structure. Its large and solid wheels, which are motorized, allow easy movement in a terrain

The large **Open FluorCams** (*FIGURE 11*) can be installed in different systems or constructions, such as step-in growing chamber *FytoScope*, light-isolated and gas proof chamber or height adjustable open construction. Typically, it is used to scan from small, through mid-sized to large plants or it can monitor plants affected by stress gradient. It can also be favorably used for analysis of multiple samples at once.

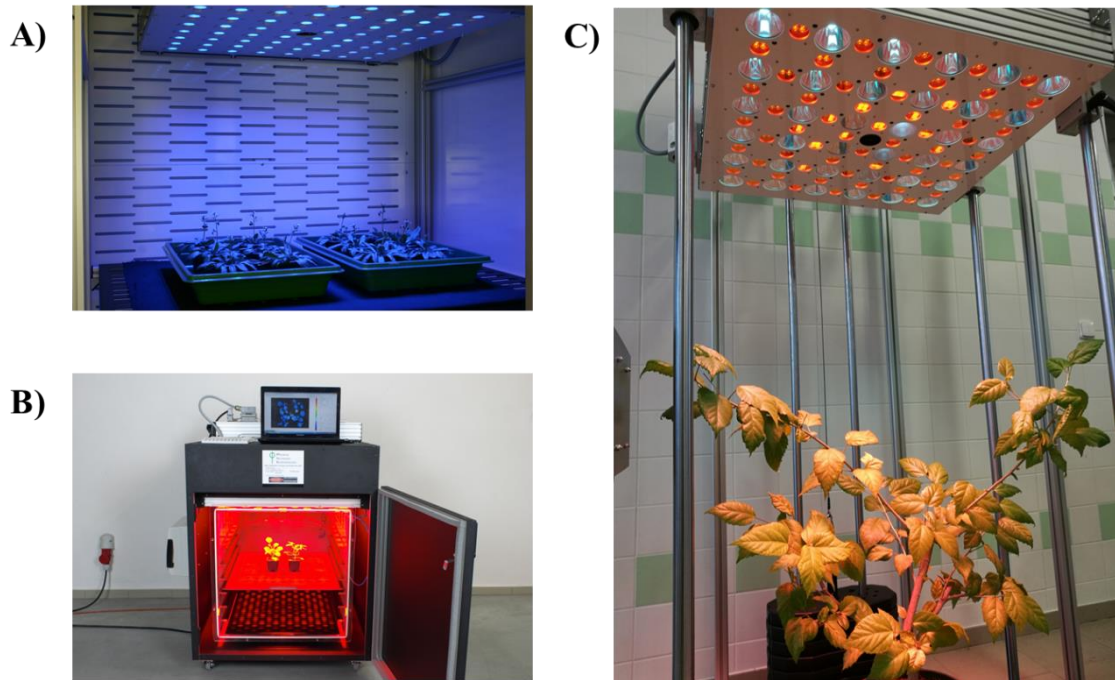


FIGURE 11. Open FluorCams installed in A) FytoScope step-in growing chamber, B) Light-isolated and gas proof chamber, C) open height-configurable construction.

The **Arch FluorCam** (*FIGURE 12*) is a customized fluorescence imaging system for three dimensional studies. The stand and the frame are exceedingly steady, yet the whole structure provides very flexible viewing angles. Large plants can be analyzed from various positions without the need to move them. Three dimensional image data are collected and the software can generate 3-D images of Chl-fluorescence emission.



FIGURE 12. Customized Arch FluorCam

IV. Installation and Assembly Instructions

Most FluorCam versions (Closed, Handy, etc.) are delivered to customers in fully composed forms and they do not require any special assemblage. Basic installation tips for the Closed FluorCam are depicted in Chapter IV.A. These tips can also be used for the Handy FluorCam version.

The Open FluorCam is a versatile model; its assemblage is therefore described in detail in Chapter IV.B.

IV.A. Assembly Instructions for the Closed FluorCam

Recommended manipulation: Due to the weight of whole device is recommended to manipulate with it in two persons.

Step 1:

- Carefully unpack the parcel.
- Place all components on a flat and firm surface. Keep them away from wet floors and counters.
- Check the contents of the package and compare it with the enclosed package list.
- Make sure that both the FluorCam and the power supply are switched off.

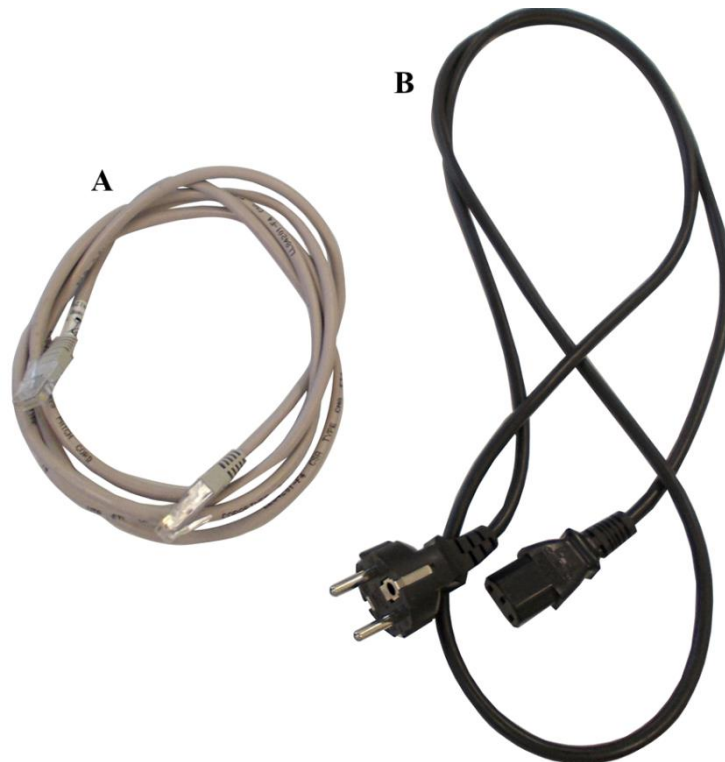


FIGURE 13. Cables. A) Ethernet cable for camera connection with PC, B) Camera power supply

Step 2:

- Using the provided thick power cord, connect the FluorCam and the power supply. The socket is on the back side of the FluorCam device and marked as POWER (*FIGURE 13-B, FIGURE 14*).
- Connect the power supply to a 110/230V outlet.



FIGURE 14. Power cable connection

- Connect the FluorCam to the computer using the provided Ethernet cable (*FIGURE 13-A, FIGURE 15*).



FIGURE 15. Ethernet cable connection

- Switch on the power supply, the FluorCam device and the computer.

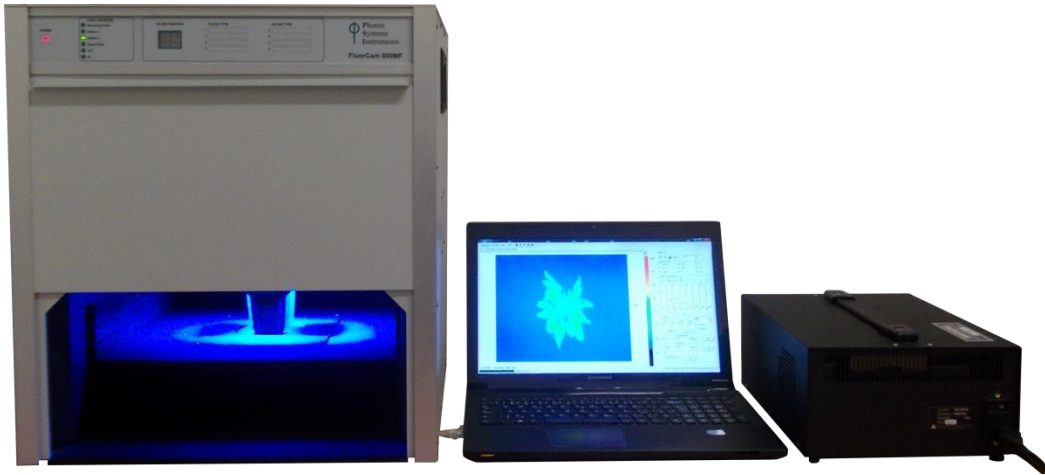


FIGURE 16. Closed FluorCam

IV.B. Assembly Instructions for the Open FluorCam

Recommended tools: Allen key (provided by the manufacturer), Phillips screwdriver.

Assembly recommendation: Due to the weight of whole device is recommended to manipulate with it in two persons.

Step 1:

- Carefully unpack the parcel.
- Place all components on a flat and firm surface. Keep them away from wet floors and counters.
- Check the contents of the package and compare it with the enclosed package list.
- Make sure that both the FluorCam and the power supply are turned off during the assemblage.

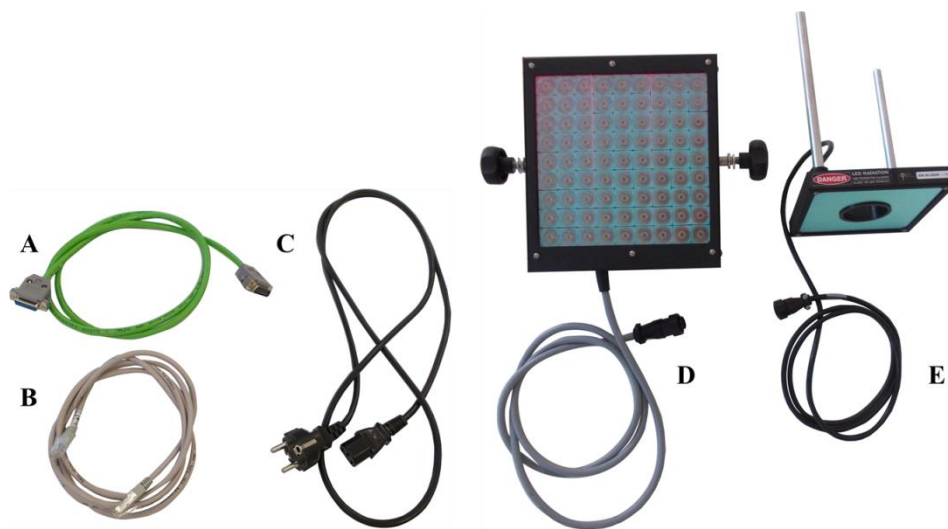


FIGURE 17. Cables and LED light types. A) Camera power supply, B) Ethernet cable for camera connection with PC, C) Power supply cable for Control unit, D) LED light panel (4 pcs in total), E) Additional LED light panel

Step 2:

- Place the four stainless steel rods into the support sockets on the stand and tighten them in place (FIGURE 18-A) using the Allen key provided.

Step 3:

- Place the four LED light steel supports to the rods (each support to each rod) and lock them with the steel clamp to prevent the unwanted movement (FIGURE 18-B).

Step 4:

- Place the upper support bracket above the four upright posts and lock it into place using the Allen key (FIGURE 18-C).



FIGURE 18. Open FluorCam stand assembly

Step 5:

- Attach each LED panels to an upright steel support and tighten the knobs on the side of the LED panels to fix them in place (FIGURE 19). Make sure that the two white/blue LED panels (for the actinic light and saturating pulses, their cables are marked ACTINIC 2A and ACTINIC 2B) are placed opposite each other. This will ensure that the red-orange LED panels (for actinic light and measuring pulses, their cables are marked ACTINIC 1A and ACTINIC 1B) are also placed opposite each other. The LED panels may be placed at any height on the upright supports, but during initial installation it is best if they are set at the position app. 1 cm from bottom. Lock the panels into this position using the steel locking clamp.
- Ideally, all four LED panels should be positioned at a 45 degree angle with respect to the plane of the sample, with similar LED panels aligned directly opposite to one another. A light meter should be used to ensure that the position of the LED panels creates homogeneous irradiance across the imaged area. The LED panels may be elevated on the steel support rods and swiveled to create different lighting configurations for complex samples. However, the user should check for homogeneity of light level at the sample position, especially when measuring fluorescence parameters that vary with actinic light level (e.g. F_v'/F_m').
- When the LED panels are placed at 45 degrees to the plane of the base plate, they create homogeneous actinic light across an area of approximately 9×9 cm. This area may be expanded a little by increasing the height of the panels and raising the camera to create a wider viewing angle. However, it is always essential to use a light meter to ensure that the homogeneous lighting area is defined when the position of the panels is changed.

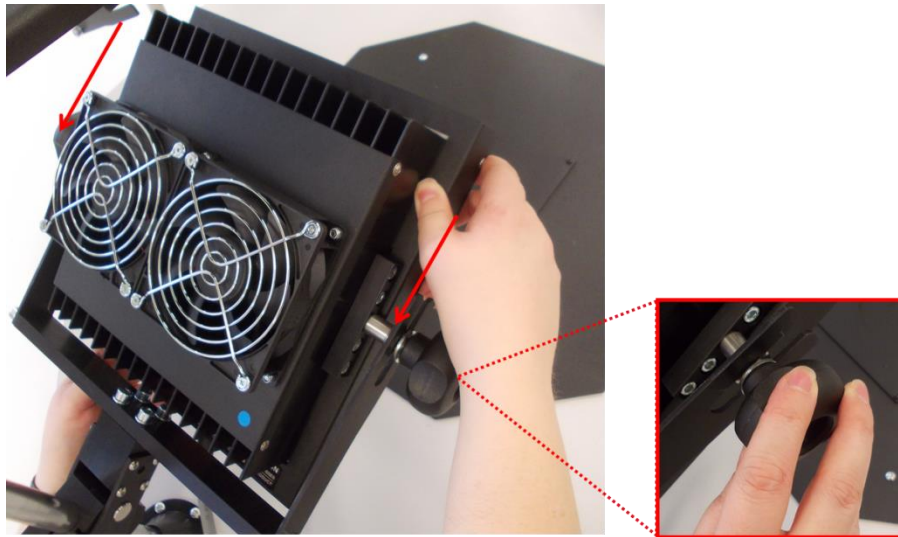


FIGURE 19. LED light panel attaching

Step 6:

- Connect the LED lights cables with relevant sockets on the front side of the Control unit. Please check the cable labels for proper connection (e.g.: cable ACTINIC 2A to socket ACTINIC 2A). Push the connector to the socket gently (please note, that the connector must be connected in exact position regarding to the stumps) and screw them clockwise. Repeat for all four LED light connectors (FIGURE 20).
- Please note, that without the connection of LED panels with control unit, the lights will not work.

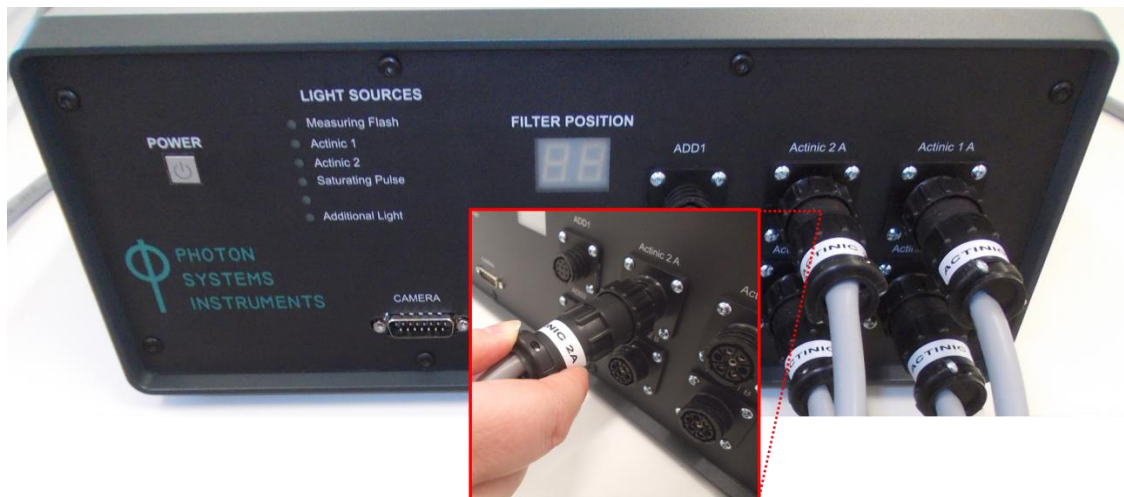


FIGURE 20. LED light panels power connection

Step 7:

- Mount the camera on the support bracket. Insert the camera between the sides of the central support so that the sockets for the camera adjustment knobs are aligned with the track in the bracket. Screw the knobs into the side of the camera body and tighten. The knobs can be loosen and the track used to adjust the camera height (FIGURE 21-A).
- Optionally, the part of the camera would be filter wheel (with up to 7 different filters), which is no necessary to adjust.



FIGURE 21. FluorCam camera with filter-wheel. A) Mounting of camera to the support brackets and tightening with the knob, B) Connecting camera with Control unit, C) Connecting camera with PC

Step 8:

- Connect the camera by green cable (FIGURE 13-A) with the Control unit. The camera socket is placed on the side of the camera and must be connected with the Control unit socket named CAMERA. For fastening of both connectors use Phillips screwdriver (FIGURE 21-B, FIGURE 22) (please note, that screwdriver is NOT part of the device accessories).
- Connect the camera to the computer using the Ethernet cable (FIGURE 13-B), which is part of the FluorCam device package. Socket for Ethernet cable is placed on the upper side of the camera (FIGURE 21-C).
- Please note, that without these two cable connection the FluorCam camera will not be working.



FIGURE 22. Camera connection with Control unit

Step 9:

- Connect the Control unit power cable to the backside of the Control unit and to the electrical socket (*FIGURE 23*).
- Press power button on the Control unit and start FluorCam7 application on your notebook. If the camera connects successfully, the Live scene will be dark and on the left lower corner will be green message with camera UC (in the camera is not connected, the scene will be noisy and the connection message will be yellow).
- Remove the lens cover, which protects lens optics during the FluorCam shipping.
- Try the light functionality by turning on and off all lights one by one in FluorCam7 Live window, Light Sources box, with plant sample placed under the camera to observe real-time fluorescence signal.



FIGURE 23. Backside of the Control unit - power supply cable connection

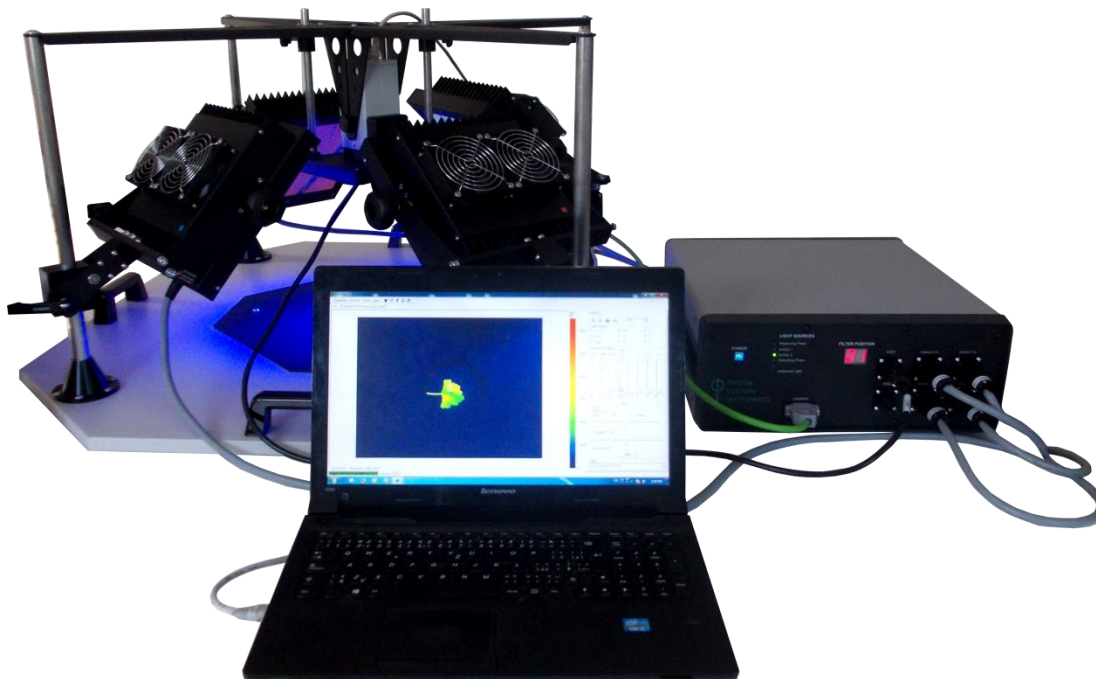


FIGURE 24. Open FluorCam

V. FluorCam Software

V.A. Quick Start Guide

It is important to find correct settings for light and camera before you start a real experiment. Follow next steps for proper FluorCam settings prior measurement initiation.

- 1) **FOCUS & ZOOM:** Place a testing plant under the FC (the testing plant should be similar to the plants that will be used in experiment – same size, same cultivation condition, same age...) Switch on measuring flashes -> mark check-box **Flashes** in **LIVE WINDOW**. Increase El. Shutter and Sensitivity to see a nice image. Change **False- color scale** (right mouse click on the scale right to the image) to Black & White. **Zoom** and **Focus** the objective to see sharp image. **FOCUS** and **ZOOM** should be kept during the whole experiment otherwise the level of absolute fluorescence signal could be changed. Then arrange the plant to final position, leaves of interest perpendicular to camera.
- 2) **CAMERA SETTINGS:** Let measuring flashes switched on and adjust **El. Shutter** and **Sensitivity** in **LIVE WINDOW**. Change **False- color scale** to Extended spectrum or Extended spectrum 3_0_3 (the most sensitive color scales for human eye). Keep El. Shutter as low as possible (low resolution CCD between 0-1, high resolution CCD between 1-2), otherwise measuring pulses would be too strong causing actinic effect. Adjust Sensitivity by trucking the bar to get a signal in the range of 200-500 digital units (dark blue or blue color).
- 3) **LIGHT SETTINGS – ACTINIC LIGHT:** Choose intensity of Actinic light (Act1 or Act2): (a) either desired absolute light intensity can be chosen with respect to cultivation conditions, or (b) it can be adjusted according to the fluorescence transient.
 - (a) Place a light meter under FC to the position and distance from the camera where the sample leaf will be located. Switch on Actinic light by marking a check-box Act (1 or 2) in **Light Sources** panel in **LIVE WINDOW**. Choose desired intensity by trucking corresponding bar in **Light Intensities** panel.
 - (b) Let measuring flashes switched on and adjust Actinic light intensity by trucking the bar. The intensity between 20-50 % is usually strong enough for plants grown in chambers.

Test image quality after the Actinic light intensity was chosen: switched on measuring pulses and desired actinic light and check if the signal is not too low or saturating. If too low fluorescence signal increase Sensitivity to use the best a dynamic scale. CCD camera is 12 bits, so fluorescence signal can be measured between 0 – 4096 or 16 bits with range 0 – 65 536. If saturating, the error message will warn you. It is necessary to decrease Sensitivity or both Sensitivity + El. Shutter.
- 4) **LIGHT SETTINGS – SATURATING PULSE INTENSITY:** Mark check box **Super** in Light Sources panel in **LIVE WINDOW**. Truck the **Super** bar in **Light Sources** panel in **LIVE WINDOW**. The saturating pulse is only switched for limited time (800 ms), so it must be switched on each time the intensity is changed. Finally, test image quality with all 3 lights (Flashes + Act + Super) switched on as described above.
- 5) **PROTOCOL:** Click on the magic hat pictogram in top panel – **Protocol & Menu Wizard** and choose measuring protocol. There are some predefined protocols on the left side. User alone can define own protocols by using Wizards on the right side. The predefined protocol Fv/Fm is a simple protocol determining Fo, Fm and Fv/Fm. This can be used to check if **Saturating pulse** is strong enough before running any other more complicated protocol.

- 6) **IMPORTING SETTINGS:** Click button **Use** in the bottom of **LIVE WINDOW** to import camera and light settings to the protocol.
- 7) **MEASURE:** Click red flash icon, **Start Experiment**, in the top panel to run the measurement.

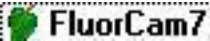
V.B. Installation and Start

V.B.1 Installation Instructions

Connect FluorCam instrument and PC using an Ethernet cable. The RJ-45 port is required. Please log in as the Administrator during the installation.

FluorCam7 runs on Windows XP, Windows Vista, Windows 7 and Windows 10 (32-bit edition only).

V.B.2 Fluorcam7 Start

- Most customers do not need to install the FluorCam software themselves. In most cases, the FluorCam device includes its own PC and the software has been installed by the manufacturer.
- Start the FluorCam operation by switching on the power supply, the FluorCam, and the computer (see Chapter IV of this Manual for the device installation).
- Start the FluorCam 7 software by clicking the FluorCam icon on the desktop of the computer or by starting the FluorCam7.exe file.
- Look for this icon: 



V.C. Testing the Instrument in the Live Window

- On start up, the FluorCam software shows the **live window in auto mode** that automatically adjusts the camera electronic shutter and the sensitivity to ambient light level.
- To begin an experiment, 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. The image is transmitted by ambient light photons scattered on the surface of the leaf.
- **The brighter the ambient light, the lower the automatic shutter setting and the camera sensitivity.**

V.C.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 720×560 pixel images with **12-bit resolution**, i.e.: discriminating a maximum of 4096 light levels, or 1360×1024 with 16-bit resolution. 50 or 20 images are taken per second..

Fig. 25 shows the live window with software control features marked by red labels:

- The size of the image window can be adjusted using (de-)magnifying icons – (1) in *FIGURE 25*.
- Details of the image can be viewed by adjusting the **zoom feature** – (2) in *FIGURE 25*.
- The **numbered scale** can be viewed after clicking the corresponding field – (3) in *FIGURE 25*.
- The **color scheme** can be changed by clicking the color / gray shade **scale bar** – (4) in *FIGURE 25*.

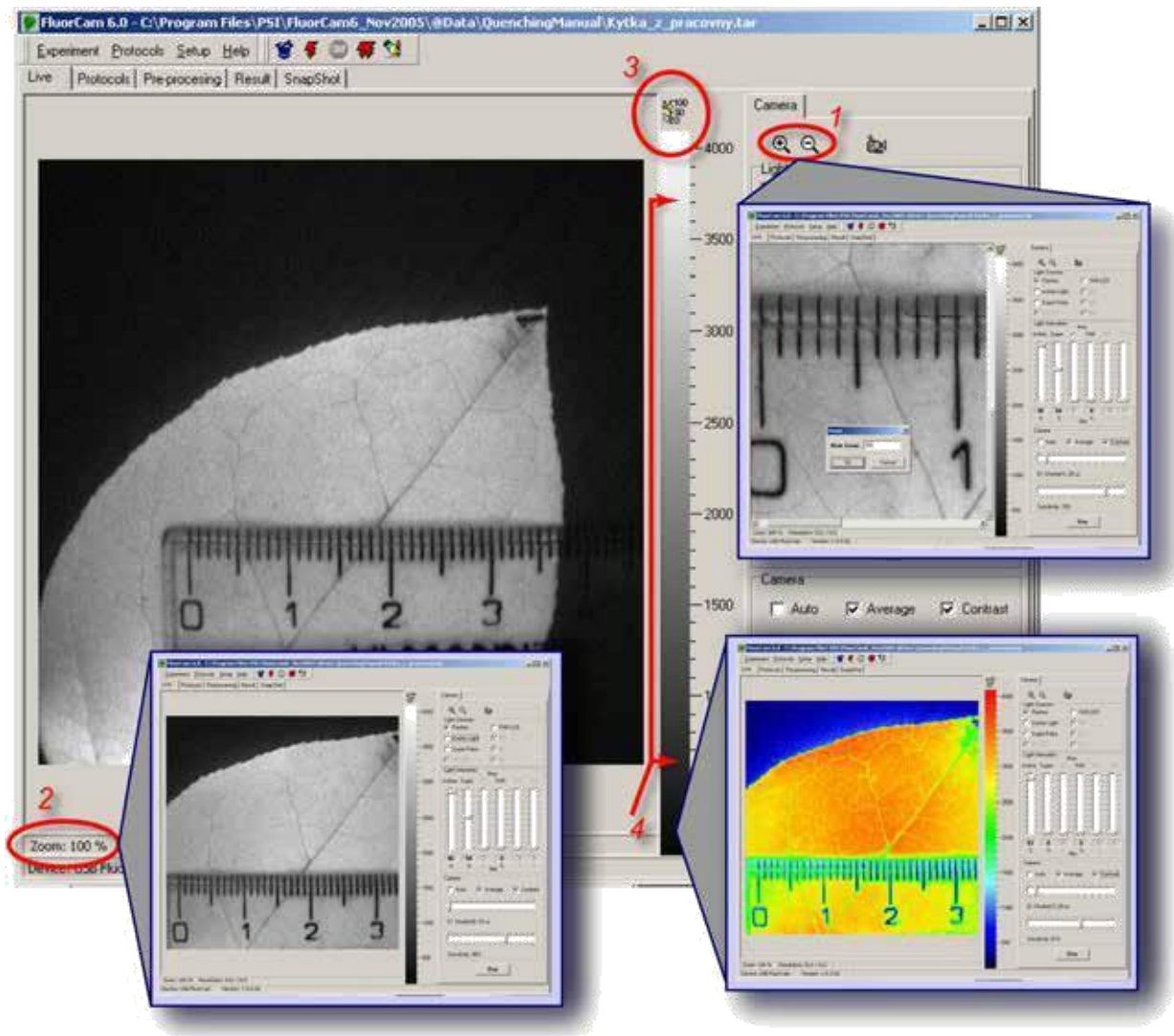


FIGURE 25. Initial adjustment with the Live FluorCam window. By clicking the magnifying and de-magnifying icons (1), one can increase and decrease the size of the image window on the computer screen. By clicking the zoom field (2), one can zoom while preserving the size of the image window. By clicking the numeric scale field (3), the scaling bar is supplemented by a numeric scale. By clicking anywhere on the color / gray scale bar (4), one can change the color scheme of the image.

V.C.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 *FIGURE 26*.
- 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.

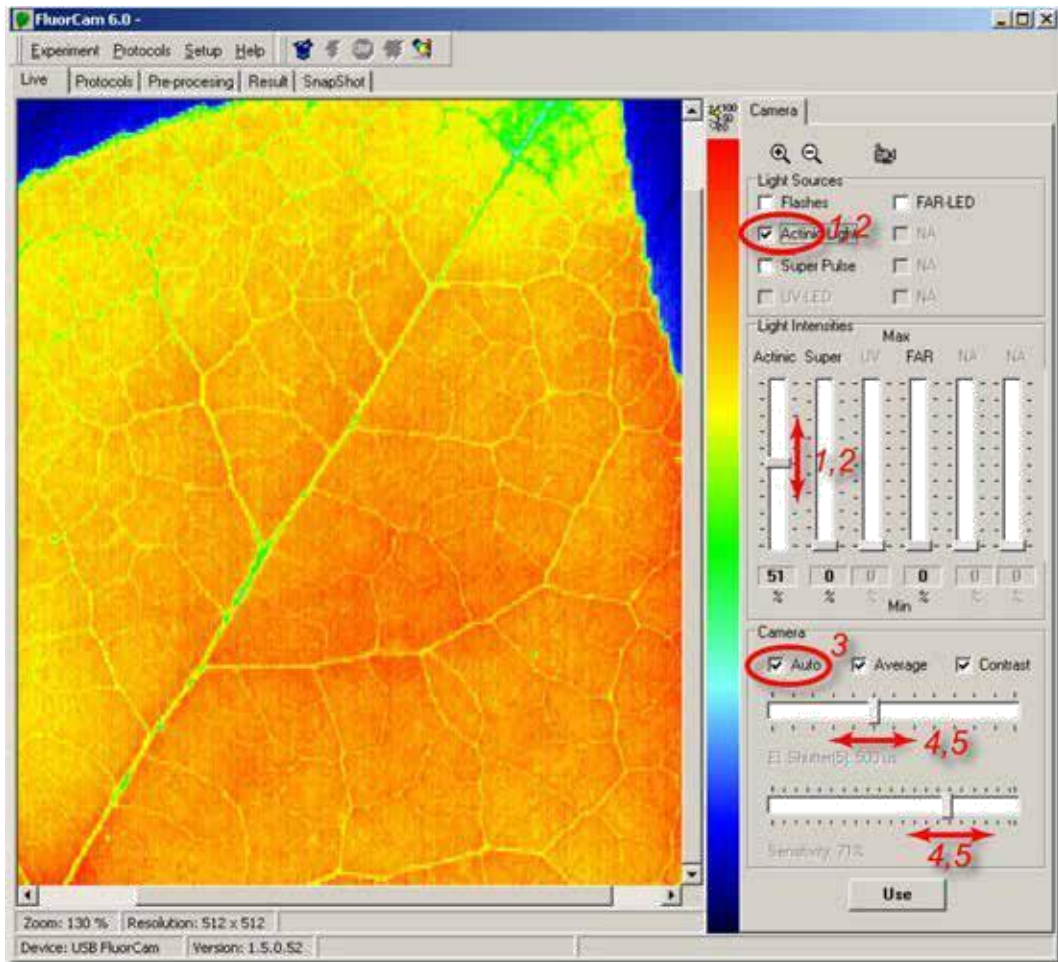


FIGURE 26. Testing continuous actinic light mode with the live FluorCam window. The actinic light is switched on and off, and its power is set by the controls (1) and (2). In the 'Auto' mode, the electronic shutter and the camera sensitivity are adjusted automatically. The Auto mode can be switched off (3) and the shutter and sensitivity can be adjusted manually using the controls (4) and (5).

Keep in mind that there are different FluorCam device versions. In some of them, certain software elements may stay inactive, e.g., 'UV' or 'UV/LED' are inactive here in the fields 'Light Sources' or 'Light Intensities'. These features depend on the actual device configuration and included options.

V.C.3 Live Imaging with Excitation by Measuring Flashes

The live window can also be used to test the PAM mode of the instrument. In this mode, the measured fluorescence that is excited by very short flashes of light can be discriminated from fluorescence due to saturating pulses, or due to continuous light. Also, the signal originating from ambient light that has the same wavelength as the chlorophyll fluorescence can be subtracted. The operation controls are shown in *FIGURE 27*:

The PAM mode can be tested by **switching on the flashes** with auto mode on - (1) and (2) in *FIGURE 27*.

The auto mode is switched off thereafter - (2) in *FIGURE 27*.

Shutter opening time and **camera sensitivity** can be adjusted by the two bars in the right bottom part of the window - (3) and (4) in *FIGURE 27*.

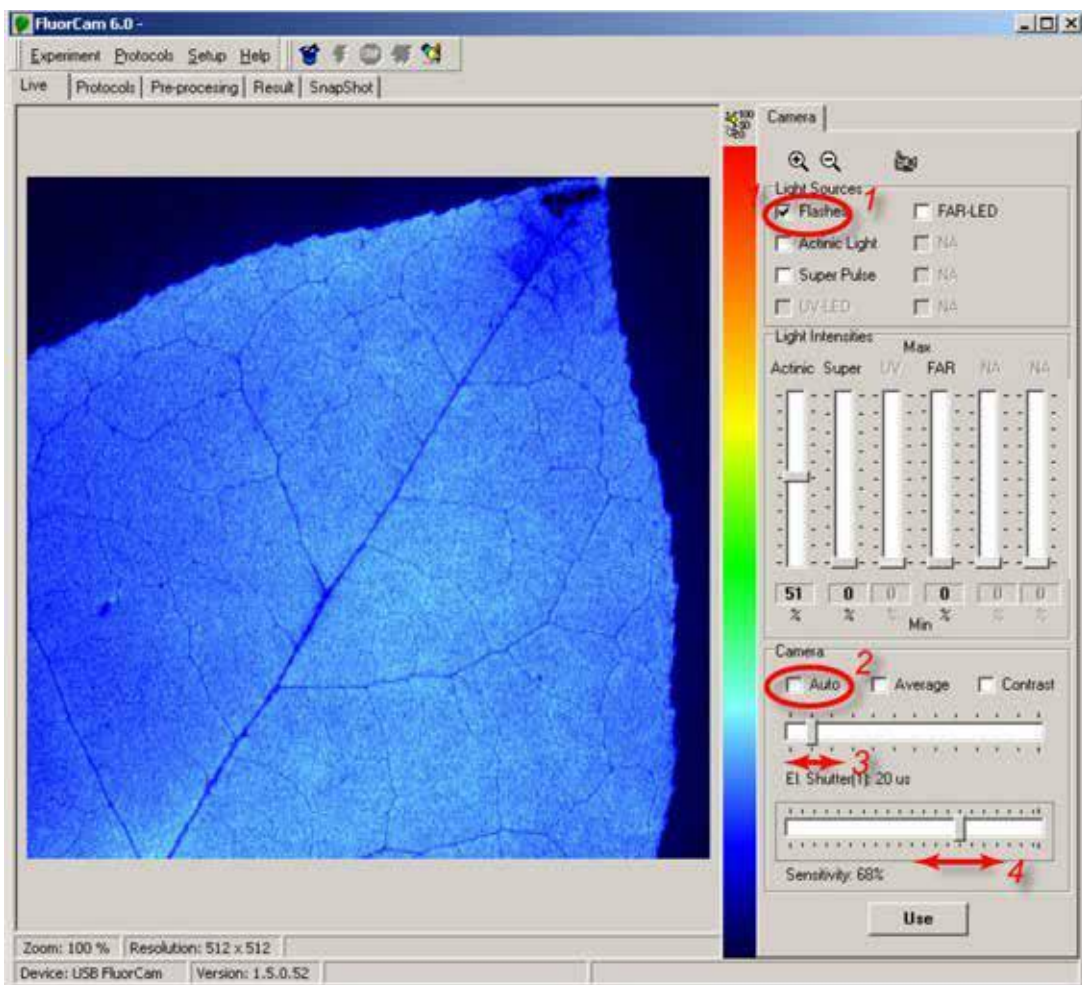


FIGURE 27. Live imaging in PAM mode with measuring flashes. The flashes are switched on by checking (1). The auto mode automatically adjusts the shutter and sensitivity to yield optimum signal. The auto mode can be switched off by (2). With the auto mode off, the shutter and sensitivity can be adjusted manually by (3) and (4). Please, note that the shutter opening time controls also duration of the measuring flashes.

The shutter opening controls also the **duration of the measuring flashes** and that cannot be longer than 20 ms. 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 by 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).

V.C.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) in *FIGURE 28*.

Observe the Test the effects of changing actinic light intensity. Kautsky effect on the computer screen. Switch the actinic light off.

In the hardware versions containing the super pulse feature, set the **pulse intensity to ca. 50%** and **activate the super pulse** - (2) in *FIGURE 28*. Observe the transition from F_0 to F_M and back on the computer screen. Repeat with changing super pulse intensity.

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** - (4) in *FIGURE 28*. 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 (low but visible on the screen). Contrast of the images will be low. To increase the contrast, **switch on the contrast mode** - (5) in *FIGURE 28*. Note the improvement of the contrast that is achieved by re-scaling of the image window from 0-to-4096 (65 536).

The FluorCam is typically operated using a protocol. The protocol specifies intensity setting of an experiment. One can find the optimum setting in the live window and **transmit this setting to the protocol**. Keep in mind that it is necessary to confirm the choice of protocol - (6) in *FIGURE 28*.

Fluorescence transients are recorded using protocol specifications. As an alternative, a fluorescence image can be recorded also in live mode by clicking the **snapshot** icon - (7) in *FIGURE 28*.

The FluorCam **device ID** and **hardware version** are automatically recognized - (8) and (9) in *FIGURE 28*. According to your particular device ID and hardware version, certain software elements may stay inactive, for instance, in the 'Light Sources' or 'Light Intensities' fields.

Any signal exceeding the maximum CCD well capacity (4096 levels) is indicated by a warning - (10) in *FIGURE 28*. This is especially important when using a combination of measuring flashes with strong actinic light and strong super pulse intensities that can generate **background signal** comparable to fluorescence. The CCD capacity can be exceeded by such a strong background 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.

The picture may be magnified or de-magnified and the resolution is also displayed – (11) and (12) in *FIGURE 28*.

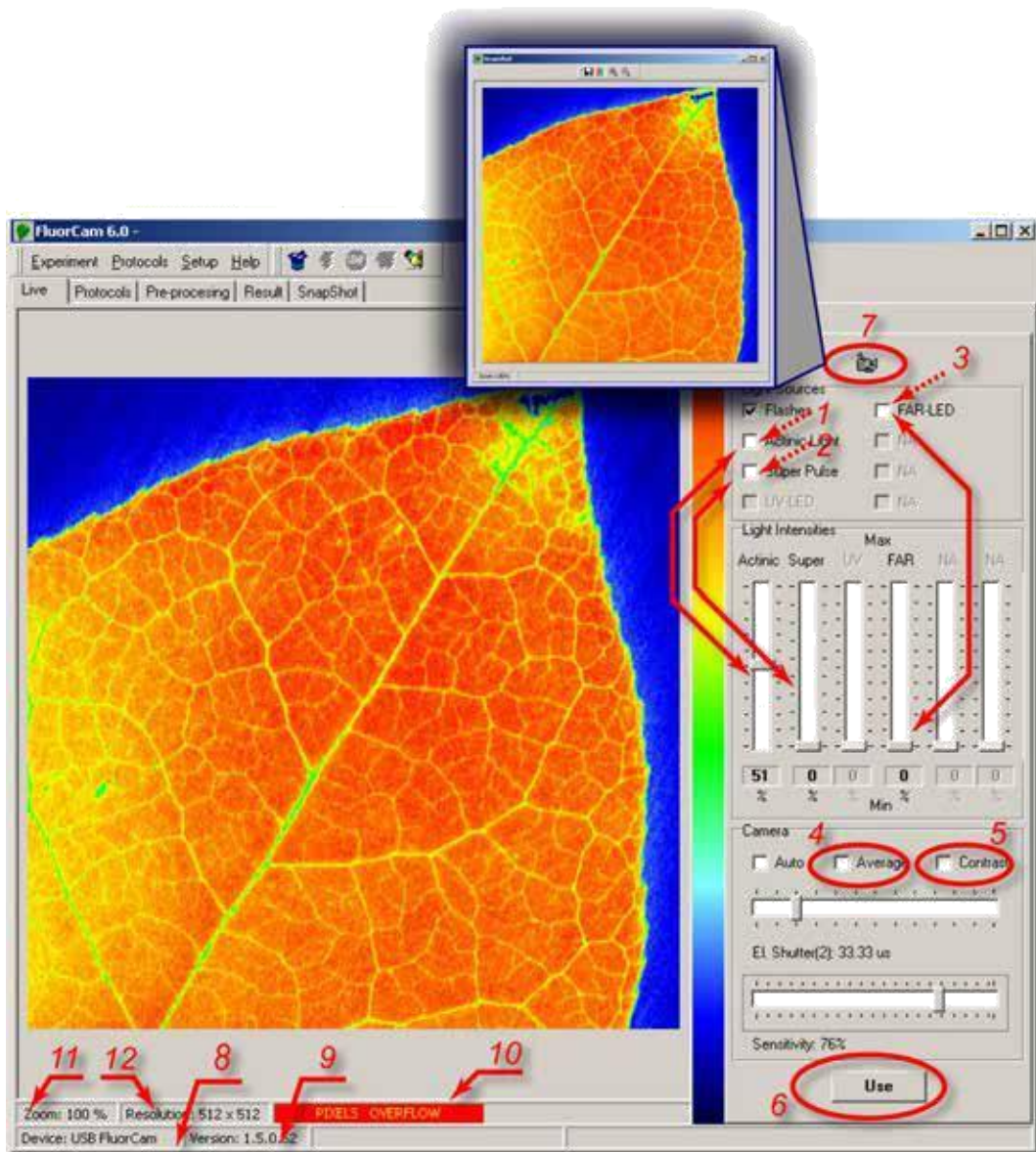


FIGURE 28. More features in the Live window: The actinic light is switched on and off and its intensity is set by (1). The super pulse is triggered and its intensity is set by (2). The far-red light is switched on and off and its intensity is set by (3). The average mode and the contrast modes are controlled by (4) and (5). The setting of the live window can be transmitted to the protocol settings (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). Any signal exceeding the maximum CCD well capacity (4096 levels) is indicated by (10). The picture may be zoomed in/out (11) and its resolution is also displayed (12).

V.D. Designing a Protocol to Capture Fluorescence Transients

The **protocol** is a list of instructions by which the user specifies what, and when, the FluorCam instrument does during a measurement. Chapter V.C.1 depicts how to use the protocols that are part of the installed software. These protocols have been pre -designed for the user's convenience. We recommend that the users who start working with the FluorCam device use these pre-designed protocols first.

More advanced users of the FluorCam device may design their own protocols. Basically, there are three modes of designing experimental protocols. All three methods are described in detail in chapters V.C.2 – V.C.4.

V.D.1 Protocols à la Carte.

First click the wizard icon - (1) in FIGURE 29. This opens the Protocol Menu. Make your choice by clicking the selected protocol or graph window (Wizard) – (2) and (3) in FIGURE 29.

Upon clicking the selected name, the corresponding protocol is loaded into the **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 intend to modify existing protocols, or create new protocols, you can skip reading the next 3 sections V.C.2 – V.C.4.

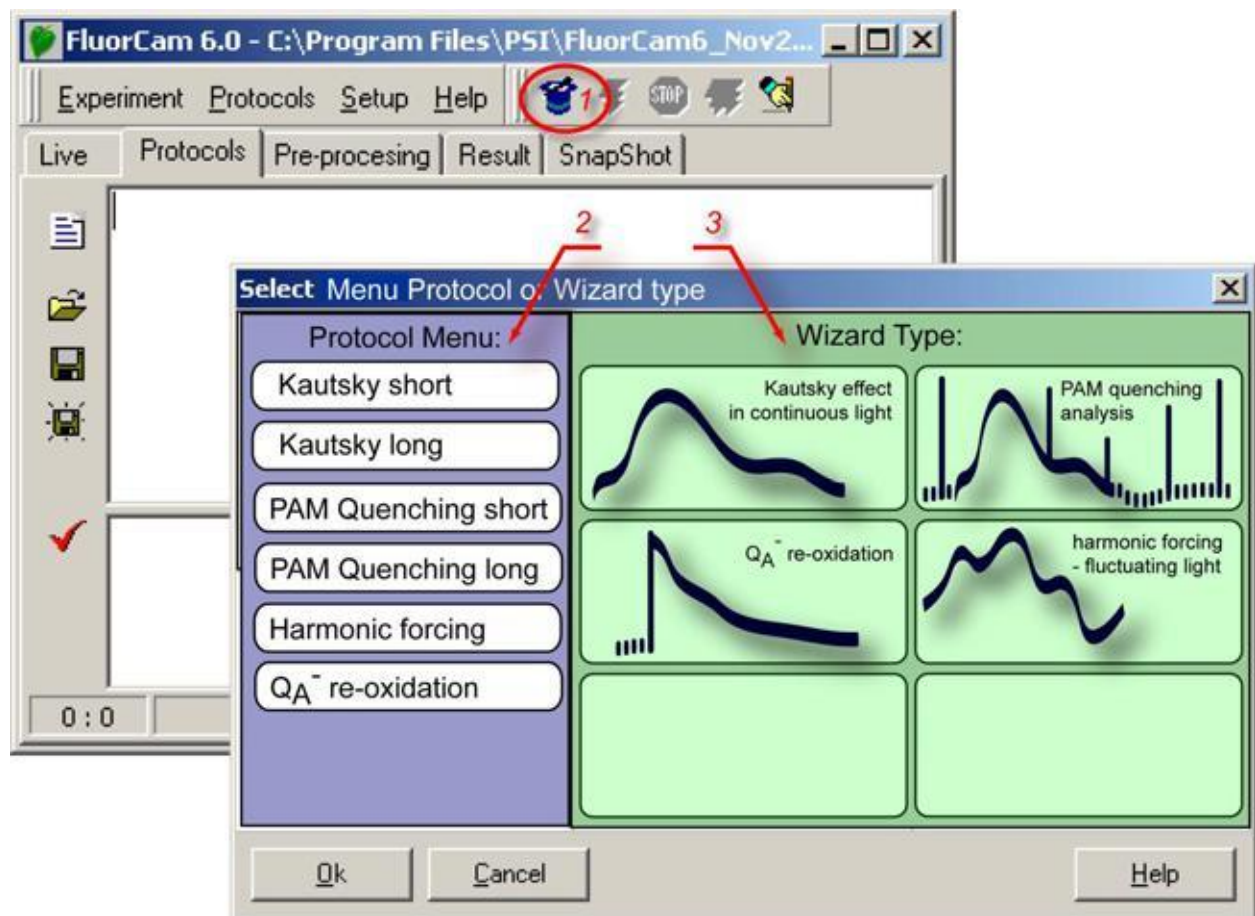


FIGURE 29. Protocol Menu and Wizard window.

* Note: Pre-defined protocols can be subjects of change without notice.

V.D.2 Protocol Template Filled by the Wizard.

The wizard offers flexibility in designing protocols without requiring that the user has expertise in the protocol syntax. This 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 the wizard template or by modifying an existing protocol (see V.C.3).

(I.) The wizard that constructs protocols for **Kautsky effect measurements** (*FIGURE 30B*) in continuous light 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.

(II.) The wizard for **PAM quenching analysis** (*FIGURE 30A*) 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;
- ‘F0 duration’ defines (in seconds) the period over which the *F0* measurement is repeated once per second.
- FM ‘Pulse duration’ defines (in milliseconds) the duration of the flash that transiently reduces the plastoquinone pool and the QA acceptor.
- FM ‘Pulse intensity’ sets relative power of the plastoquinone / QA reducing flash.
- ‘Dark relaxation period after Fm’ defines (in seconds) duration of the dark relaxation following the FM measurement.
- ‘Actinic light exposure’ determines (in seconds) duration of the period in which the sample is exposed to actinic light.
- ‘Actinic light intensity’ determines (in percent) the relative power of the actinic light.
- ‘Number of pulses’ defines the number of saturating flashes, and corresponding number of 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 corresponding number of Fm’ measurements, during the dark relaxation following the Kautsky effect measurement.

(III.) The wizard constructing protocols for **QA re-oxidation kinetics** (*FIGURE 30C*) requires the following parameters:

- ‘Dark interval separating ST flashes’ defines the period of 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 a logarithmic time scale. Keep in mind that re-oxidation kinetics are 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 a dark interval of 5 s separating the measurements, 4 data points per decade and 4 decades of time scale covered (100 μ s, 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 the period of the single-turnover flash. The flash should be short to prevent double-turnovers. At the same time, the flash should be saturating. The saturation requires high power and long duration. A compromise between the two requirements must be found.

FIGURE 30. Wizard windows. (A) The PAM quenching analysis consists of three phases: measurement of dark-adapted levels F_0 and F_M , measurement of the Kautsky effect and of non-photochemical quenching in the light.

(B) 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.

(C) The Q_A re-oxidation kinetics is measured in pump-and-probe with short saturating flashes repeated at a low frequency and with measurements taking place with a pre-defined delay after the Q_A reducing flash

V.D.3 Modification of the Menu and Wizard Protocols

The pre-designed protocols offered in the menu, or the protocols generated by the wizard, are displayed in the protocol window in the form of a text file that can be edited (FIGURE 31). The protocols can be saved under their current name (see (3) in FIGURE 31) or saved under a new name -

(4) in FIGURE 31. The syntax of the modified protocol can be checked - (9) in FIGURE 31. The FluorCam program displays potential warnings and errors revealed by syntax check in a separate text window - (10) in FIGURE 31. Several lines of a protocol source code that are frequently and easily modified are shown in FIGURE 31 (5-8).

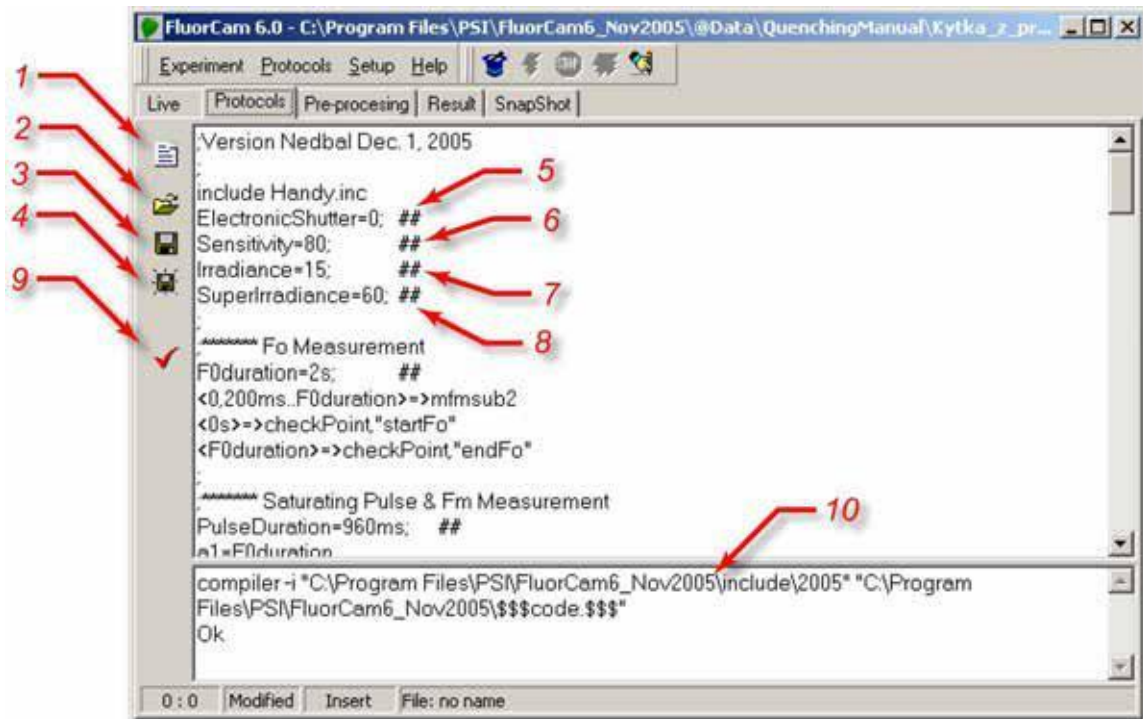


FIGURE 31. Protocol window. (1) New protocol icon erases the protocol memory. (2) Open an existing protocol.

(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 checks of the protocol source file and protocol compilation. (10) Syntax check report with identification of potential warnings and errors.

V.D.4 Designing 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. 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 that covered by wizards. Full versatility of the hardware can be exploited by user-designed protocols. The protocol syntax is explained here using the protocol for quenching analysis offered in the protocol menu.

The protocol starts with the header section

```

;Quenchin analysis Dec. 1, 2005
include default.inc
include Handy.inc
ElectronicShutter=0; ##
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 during which the electronic shutter is 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 a string (##), which is a comment ignored by the compiler, and that serves to mark an easily modified line. 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 μ s	20 μ s	33.3 μ s	100 μ s	250 μ s	500 μ s	1 ms	2 ms	4 ms	6.3 ms	10 ms	16.7 ms	20 ms

The sensitivity of 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 lead to a relatively low signal-to-noise ratio.

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 with any of the widely available PhaR meters. Absolute irradiance is determined by the actinic light settings, 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 as well as 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 ($F_0\text{duration}=2\text{s}$). The next line ($\langle 0,200\text{ms}..F_0\text{duration}\rangle\Rightarrow\text{mfmsub2}$) determines the F_0 measurement sequence.

$\langle 0,200\text{ms}..F_0\text{duration}\rangle$ defines the time series that is interpreted by the compiler using the following syntax elements: Parenthesis $\langle \rangle$ represent linear scale of the timing. The first event of $\langle t_1, t_2, \dots, t_N \rangle$ 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 \Rightarrow 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. A second image is taken 40 ms later synchronous with a measuring flash. The signal in the second image consists of both the background signal and the fluorescence signal excited by the measuring flash. By subtracting the first image from the second image subtracts the background and obtains the net fluorescence signal. The technique requires that the background is not changing on the time scale of tens of milliseconds (avoid measuring in bright fluorescent light). Also, neither of the two measurements should reach saturation (4096 or 65 536).

The next two lines determine that all the images taken between $\langle 0\text{s} \rangle$ and $\langle F_0\text{duration} \rangle$ can be jointly addressed under the fluorescence feature name "Fo". The images, under this name, can be 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, a_1 renames the end of the previous series, and a_2 defines the start of the F_M measurement.

The SuperPulse is triggered ($\Rightarrow\text{SatPulse}(\text{PulseDuration})$) 40ms after the end of the preceding series $\langle a_1+40\text{ms} \rangle$. The Pulse is on for an interval specified in PulseDuration . The actinic light is switched on in parallel ($\langle a_1+40\text{ms} \rangle\Rightarrow\text{actinlightOn}(\text{PulseDuration})$). The first measurement is taken 120 ms after the saturating flash is triggered ($\langle a_2,a_2+160\text{ms}..a_1+\text{PulseDuration} \rangle\Rightarrow\text{mfmsub2}$). 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 a_2 ($= a_1+160\text{ms}$) and $a_1+\text{PulseDuration}$ to a single collective name of the fluorescence parameter - F_m .

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 the start of the measurement of 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 measurement when the sample is still in the 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)

```

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 on 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 (*FIGURE 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"
```

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
```



```

gg3+FRPeriod=>checkPoint,"endFo_D2"
.^AAAAAAAAAAAAAAAAAAAA
,
;***** 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"
.^AAAAAAAAAAAAAAAAAAAA
,
;***** 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 *****

```


V.E. Experiment and Data Pre-Processing

The experiment encoded in the protocol is executed upon clicking the trigger - (1) in FIGURE 32. 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 FIGURE 32².

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 FIGURE 32), or manually by the bar code reader activation (4 in FIGURE 32).



FIGURE 32. 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.

V.E.1 Experiment

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

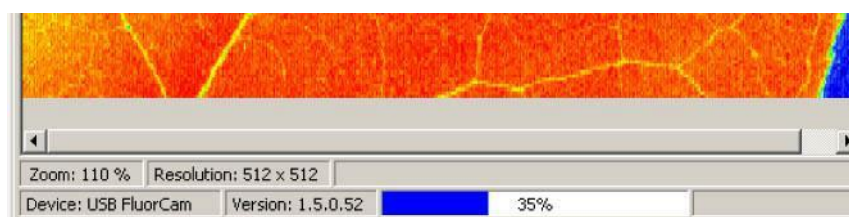


FIGURE 33. The bottom bar shows (in percent) the progress of the experiment.

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

V.E.2 V.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 a 'psitemp' folder. Simple automatic image segmentation is proposed by the FluorCam software to separate the image regions with a fluorescence signal from the dark background. This segmentation is only tentative and can fail, for example, when there is no background or when the signal is too low.

FIGURE 34 shows the FluorCam window offering more pre-processing features. The green area in the image indicates the region-of-interest that is distinct from the background - (1) in FIGURE 34. The signal integrated over all pixels of this region is represented in the lower panel by the green kinetic trace³. Using the computer mouse, one can place a cross cursor anywhere in the image window, and the fluorescence transient corresponding to the pixel selected by the cursor is shown by the red kinetic trace - (2) in FIGURE 34.

The displayed image can be changed by sliding the cursor along the time axis - (3) in FIGURE 34. By clicking the view icon one can toggle between the 'show-all', 'show-image', and 'show-kinetics' display modes - (4) in FIGURE 34.

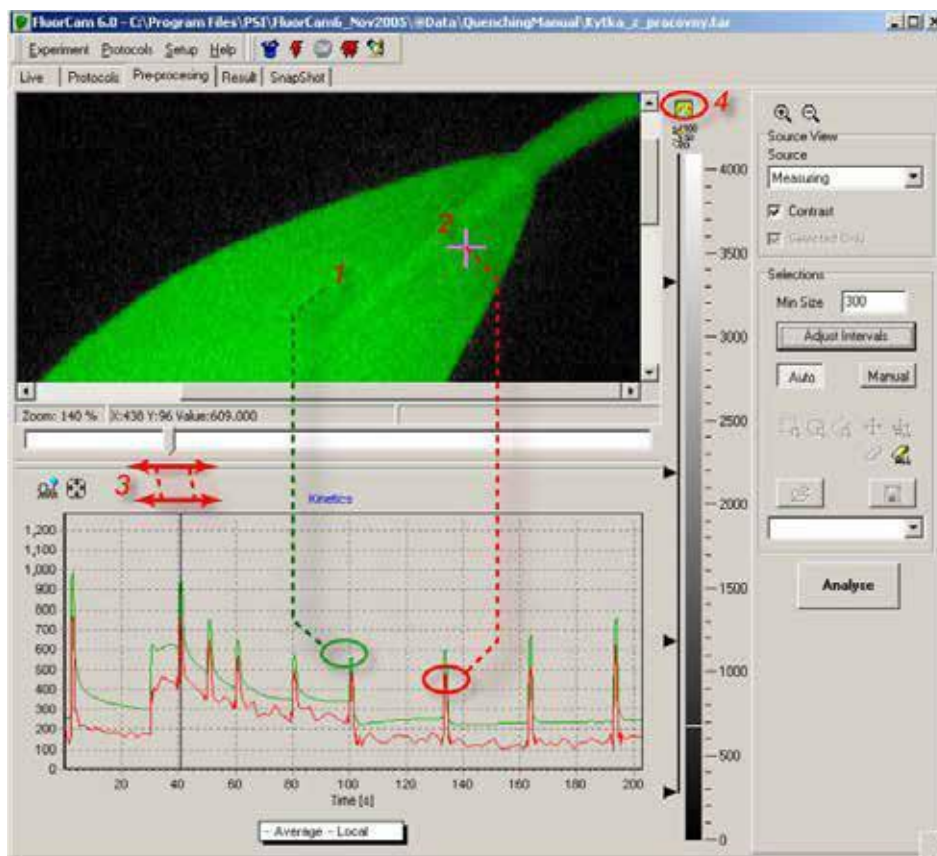


FIGURE 34. The pre-processing window shows a tentatively image segmented fluorescence image in the top panel. The green shading shows the region-of-interest that most likely represents the fluorescing object. The surrounding area is tentatively identified as background. The signal in the region-of-interest 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 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).

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

FIGURE 35 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 FIGURE 35. The scale bar can be shown with or without the numeric scale - (2) in FIGURE 35. The segmentation of the image can be assisted by the four scale dividers - (3) in FIGURE 35 - 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 FIGURE 35. This corresponds to the black segment of the image window showing the leaf background. By moving the scale divider from 300 to lower values, pixels in the background will start to be included in the image processing and averaging. The image that is used for segmentation can be any frame that is selected by the cursor from the measured sequence - (3) in FIGURE 34, 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 FIGURE 35. 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).

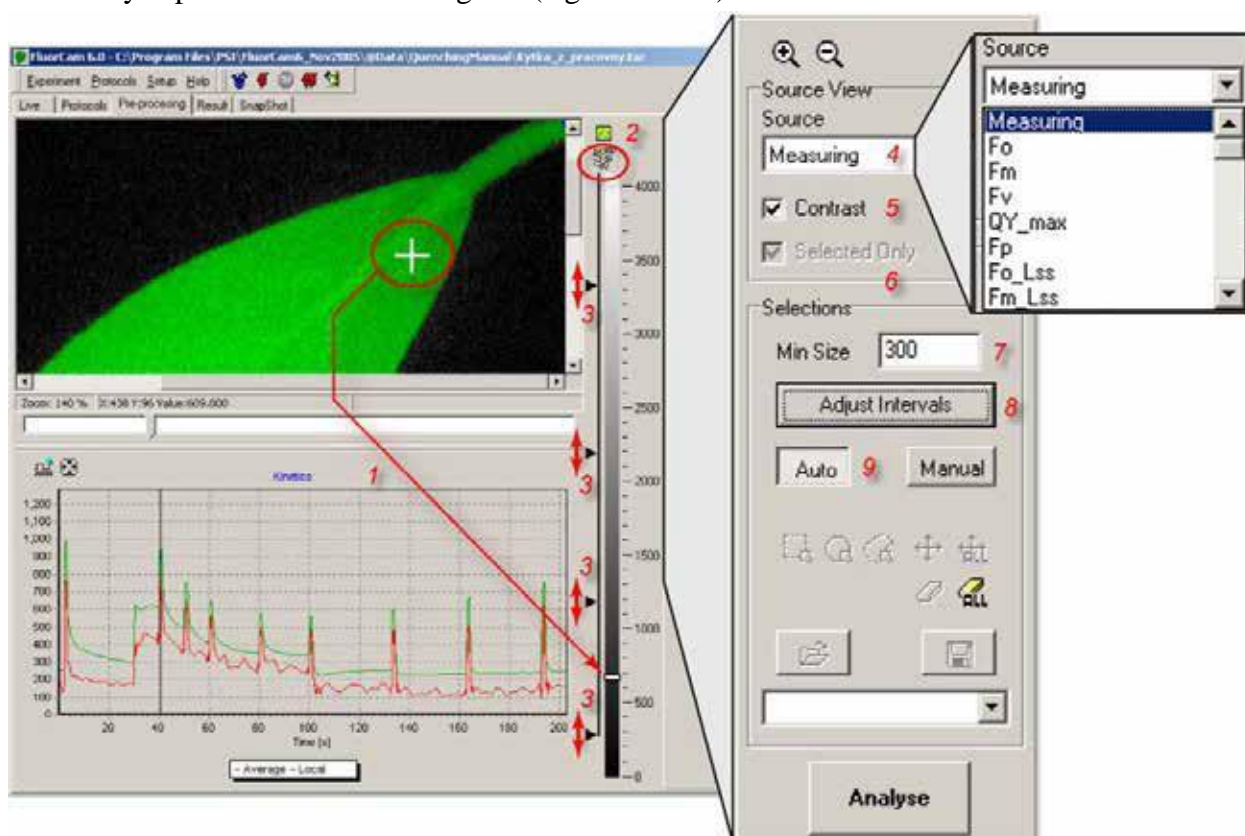


FIGURE 35. More pre-processing tools. The signal at the position of the cross cursor (1) is shown by the indicator on the scale bar (1 arrow). The scale bar can be displayed with or without a numeric scale (2). A finer segmentation of the image is possible by using and moving the scale dividers (3, see 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 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 the 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).

Sometimes (e.g. with F_0), the measured image can be too dim for segmentation. Then, one can use Contrast to re-scale the signal between the maximum and the minimum signal levels - (5) in FIGURE 35.

The 'Selected Only' field is active for secondary image segmentation - (6) in FIGURE 35. One can, for example, use one fluorescence parameter to define the primary region-of-interest, and perform secondary image segmentation only within this primary region-of-interest using another fluorescence parameter.

The 'Minimum Size' field limits the size of the regions-of-interest that are included in the image processing - (7) in FIGURE 35. By setting this value at a low level one can include small objects but can, unintentionally, also include small groups of pixels that are separated by the artifact of a noisy signal.

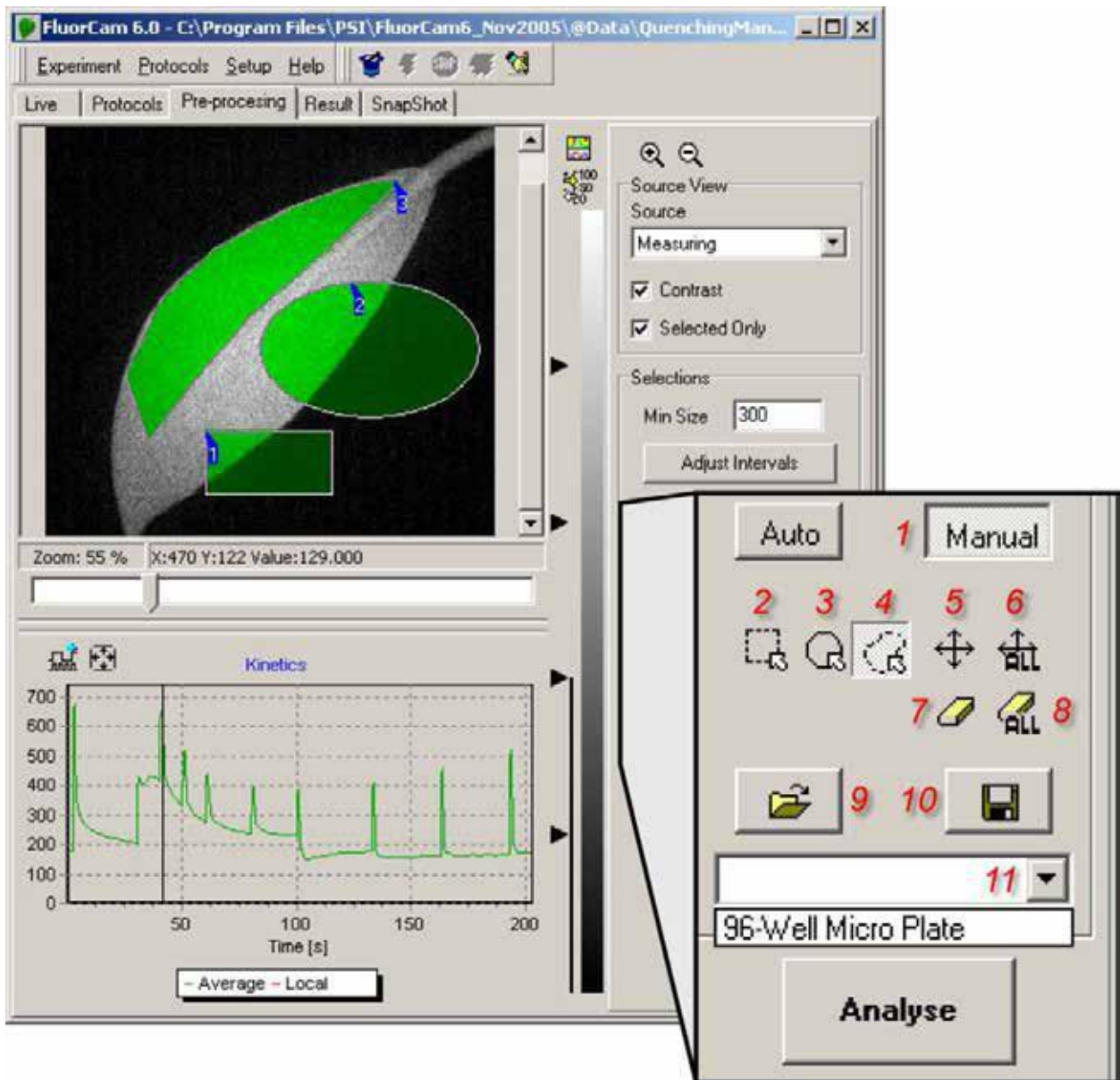


FIGURE 36. The manual image segmentation is selected by (1). The segments are rectangular (2), elliptic (3) or polygonal (4). The segments can be moved individually (5) or in a group (6). The segments can be erased by (7) and (8). The segments can be saved (10) and loaded from memory (9 and 11). The signal in the selected regions-of-interest can be integrated by 'Analyse'.

The ‘Background exclusion’ trigger (see (8) in *FIGURE 35*) re-sets the lowest scale divider to a minimum value of the image histogram so that the background can be tentatively de-selected - (3) in *FIGURE 35*.

The ‘Auto’ mode (see (9) in *FIGURE 35*) relies on image segmentation by the scale dividers (see (3) in *FIGURE 35*) and by the ‘Adjust Intervals’ routine.

The ‘Manual’ mode of image segmentation is selected by – (1) in *FIGURE 36*. The regions-of-interest can be square (see (2) in *FIGURE 36*), elliptic (see (3) in *FIGURE 36*), or polygonal (see (4) in *FIGURE 36*).

Individual region of interest can be moved – (5) in *FIGURE 36*. Multiple regions-of-interest can be moved as a group – (6) in *FIGURE 36*. Individual, or all regions-of-interest, can be erased - see (7) and (8) in *FIGURE 36*.

The regions-of-interest can be saved and reloaded - see (10) and (9) in *FIGURE 36*. This is a particularly handy feature in those situations when a reproducible pattern, e.g., of multi-well plates, is repeatedly imaged - (11) in *FIGURE 36*.

The segmentation of the image by scale dividers is explained in detail in *FIGURE 37*.

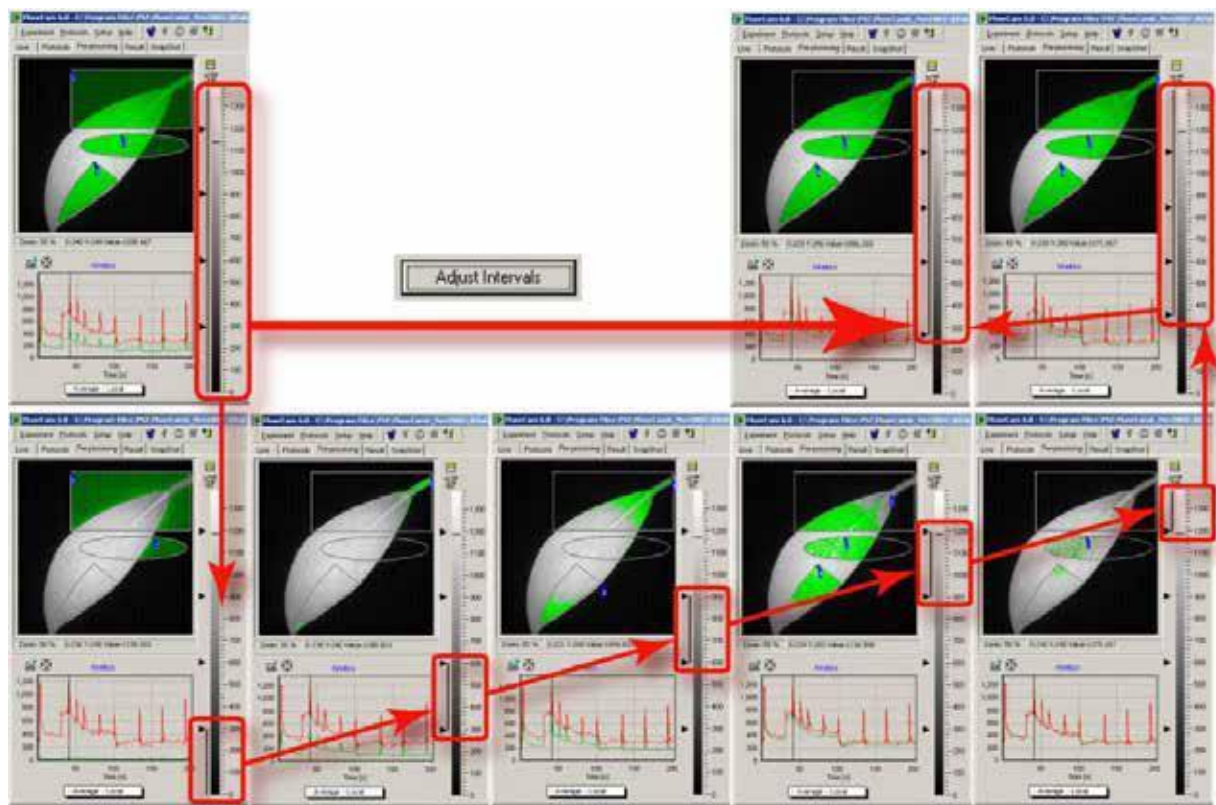


FIGURE 37. Image segmentation by scale dividers. In the top left panel, the space dividers are distributed uniformly from 0 to the maximal signal level, and all five scale ranges are selected as indicated by the vertical line along the scale bar. With the entire signal range selected, the square, elliptic, and polygonal shapes (1- 3) are fully selected, including the segments that show the background. Upon pressing ‘Background Exclusion’, the lowest scale divider is moved to the histogram minimum that typically separates background from the useful signal range (thick red arrow). The function of the scale dividers can be demonstrated by sequentially 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 the 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.

V.F. Results Analysis

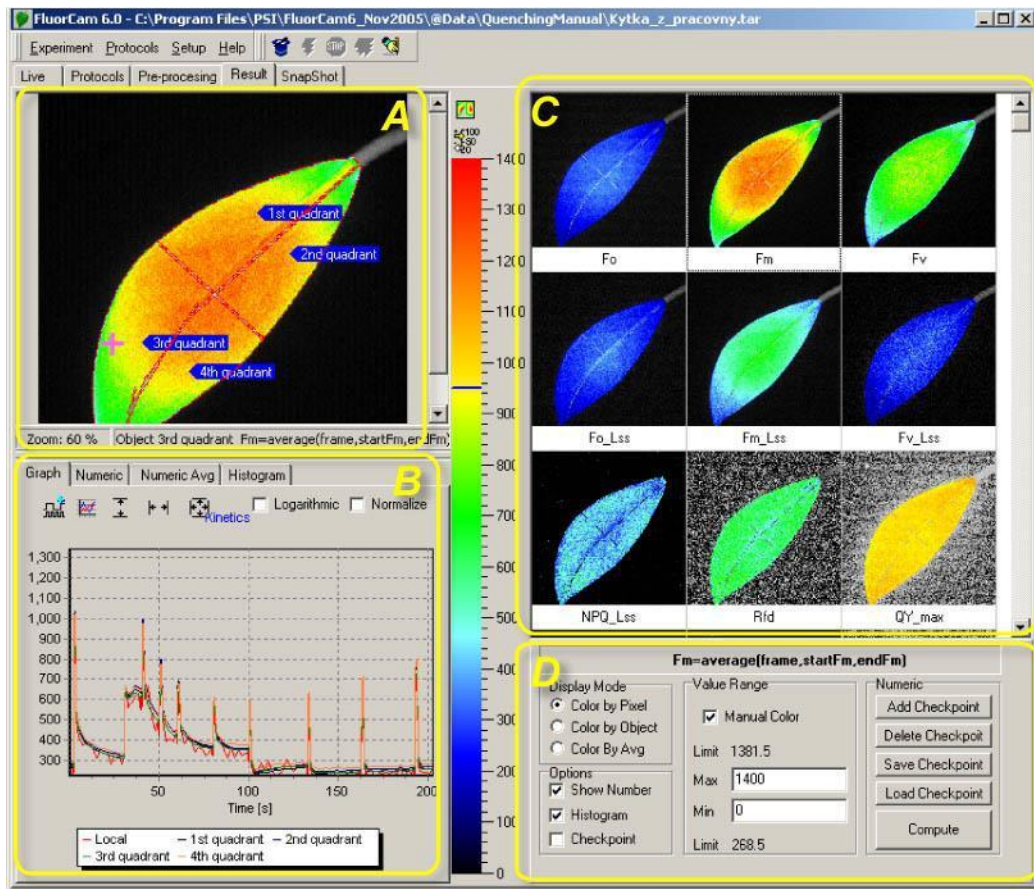


FIGURE 38. The Results window consists of four panels (marked by yellow color). Panel A represents spatial dimensions by one image of the fluorescence transient sequence. Panel B shows kinetics of the fluorescence signals averaged over the image segments that were selected prior to analysis in the Pre-processing window and that 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. 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 selected, and about the image that is selected by clicking on any of the frames in 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.

V.F.1 Spatial Heterogeneity of the Measured and Calculated Parameters

The Results window (FIGURE 38) shows, in panel C, all images that are calculated by analysis of the pre-processing window (FIGURE 36). These images are directly captured and averaged frames (e.g., F_0 , F_M , F_t) or they are calculated from the captured frames (e.g., $F_V = F_M - F_0$, $NPQ = (F_M - F_0) / F_M$). Any of the images in 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 actions made in the pre-processing window (FIGURE 36). The signals from all pixels of each segment are integrated, and the averaged value represents the segment fluorescence at given time point. Time sequences of these fluorescence levels constitute fluorescence transients shown in panel B.

A trace is also shown that represents values of a specific pixel selected by the mouse cursor (the noisy red line in the panel B). Panel D is used to customize visualization of the results.

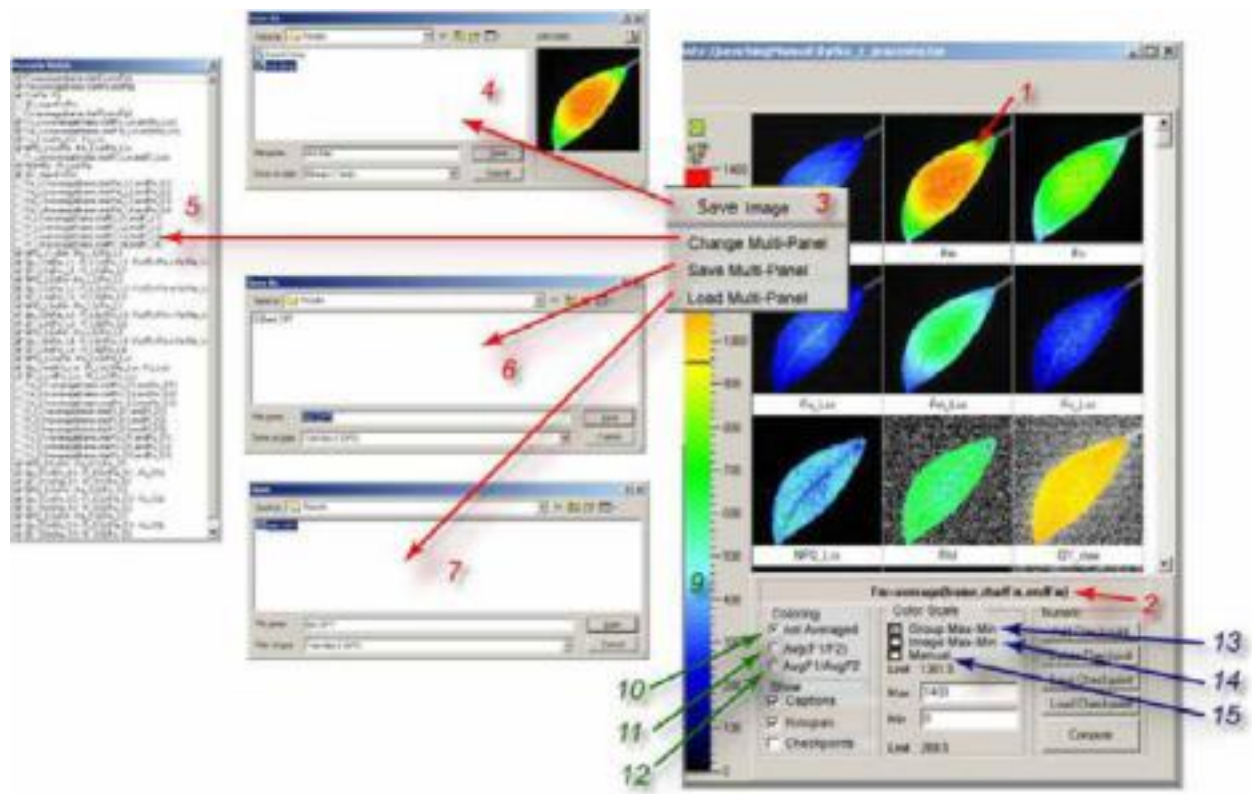


FIGURE 39. Visualization of the spatial heterogeneity of fluorescence emission. Any image from the multi panel window can be selected for closer inspection by left -clicking it (1). The formula by which the selected image is calculated is shown in field (2). Right clicking opens a sub-menu (3). By selecting ‘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). Only a sub-set of the images that are calculated in the analysis window 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 a mode in which the values are calculated for each pixel, and averaging is done after the calculation, e.g., Average (F_V/F_M). By selecting (12), one switches to a mode in which values are first averaged and then used for a calculation, e.g., Average (F_V)/ Average (F_M).

Visual perception of the images is 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 the greatest resolution between maximum and minimum values of the signal. Sometimes it can be advantageous to define the scale limits manually by clicking (15) and typing the chosen minimum and maximum limits.

Saving of a particular image, as well as changing, saving and loading a customized configuration of the multi panel Results window is described in **FIGURE 39** (red numbers and arrows).

Visual presentation can be pixel-by-pixel without any averaging - (10) in **FIGURE 39**, or one can select only the averaged values of measured or calculated signals. When (11) in **FIGURE 39** selected, the software first calculates the requested parameter for each pixel and averages the calculated parameters over the selected segment of the image. For example, assuming that the fluorescence signal in a hypothetical 2-pixel segment is: $F_{01} = 1.5$ and $F_{02} = 0.5$, then $F_{M1} = 2$ and

$F_{M2} = 8$, the parameter defined as $F_{ratio} = (F_M - F_0) / F_M$ is first calculated for each pixel: $F_{1ratio} = (2 - 1.5) / 2 = 0.25$ and $F_{2ratio} = (8 - 0.5) / 8 \approx 0.94$. The average value of F_{ratio} in the 2-pixel segment is, therefore, 0.59. All three pixels are assigned a color that corresponds to 0.59.

The mode selected by (11) in FIGURE 39 yields mathematically correct averages, but has one severe limitation in that the signal-to-noise ratio is low. In some cases, 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 calculated averages by disproportionately large parameter values. This noise artifact is limited in mode (11) in FIGURE 39 by eliminating pixels that would contribute to averaging by a value that is outside of pre-set limits.

Alternatively, the user can avoid this noise artifact by choosing mode (12) in FIGURE 39. In this case, the software first calculates the average values and 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 ($F_{1ratio} = 0.25$, $F_{2ratio} \approx 0.94$) 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 more correct one $Average[(F_M - F_0) / F_0]$ is very low. It is up to the user to choose the best option for a particular experimental situation. With high signal-to-noise ratio it is typically possible to use the mathematically correct mode - (11) in FIGURE 39, whereas in experiments with low signal-to-noise it is certainly better to use mode (12) in FIGURE 39. These two modes are available for the tabulated numerical values of the fluorescence parameters as described in FIGURE 41.

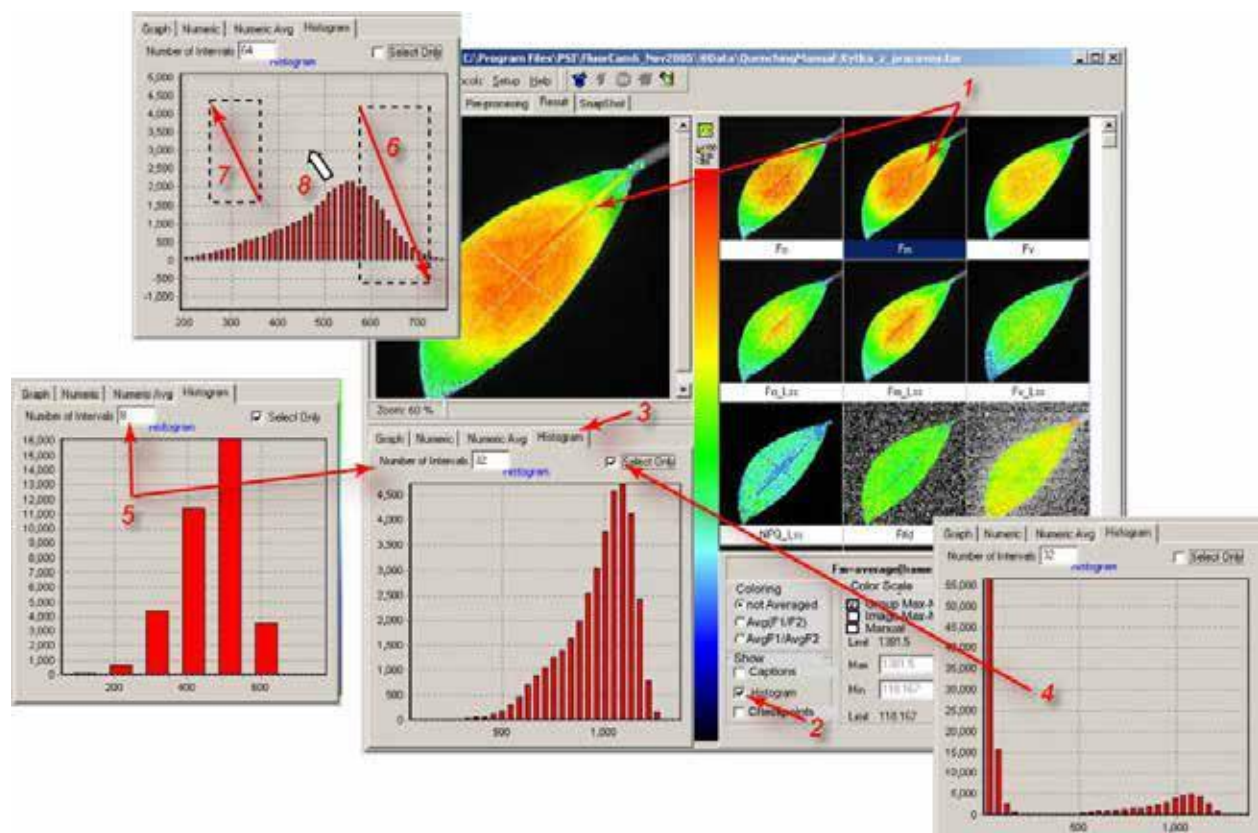


FIGURE 40. Histogram. The histogram of any selected image (1) can be enabled / disabled by (2). If enabled, the window (3) can be opened to see number of pixels corresponding to a given interval of signal levels within the selected segment, or within the entire image (if 4 is not checked). The number of signal level 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 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.

V.F.2 Kinetics of Measured and Calculated Parameters

The kinetics of fluorescence parameters can be qualitatively inspected in the multi-panel window described in *FIGURE 38 and FIGURE 39*. The quantitative assessment of kinetics can be obtained using the Graph window in Results (*FIGURE 40*).

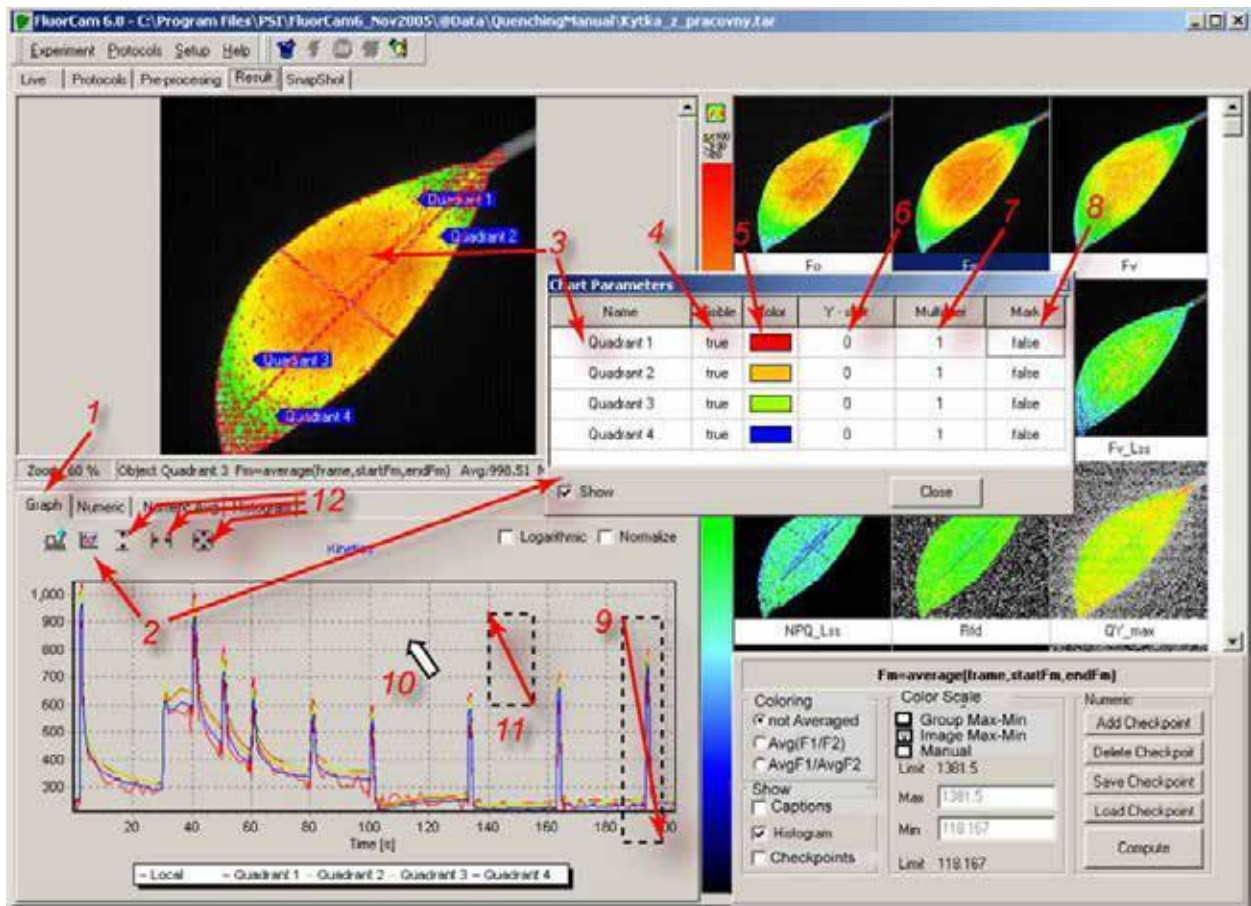


FIGURE 41. Kinetics of the fluorescence transient is displayed by selecting (1). The traces for sample segments defined in the Pre-Processing window are shown in format defined in the Chart Parameters window (2). The caption of individual segments can be modified either by clicking the segment in the 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 and the numerical values of individual data points observed (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 dragging with the right mouse button pressed (10). The graph can be zoomed out by dragging with the left mouse button pressed 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 *FIGURE 40* 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 *FIGURE 41*. The zoomed-in graph can be moved around by dragging with the right mouse button pressed - (10) in *FIGURE 41*. The original show-all zoom can be re-established by dragging with the left button clicked in the bottom-right to top-left direction.

FIGURE 42 demonstrates additional features that are available for kinetic analysis of the fluorescence transients.

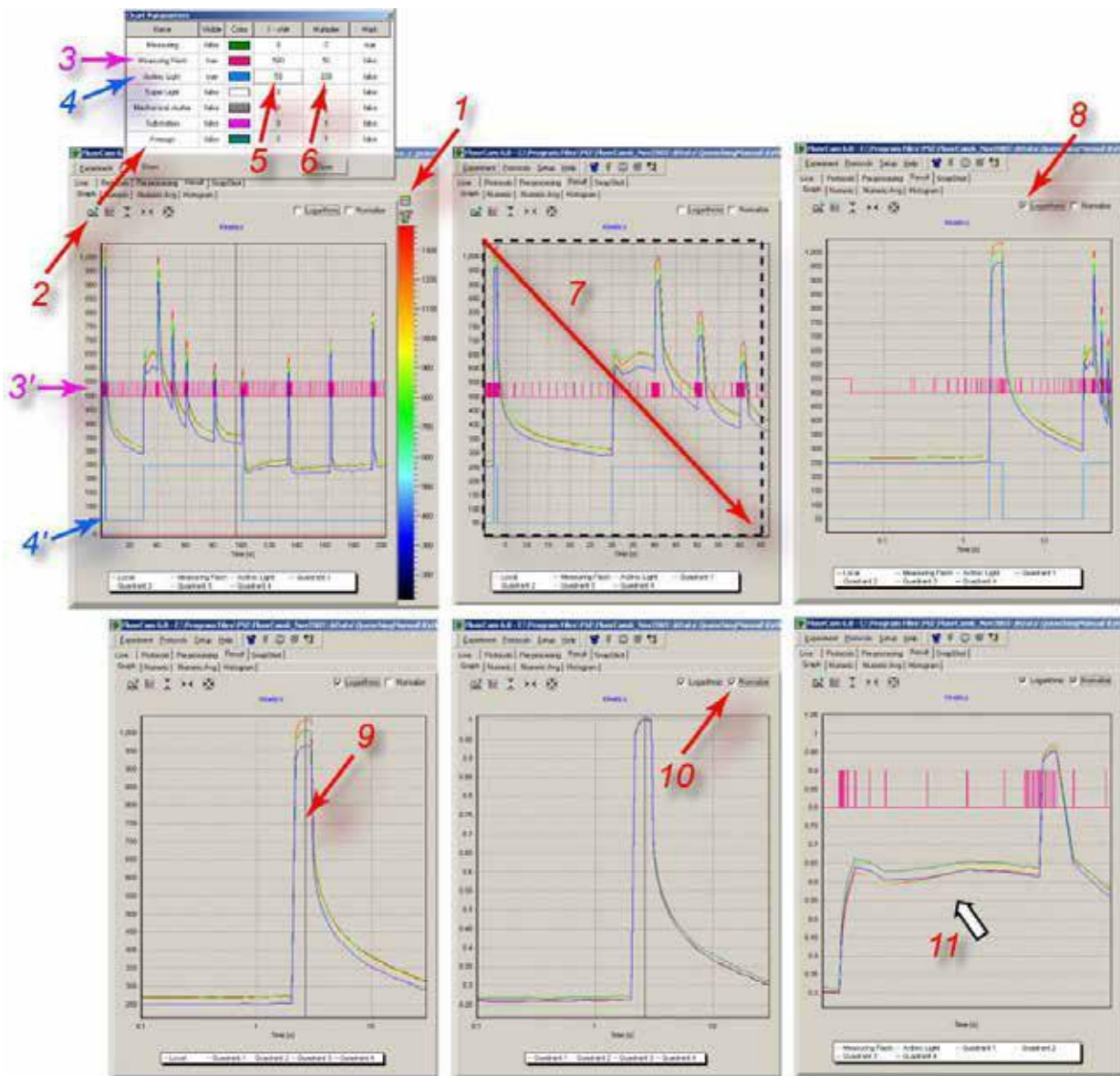


FIGURE 42. Additional visualization features available for kinetic analysis. The panel showing the fluorescence transient can be expanded by (1). By clicking (2), one can open the window to visualize the time course of controlling signals defined in the protocol. The magenta arrow (3) shows the line that denotes 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 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 best visualized using a 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 the right mouse button pressed.

FIGURE 43 shows a zoomed-in detail of a kinetic trace showing the measurement of F_M .

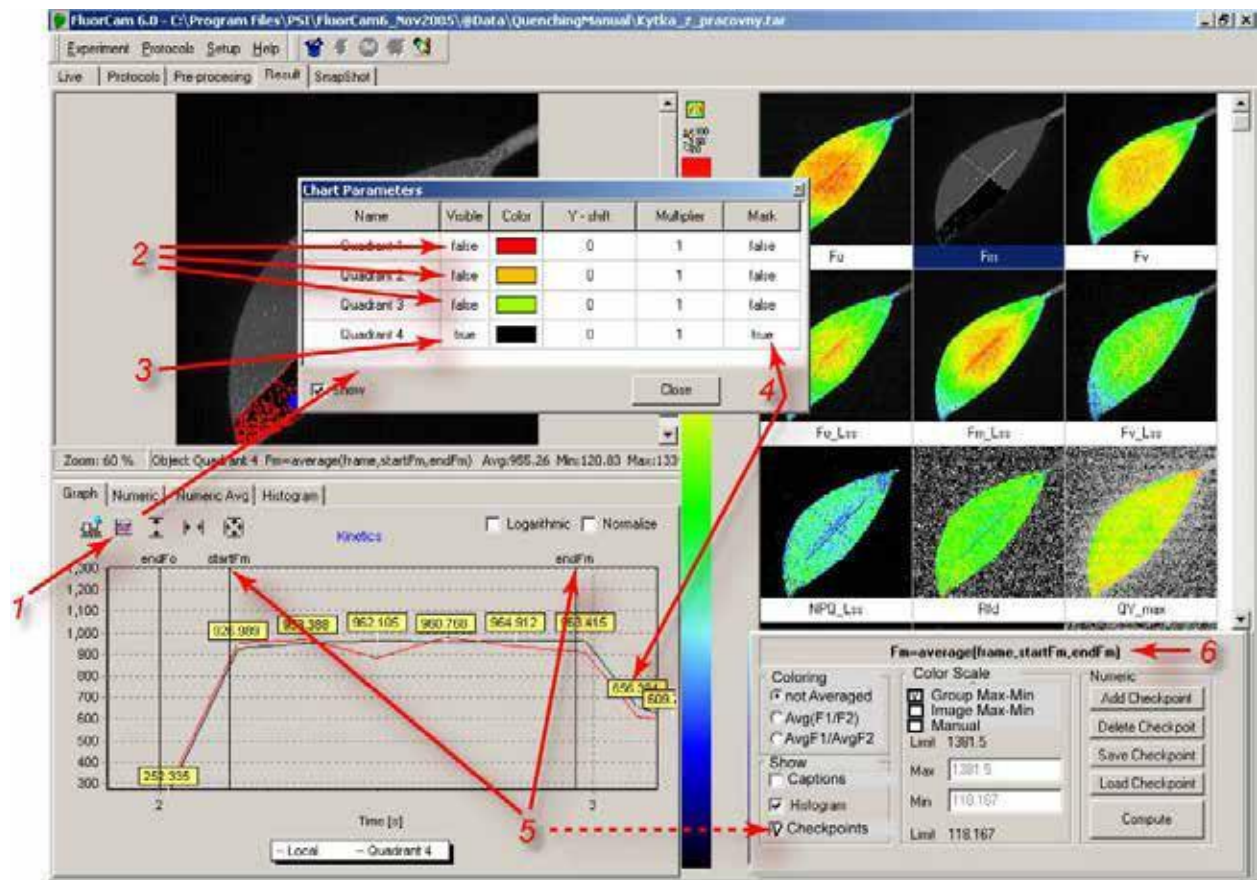


FIGURE 43. The attributes of the zoomed fluorescence transient can be set in the 'Chart Parameters' window (1). The signals of the first 3 segments are hidden (2) and only the fourth transient shown (3). The line color is black. The red noisy line corresponds to a pixel that is selected by the cursor. The black kinetic trace of the fourth segment is shown with marks (4) that indicate 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). These 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.

V.F.3 More on Checkpoints

Checkpoints are marks separating and naming different phases of the fluorescence transient. Paragraph V.C.4 describes how the checkpoints are 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 0 s
- 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 typically, to operate with 2 sets of frames, each limited by *start* and *end* timing.

The use of 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"/>`

b: `<NOEXIST name="endFo"/>`

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

These 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 the "Fo" frame variable with 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, a block of *numerix* file exists:

`<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"/>`

In this way 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 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.

V.G. Snapshot Mode - Imaging of Fluorescence Proteins and Fluorescent Dyes

In addition to ChlF kinetics, FluorCam, in principle, allows to capture images of any fluorescence signal, e.g. blue-green plant auto-fluorescence, fluorescent proteins or samples stained by fluorescent dyes. The system must be equipped with:

- appropriate wavelength of **excitation light**.

Fluorochrome	LED panel type	Excitation wavelenght	Emission wavelenght	Bandpass
DAPI	UV 365 nm	358 nm	461 nm	469/35
	UV 385 nm			
EBFP	UV 385 nm	383 nm	445 nm	469/35
CFP	Royal Blue 450 nm	434 nm	477 nm	500/10
				517/20
EGFP	Royal Blue 450 nm	489 nm	508 nm	517/20
	Blue 470 nm			
YFP	Royal Blue 450 nm	514 nm	527 nm	517/20
	Blue 470 nm			
	Green 530 nm			593/46
DsRed	Green 530 nm	558 nm	583 nm	593/46
ChlF	Royal Blue 450 nm	400-660 nm	650-800 nm	695-770
	Blue 470 nm			
	Orange-red 620 nm			
	White			

- **Filter wheel** with appropriate **emission filters**.
- **High resolution camera**. The most of the fluorescence dyes and fluorescence proteins require longer integration time to acquire reasonable signal, typically hundreds of milliseconds. The high resolution camera allows to integrate from 1ms up to minutes in “Snapshot Measuring Mode”. The disadvantage of the Snapshot mode is the frame rate that is restricted to 2 fps (frame per second) in contrast to the Live Mode (10-50 fps regarding selected image size).

V.G.1 Switching to the Snapshot Mode

Click **Setup** button in the top panel (1 in FIGURE 44) and select **Device** (2). This opens the Setup Menu with the advanced camera settings (3, 4 in FIGURE 44).

a) Resolution:

The dropdown list provides available display resolutions. Various sizes of an image are defined with regard to subwindowing and binning options.

Subwindowing, also known as region of interest (ROI), is the ability of camera to output the smaller image size than the whole image array. Subwindowing is actually a smaller field of view than the maximum resolution available.

Binning is a process of combining charge from adjacent pixels in a CCD during readout. The primary benefit of binning is to improve signal-to-noise ratio (SNR). Moreover, both subwindowing and binning increases the frame rate, albeit at the expense of reduced spatial resolution.

Image size	Subwindowing	Binning	Frame rate
1392 x 1040	full resolution	-	2 fps
1024 x 768	ROI 1024 x 768	-	10 fps
696 x 520	ROI 1024 x 768	2x2	26 fps
464 x 344	ROI 1024 x 768	3x3	20 fps
348 x 256	ROI 1024 x 768	4x4	25 fps

b) Measuring Mode:

Measuring Mode allows choosing between uncompressed image live streaming in Video mode and manual image capture in the Snapshot mode. When changing the mode, be aware of keeping the same image size. The image size can be defined for Video and Snapshot modes independently.

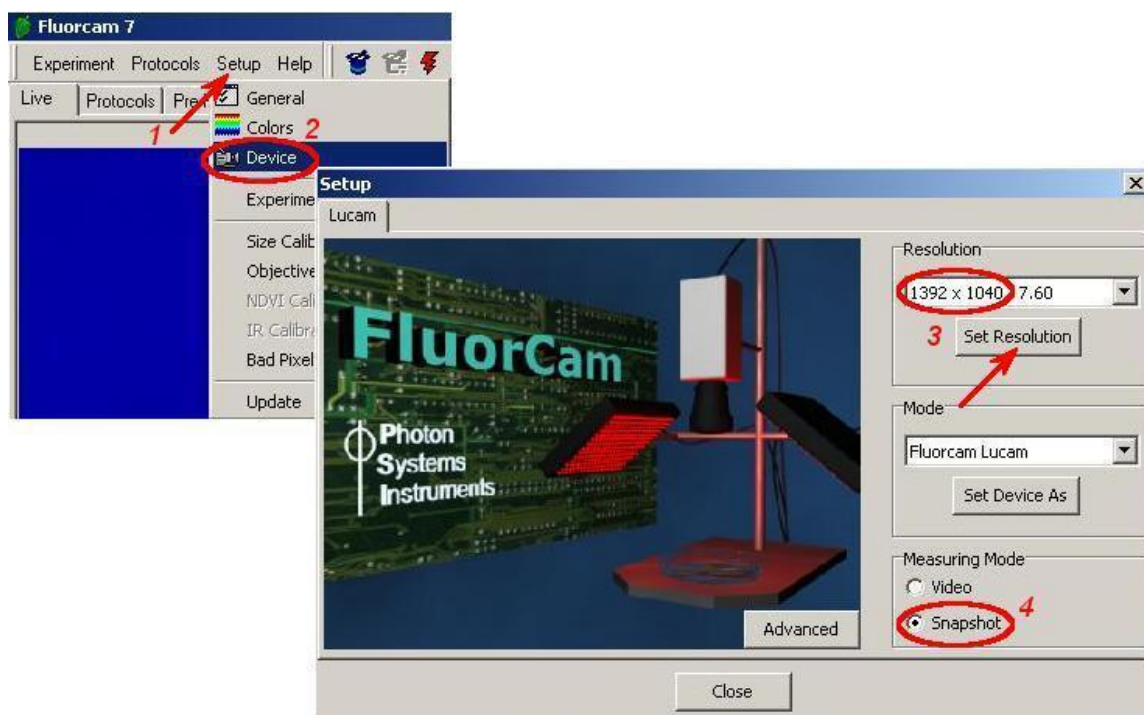


FIGURE 44. Advanced camera control settings, Resolution (3) and Measuring Mode (4) are available through the Setup Menu. To open the Setup Menu, click Setup (1) and Device (2).

V.G.2 Live Imaging in the Snapshot Measuring Mode

The live window in the Snapshot Mode does not show live video streaming. The image is displayed only if it was captured manually by clicking **Get Frame** button (5 in FIGURE 45).

The major advantage of the snapshot mode is “unlimited” shutter opening time (camera integration time, 4 in FIGURE 45) that can be defined from 1 millisecond up to minutes.

In the snapshot mode, it is not recommended to apply Average filtering on displayed images. Average filter averages 3 consecutively captured frames together to reduce random noise. However, this option will produce undesirable results when the field of view contains object in motion or fluorescence signal changes in time or the camera settings of 3 consecutive images were not kept the same.

In contrast to ChlF, the fluorescent signal corresponding to the blue-green plant auto-fluorescence, or fluorescent proteins and dyes, is static. These signals are not measured in the PAM mode using measuring pulses, but simply under the continuous illumination. So, the scattered ambient light interferes with this measurement. It is recommended to place the equipment to a dark room or pre-darkening box.

In most FluorCam setups, blue Actinic light 2 (1, 2 in FIGURE 45) is used to excite green fluorescence emission of GFP.

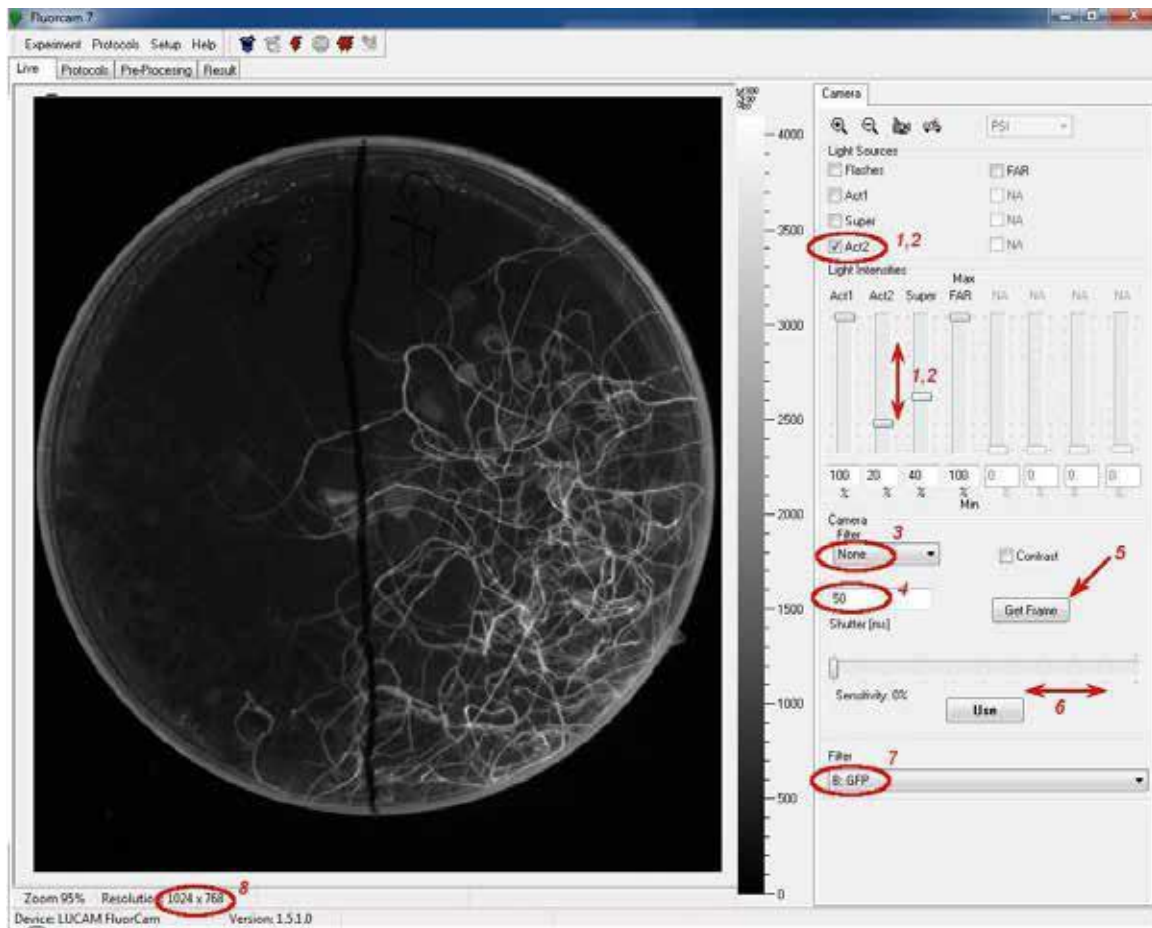


FIGURE 45. Live FluorCam window in Snapshot Measuring Mode: The actinic light is switched on and off, and its power is set by the controls (1) and (2). The camera Filter (3): None – raw data are displayed in the live window, Average – averages of 3 images captured consecutively. (4) Camera integration time can be defined from 1 up to thousand milliseconds. (5) Image is captured and displayed in live window only if clicking Get Frame button. (6) Sensitivity of the camera can be adjusted manually using the control bar. (7) The dropdown list shows emission filters available in the filter-wheel. (8) Actual size of the displayed image.

V.G.3 Taking Snapshot

In snapshot mode, a fluorescence image can be recorded in live window by clicking the **snapshot** icon - (1) in FIGURE 46. The snapshot image is displayed in a separate window together with false color scale (2), (3) in FIGURE 46. Such view can be saved as it is by clicking “Save” button in FIGURE 46. The image is saved in *.bmp format. The raw data can be “Exported” in *.fcimg file format which can be handled and processed in other image processing programs such as ImageJ.

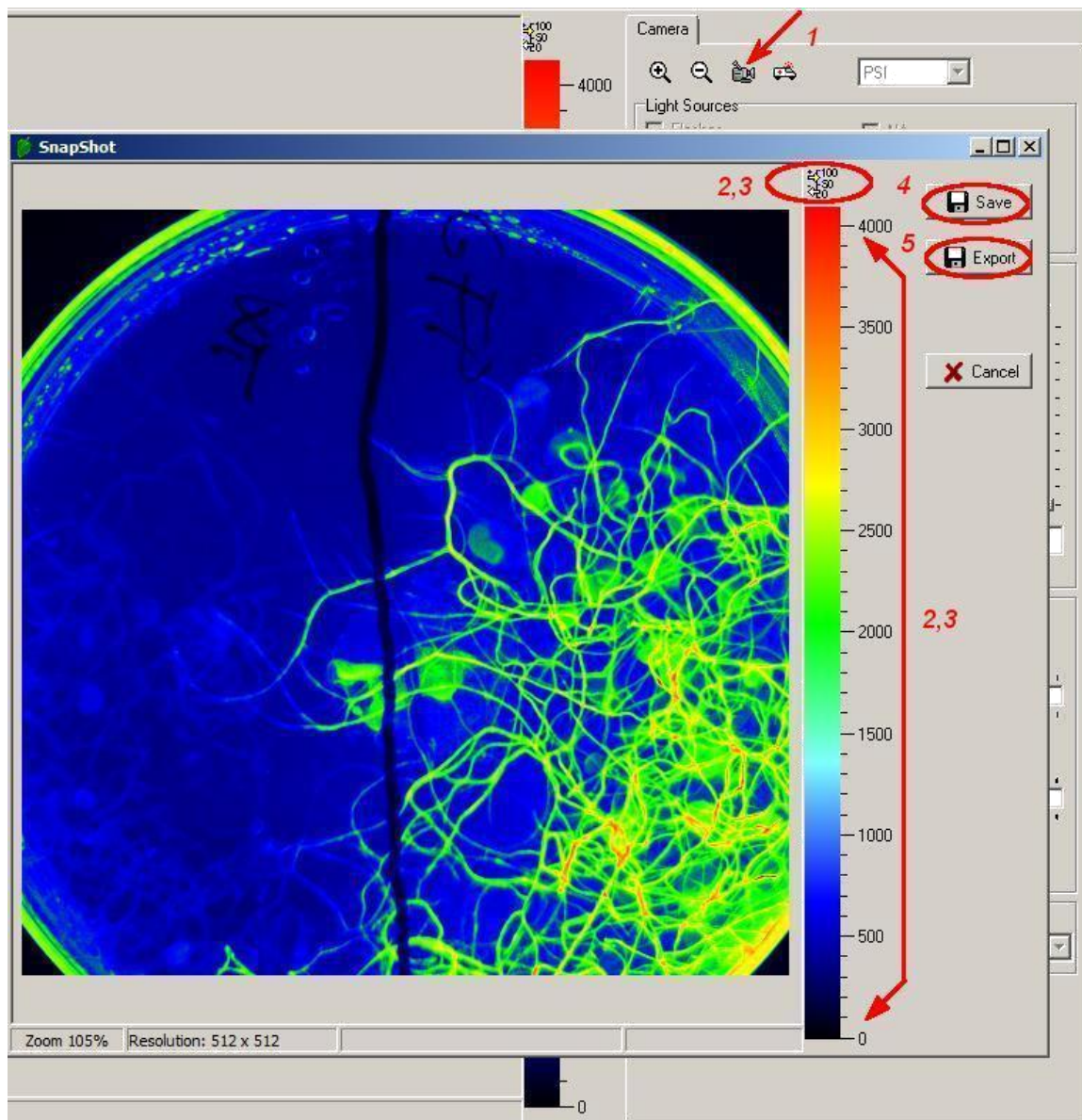


FIGURE 46. The image shown in the image window can be captured as a snapshot by (1). The image is displayed with its false color scale (2, 3). By clicking the numeric scale field (2), the scaling bar is supplemented by a numeric scale. The image view can be saved to bmp file format together with borders and false-color scale (4) or exported to raw data format *.fcimg (5).

V.G.4 Predefined Protocols and Wizard Protocols in Snapshot Mode

Alike in video mode, image capturing can be automated using predefined list of instructions – the protocol. However, the protocols must be designed specially for the snapshot mode in which the frame rate is limited to 2 frames per second (fps). Nowadays, there is available one predefined protocol (GFP) and one wizard (Dyes&FPs) to measure static fluorescence emission of fluorescent proteins or dyes.

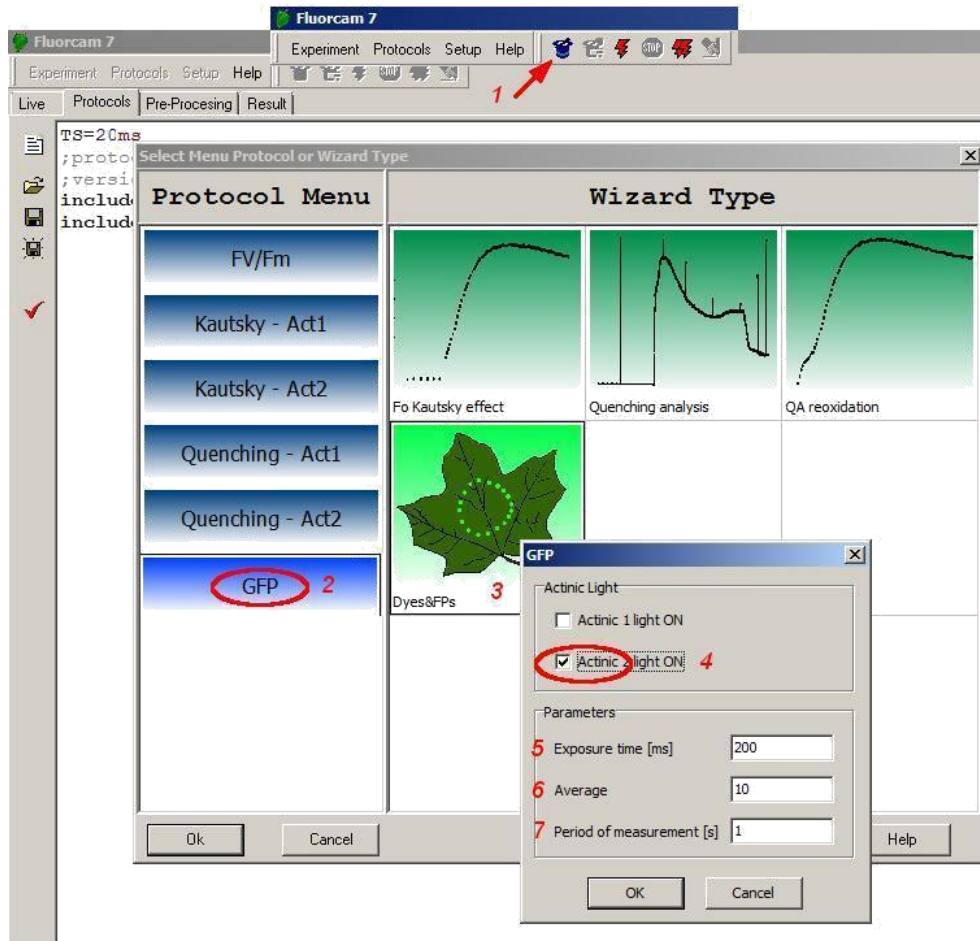


FIGURE 47. Protocol Menu and Wizard window: (1) click the wizard icon to open the Protocol Menu. (2) Predefined protocol for GFP imaging. (3) Wizard template for measuring static fluorescence signal of FPs and fluorescent dyes. The wizard allows to define certain parameters such as the source of excitation light (4), Exposure time (5), Averaging (6) ...to design own protocols.

**Note: Pre-defined protocols can be subjects of change without notice.*

a) GFP protocol

GFP protocol is a simple protocol in which blue actinic light (Act2) is switched on for 2s. During illumination period, three consecutive frames are acquired and averaged.

b) Dyes & FPs wizard

This wizard is more versatile.

It allows choosing **excitation light** Act1 or Act2 according to the wavelength needed to excite fluorescent probe or dye. In most FluorCam setups, blue Actinic light (Act2) is used to excite green fluorescence emission of GFP.

The **Exposure time** (5 in FIGURE 47) can be defined between 1 up to thousands of milliseconds.

To reduce the image noise and improve S/N ratio, a user can interactively choose number of images subjected to **averaging** (6) in FIGURE 47.

The **period of measurement** (7 in FIGURE 47) can be prolonged to avoid photo-bleaching of sensitive samples when high number of averaging is needed.

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VII. Figure captions

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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 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 best visualized using a 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 the right mouse button pressed. 61

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