Instruction Guide

PHOTON SYSTEMS INSTRUMENTS

FluorCam 1300-C/3535

Please read the Guide before operating this product

Manual Version: 2024/12

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TABLE OF CONTENT

1 WARNINGS AND SAFETY PRECAUTIONS

Read this manual carefully before operating the device. If you are not sure about something in the manual, contact the manufacturer for clarification.

	By accepting the device, the customer agrees to follow the instructions in this guide.

Always follow corresponding manuals while working with the FluorCam device or doing the maintenance.
It is forbidden to interfere with the hardware or software of the FluorCam device in any way without previous agreement with the manufacturer.

The following table presents basic highlight symbols used in this manual:

Symbol	Description
	Important information, read carefully.
	Complementary and additional information.

Tab. 1 Used symbols.

WARNING:
The FluorCam device is considered Class 1M* LED Product. LED radiation may be harmful to eye. Avoid direct and strongly reflected exposure. It is recommended to use protective glasses.
**Class 1M: Laser and LED equipment that is safe under reasonable conditions of operation for use with the naked eye. Looking directly into the source of radiation by employing optics within the beam such as magnifying glass, telescope or microscope can be potentially hazardous.*

2 INTRODUCTION

FluorCam FC1300-C/3535 is an innovative, compact system for imaging of chlorophyll fluorescence kinetics. The system provides a wealth of information about plant's photosynthetic capacity, physiological and metabolic condition, as well as its susceptibility to various stress conditions.

2.1 CHLOROPHYLL FLUORESCENCE

Chlorophyll fluorescence is a popular technique in plant physiology used for rapid non-invasive measurement of photosystem II activity. PSII activity is very sensitive to a range of biotic and abiotic factors and therefore chlorophyll fluorescence technique is used as a rapid indicator of photosynthetic performance of plants in different developmental stages and/or in response to changing environment. The advantage of chlorophyll fluorescence measurements over other methods for monitoring stresses is that changes in chlorophyll fluorescence kinetic parameters often occur before other effects of stress are apparent. The method is non-invasive, and the spread of inhibition can be observed and quantified with time. Heterogeneity in the location of inhibition is easily seen and quantified when using imaging systems to measure chlorophyll fluorescence.

Chlorophyll fluorescence imaging has become one of the most powerful and popular tools to monitor changes in the photosynthetic capacities of plants in response to biotic and abiotic stimuli or to environmental changes. Chlorophyll fluorescence is one of the processes which competes with photosynthesis for excitation light energy. Each quantum of light absorbed by chlorophyll molecule rises an electron from the ground state to the excited state. Upon de-excitation from the chlorophyll molecule, 3-5% of the excitation energy is dissipated as red fluorescence. This is based on the fact that light energy absorbed by chlorophyll molecules may undergo one of three competing fates: 1) it may be used to drive photosynthesis (83% of energy in healthy plants), 2) it may be dissipated as heat (up to 15 % of the energy) or 3) it may be re-emitted as chlorophyll fluorescence. The three processes do not exist in isolation but rather in competition with each other. Therefore, changes in fluorescence yield reflect changes in photochemical efficiency and heat dissipation or non-photochemical quenching. the yield of chlorophyll fluorescence emission provides valuable information about the quantum efficiency of photochemistry and heat dissipation (Murchie and Lawson, 2013). As photochemistry is used to provide energy and reducing power for CO2 assimilation, the analysis of chlorophyll fluorescence kinetics provides information about performance of plant photosynthesis and ultimately plant productivity. Please note that at room temperature, the fluorescence signal variations are assumed to arise from PSII only and the emission from PSI is ignored as the signal does not make a significant contribution below 700 nm (Baker, 2008).

Chlorophyll fluorescence imaging station is used to monitor monitors fluorescence kinetics in pulse-amplitude modulated mode and by using saturation pulse method., which provides a wealth of information about a plant's photosynthetic capacity, physiological and metabolic condition, as well as its susceptibility to various stress conditions. Modulating systems use the light measuring beam applied at a known frequency (modulated) to induce the fluorescence with detector set to measure at the same frequency. Chlorophyll fluorescence yield is estimated after the application of short saturating flash (saturation pulse) in dark-adapted plants or when plants are light-adapted or illuminated with photosynthetically active actinic light. The changes in chlorophyll fluorescence are used to describe plants capacities for photochemical and non-photochemical quenching of light energy supplied to plants surface.

The FluorCam device installed in this system is designed for the measuring 1) of the chlorophyll fluorescence and calculation of commonly used fluorescence parameters, as well as for the measuring of 2) different various static fluorescence signals. Depending on the type of the excitation light and combination of detection filters in the camera filter wheel, the fluorescence of different proteins/dyes can be induced and/or multi-color fluorescence can be quantified (Buschmann and Lichtenthaler, 1998). Photon Systems Instruments (PSI) has developed proprietary hardware: 1) LED light panel sources for homogenous sample illumination (measuring beam flashes, saturation super pulse, actinic lights, additional lights), 2) CCD high-resolution camera mounted in the middle of the LED panel with optionally filter wheel installed for top view sample imaging and 3) software for the acquisition and analysis of chlorophyll fluorescence kinetic and/or static measurements (Tschiers et al., 2017).

Key features of the chlorophyll fluorescence imaging unit are:

- FC imaging unit in **light isolated box** with precise positioning
- High sensitivity CCD camera
- Multi-color LED light panel
- **Pulse-modulated short duration flashes** for accurate measurement of minimal fluorescence (Fo value) determination
- Two types of **actinic lights** for light-adapted and quenching analysis
- **Saturating light** pulse for maximal fluorescence Fm value determination
- **Additional lights as** FAR (735 nm) for determination of Fo'
- **Optionally Multi-positional filterwheel**
- **Dynamic** software package for the user-friendly protocol design, acquisition and analysis of chlorophyll fluorescence kinetic and/or static measurements and advanced image processing

References:
1. Murchie, E.H. and Lawson, T. (2013) Chlorophyll Fluorescence Analysis: A Guide to Good Practice and Understanding Some New Applications. Journal of Experimental Botany, 13, 3983-3998. http://dx.doi.org/10.1093/jxb/ert208
2. Neil R. Baker (2008). Chlorophyll Fluorescence: A Probe of Photosynthesis In Vivo. Annual Review of Plant Biology 2008 59:1, 89-113
3. Buschmann C, Lichtenthaler HK. (1998). Principles and characteristics of multi-colour fluorescence imaging of plants. J Plant Physiol. 1998;152:297–314. https://doi.org/10.1016/S0176-1617(98)80144-2.
4. Brestic, M., and Zivcak, M. (2013). "PSII fluorescence techniques for measurement of drought and high temperature stress signal in crop plants: protocols and applications," in Molecular Stress Physiology of Plants, eds R. G. Rout and B. A. Das (New Delhi: Springer), 87–131
5. Tschiersch, H., Junker, A., Meyer, R.C. and Altmann, T. (2017) Establishment of integrated protocols for automated high throughput kinetic chlorophyll fluorescence analyses. Plant Methods, 13, 1–16.

2.2 CHLOROPHYLL FLUORESCENCE MEASUREMENT

The Fluorescence Imaging station uses an enhancement of the FluorCam FC-800MF Pulse Amplitude modulated (PAM) system manufactured by the Photon Systems Instruments. PSI imaging system monitors fluorescence kinetics, which provides a wealth of information about a plant's PSII photosynthetic efficiency, physiological and metabolic condition, as well as susceptibility of photosynthetic apparatus to various stress conditions.

Chlorophyll fluorescence is a measure of re-emitted light from PSII. Principle of modulated fluorescence systems is that only fluorescence resulting from excitation of the sample from the measuring beam (flashes) is measured with the detector and no ambient light interference is permitted. Pulse-amplitude modulated ChlF technique provides non-invasive assessment of photosystem II efficiency to pass electrons to photosynthetic apparatus by comparing fluorescence levels whilst exposing the plant or leaf to a combination of actinic lights (lights that drives photochemistry and photosynthesis), darkness and series of saturating pulses. Depending on the protocol used for measurement of chlorophyll fluorescence kinetics broad range of parameters can be measured and calculated, that provide important information about the performance of photosynthetic apparatus, about photochemical and non-photochemical efficiency of the plant at given developmental stage.

3 TECHNICAL SPECIFICATION

CCD Detector			
Camera	High resolution CCD camera TOMI-2		
Resolution	1 360 × 1 024 pixels		
A/D Converter Resolution	16 bit (65 536 grey levels)		
Pixel Size	6.45 µm × 6.45 µm		
Frame Rate	20 frames per second for full resolution		
CCD Detector Wavelength Range	400 – 1 000 nm		
Spectral Response	QE max at 540 nm (~72%), 50% roll-off at 350 nm and 800 nm		
Read-Out Noise	< 8 electrons RMS		
Full-Well Capacity	> 22 000 electrons		
Dynamic Range	65 dB		
Connectivity	Control and data: Gigabit Ethernet		
Operating Modes	Video (ChlF), Snapshot (long integration times for FPs detection)		
Lights		**Detection Channels – Up to 7 Emission Filters in a Filter Wheel**	
Light Sources	Red-Orange 618 ± 10 nm Cool White 5700 K Far-Red 735 ± 10 nm	Chlorophyll filter	695 - 770 nm
Super Pulse Intensity	> 5 000 µmol.m^{-2}.s^{-1}		
Actinic Light Intensity	Up to 2 000 µmol.m^{-2}.s^{-1}		
Additional Lights	UV 365 ± 9 nm Royal Blue 450 ± 10 nm Blue 475 ± 10 nm Cyan 505 ± 15 nm Green 530 ± 15 nm Amber 590 ± 40 nm	Filters for Multicolor GFP filter YFP filter mCherry	440/40, 520/28, 690/8, 747/33 nm 517/20 nm 593/46 nm 635/18 nm
Lens			
Lens type	SV-0814H (VS Technology)		
Focal length	8 mm		
Brightness	F1.4		
Technical Data			
Dimensions (W × D × H)	1 200 × 1 150 × 1 900 mm		
Weight	205 kg		
Electrical	400 V AC (3 phases)		
Power Consumption	4 100 W		
Operating Temperature	5 – 40°C		
Operating Humidity	0 to 90% (non-condensing)		
Software			
FluorCam10	• fully automated control of FC device • image acquisition *via* automated experimental protocols • numerous image manipulation tools • automated data analysis and parameters computation (F0, FM, FV, F0', FM', FV', FT, FV/FM, FV'/FM', ΦPSII, NPQ, qN, qP, Rfd, …		

4 General Information

4.1 Device Description

FluorCam 1300-C/3535 features the unique flat LED panels design which results in a highly uniform illumination across the whole imaging area (350 × 350 mm) with minimum shading effects.

The standard version of FC 1300-C/3535 contains multiple LED sources for use in excitation of chlorophyll in various ChlF protocols. The red-orange LEDs are used for measuring flashes and actinic light. The cool white LEDs are used for saturating pulse and as additional actinic light. The FAR-red LEDs are employed in estimation of F_0'. High resolution CCD camera allows both measurement of ChlF and detection of weak steady-state fluorescence signals, where long integration times are needed. Applications where high spatial resolution of the fluorescence signal is of importance will also benefit from the high-resolution CCD camera. The enhanced version of FC 1300-C/3535 is supplemented by a state-of-the-art Multi-Excitation Module combining conventional chlorophyll fluorescence measurements with detection of the various fluorescence or reflectance signals. The light source for multispectral fluorescence includes, additional to the standard cool-white, orange-red and FAR, 6 LED colors: UV, Royal Blue, Blue, Cyan, Green and Amber.

FC 1300-C/3535 equipped with Multi-Excitation Module also include a motorized, software-controlled filter wheel equipped with up to 7 different emission filters, allowing for a number of experimental protocols as required by the user:

- Detection of fluorescent proteins and dyes (CFP, GFP, YFP, RFP, mCherry, SYBR Green…)
- Multi-color fluorescence and auto-fluorescence analysis
- Measurements of chlorophyll fluorescence excitation spectra
- Quantification of pigments (flavonols, anthocyanins, chlorophyll)

The Closed FluorCam 1300 system is compact and allows easy dark adaptation of the sample. The system includes a high-performance PC and comprehensive software package which provides full device control, data acquisition and image processing. Experienced users may design new protocols with sophisticated programming language and vary the timing and measuring sequences.

4.2 Device Installation

FC 1300-C/3535 are delivered to customers as a compact device (Fig. 1) and does not require any special assemblage. Basic installation recommendations for FC 1300-C/3535 are depicted below.

Fig. 1 FC 1300-C/3535.

	Recommended tools: no tools necessary. Recommended manipulation: It is advised to move the FC 1300-C/3535 with a pallet truck only.

Unpacking:

- 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.

Connecting:

- Connect FC 1300-C/3535 to the computer using the provided Ethernet cable (Fig. 2A, C and D).
- Using the provided three-phase cable, connect the FluorCam and the 3-phase power supply. The red socket is on the front of the FluorCam device (Fig. 2B, C and D).
- Connect the power supply to a 400V outlet.
- Switch on the power supply, the FC 1300-C/3535 device and the computer.

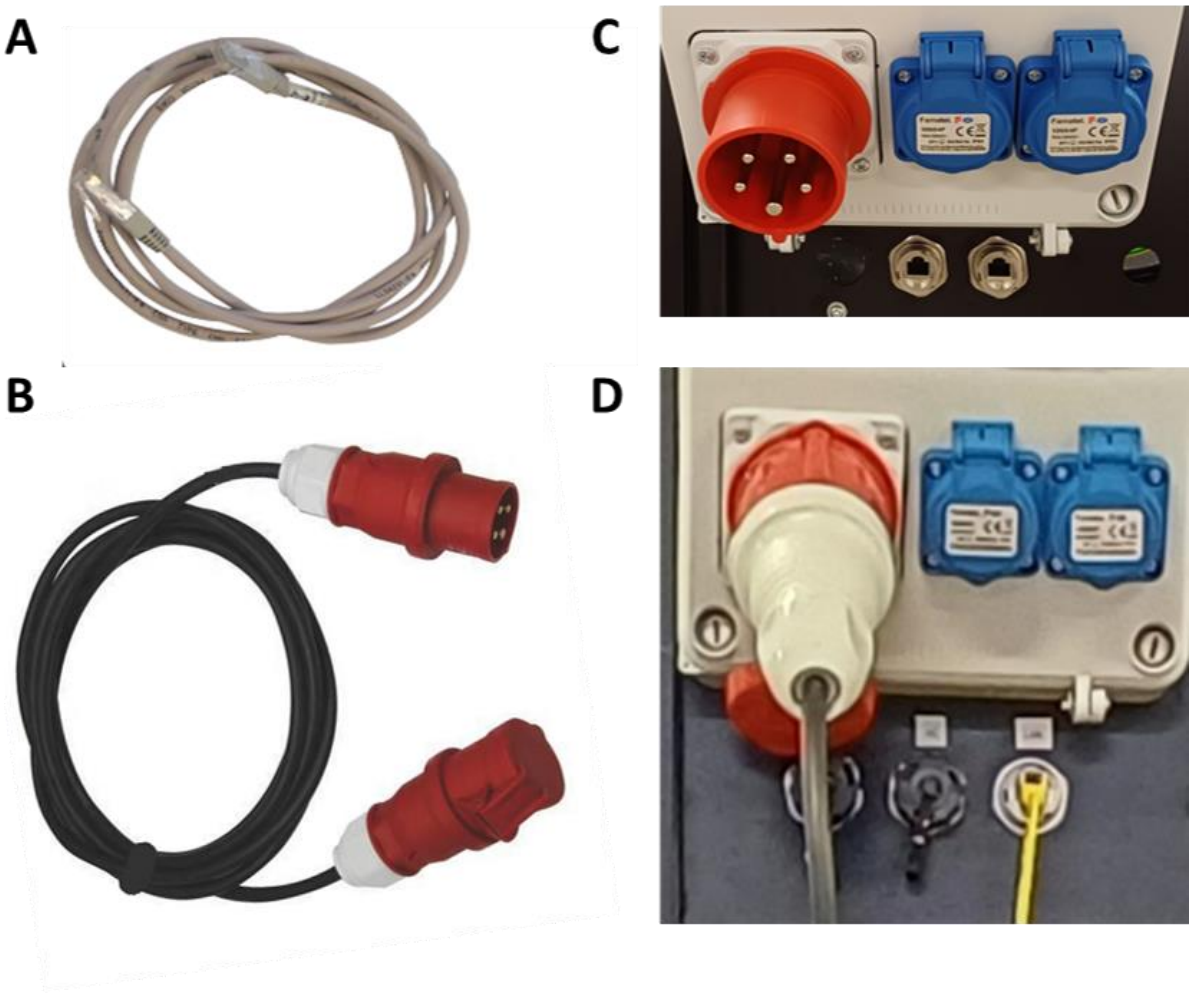

Fig. 2 Cables essential for FC 1300-C/3535 function and their proper connecting:

(A) An Ethernet cable, (B) a power cord, (C and D) corresponding sockets on the rear side of FC 1300-C/3535.

4.3 The Switch and the Fan

The device is powered on using the red switch located on the front panel of the FluorCam FC 1300-C/3535 (Fig. 3). The operating mode is indicated by a green light. To maintain room temperature inside the box, the fan can be switched on during dark incubation between measurement (Fig. 3B). However, ensure the fan is turned off during imaging, as moving air can cause the plant leaves to shift, making it impossible to accurately define the plant mask.

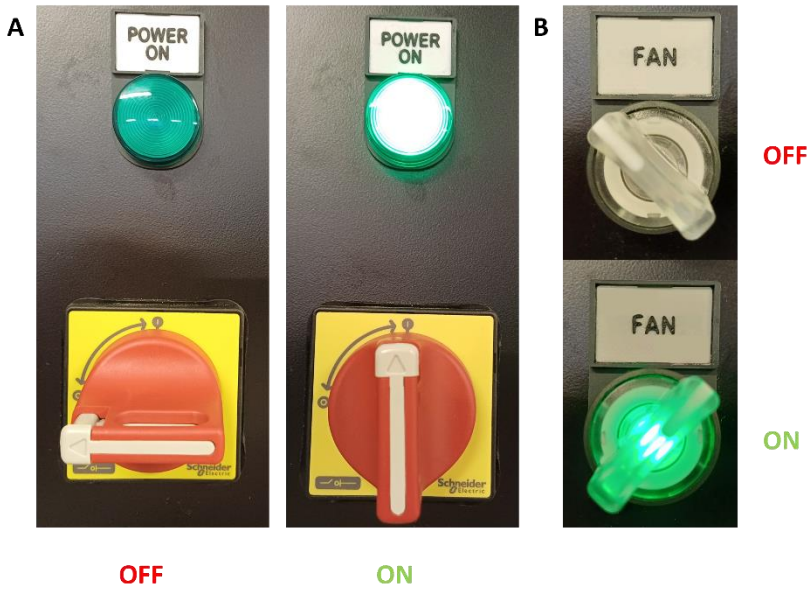

Fig. 3 Important switches:

(A) the power switch and (B) the fan switch.

4.4 The Shelving System and the Thorns

The shelving allows plants of varying sizes to be positioned at an appropriate distance from the lights (Fig. 4), ensuring uniform light distribution for measurements. The shelf's position is secured using levers located on both sides. To properly fix the shelf in place, ensure that the levers are turned perpendicular to the shelf. The proper position of tray for imaging is ensured by thorns (Fig. 5).

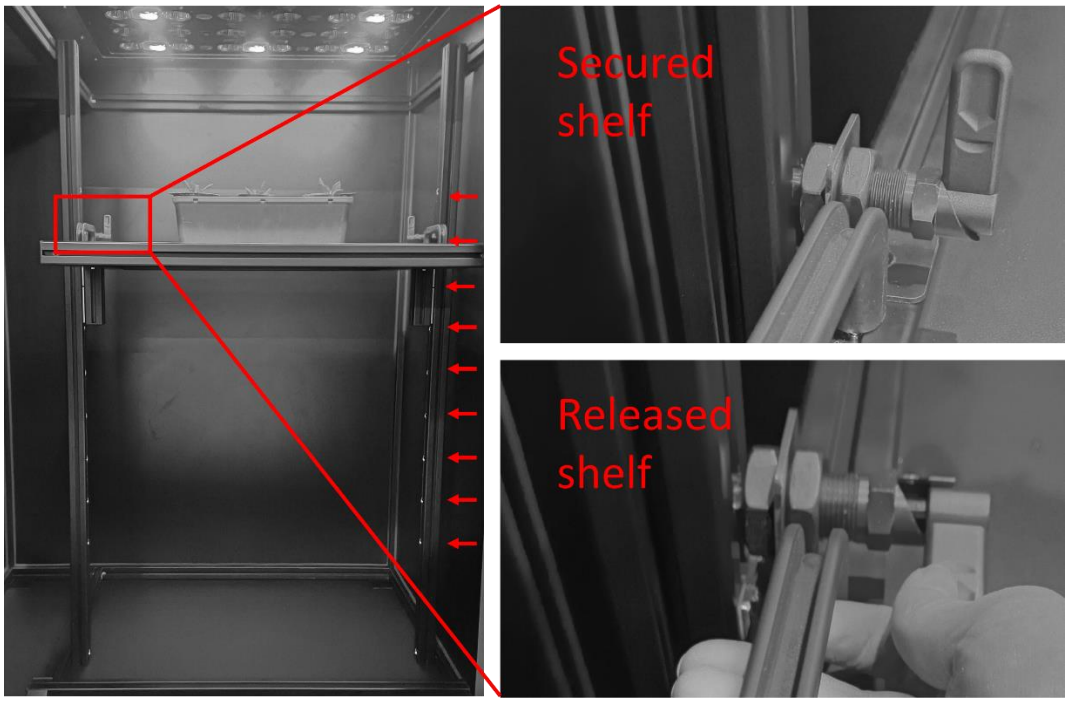

Fig. 4 The shelving system enables proper placing of plants of different sizes, the shelf is fixed by levers on both sides.

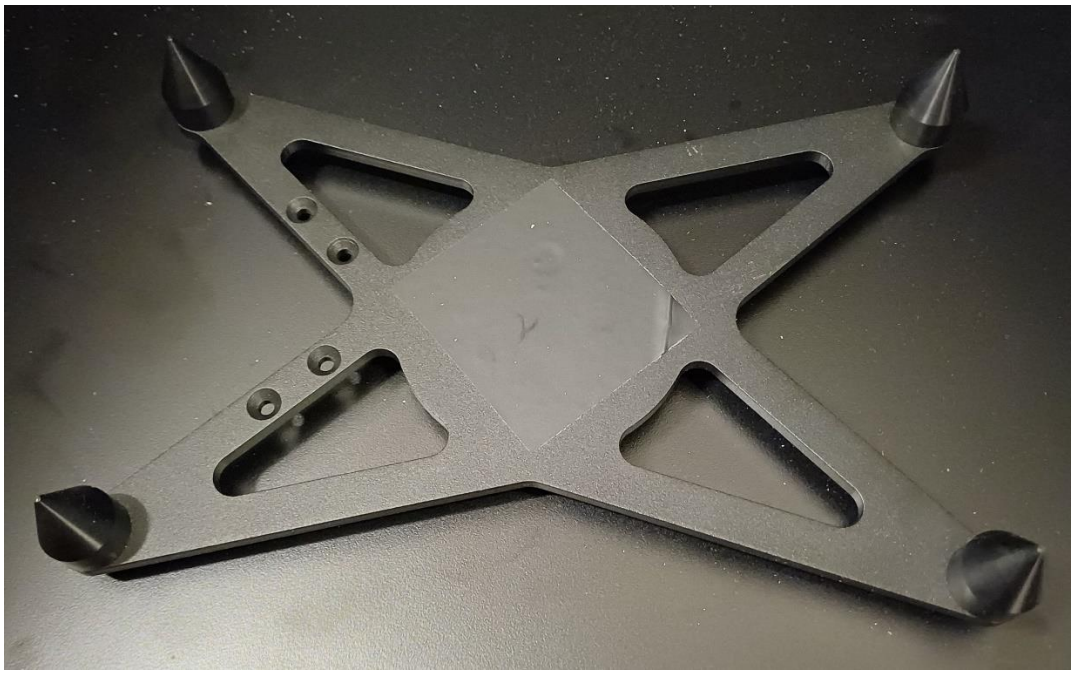

Fig. 5 Thorns for precise positioning of the tray with plants.

5 FluorCam 10 Software

The FluorCam 1300-C/3535 is compatible with the FluorCam10 software. Unlike FluorCam7, FluorCam10 is a 64-bit application. This change enables FluorCam10 to access more memory than 32-bit applications and so run complex protocols more smoothly. The setting of FluorCam10 resembles FluorCam7 and is quite intuitive. The useful functions of FluorCam7 have been preserved so the details can be found in the FluorCam7 instruction guide if necessary.

5.1 Device Connection

First of all, the proper connection of the switched-on FluorCam 1300-C/3535 with the computer through the Ethernet cable should be checked. If the device is properly connected, the "Live" tab of FluorCam10 contains information about the device in the bottom left corner (highlighted by the green color) as shown in the Fig. 6. If the device is not connected, check Network & Internet Settings and set proper IP address via Internet Protocol version 4 (**TCP/IPv4: IP address: 172.23.0.1, Subnet mask: 255.255.240.0**).

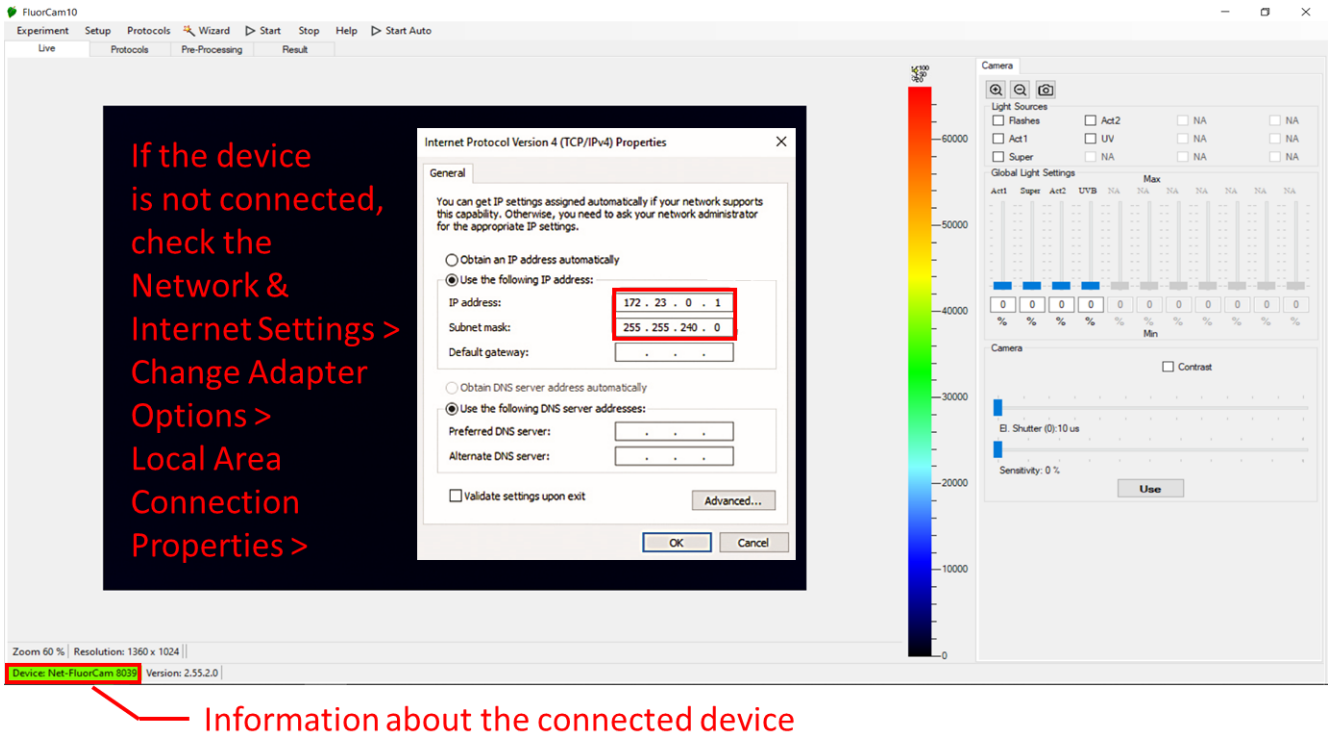

Fig. 6 Indication of properly connected FluorCam 1300 in FluorCam10 and Ethernet settings.

5.2 Software License and Automation License

Unliked FluorCam7, the FluorCam10 is a licensed software. It is important to insert **FC10 License Code** into the software to activate most of functionalities of FluorCam10. The unique FC10 License Code is put onto the delivered USB flash drive in the folder "User Keys". The FluorCam10 software will be licensed (Fig. 7) after pasting FC10 License Code in the following way: Help > About > Not Licensed > License Code > OK.

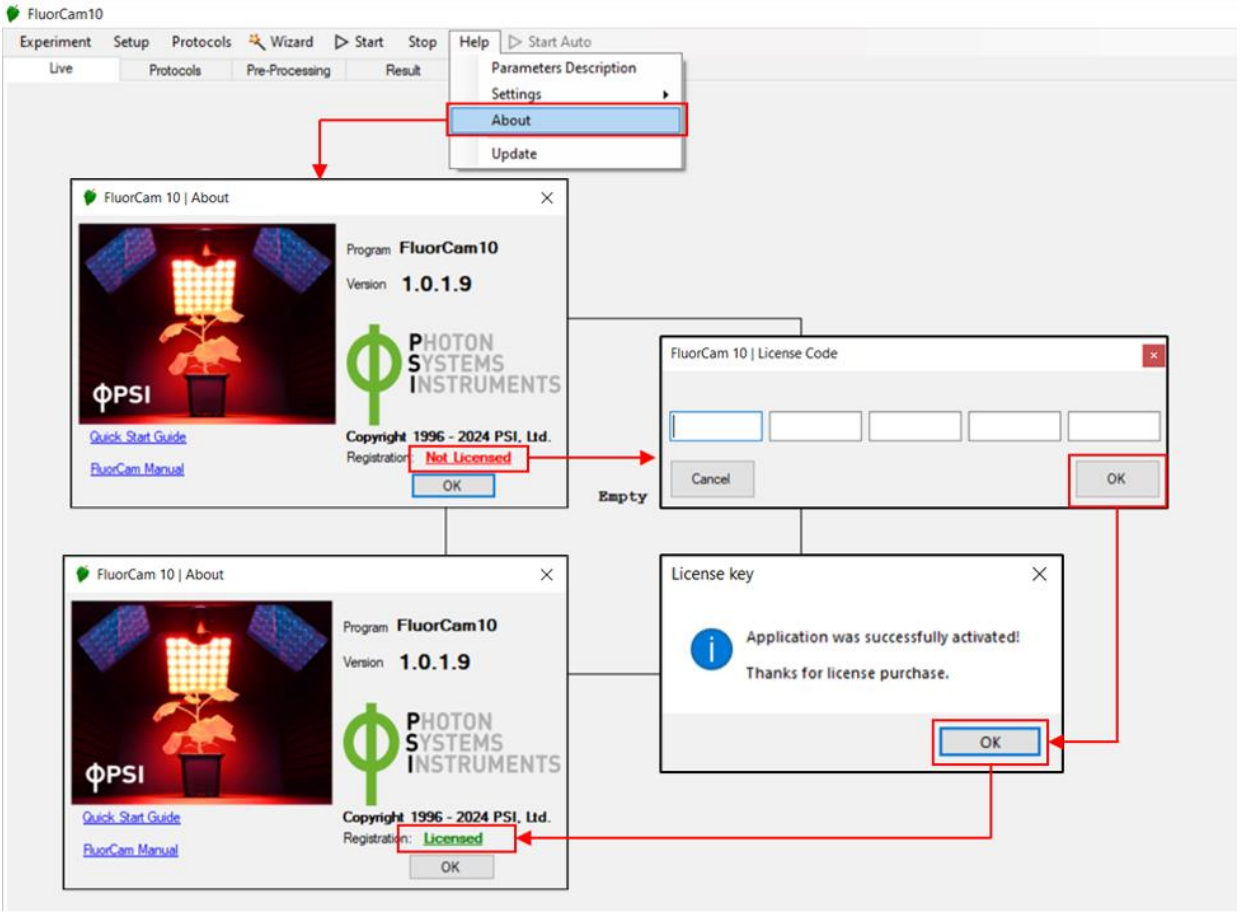

Fig. 7 Activation of FluorCam10.

To activate the purchased **Advanced multiple function/Automation license,** the file with FC10 Automation License key has to be inserted into the software in the following way: Setup > General > Advanced > Select file > Close as described in the Fig. 8. Then, the multiple protocols can be triggered by a click on "► Start Auto".

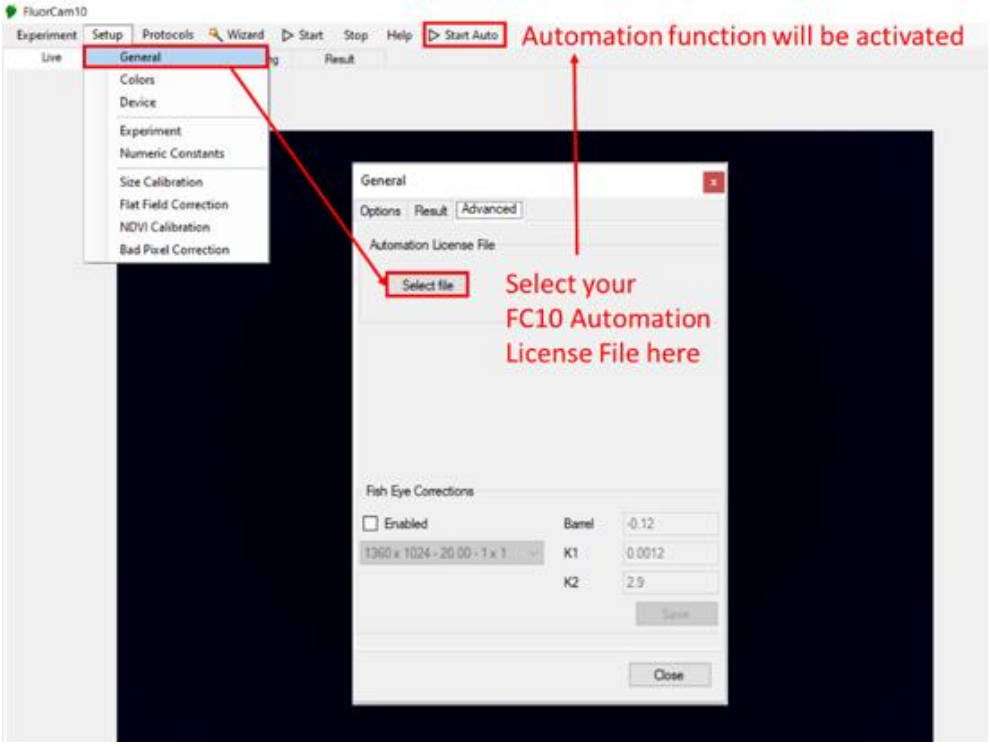

Fig. 8 Activation of the purchased Automation license.

5.3 The Live Tab

Then, the **"Live" tab** can be used for focusing the sample of interest (this is done manually) and selection of a suitable filter and right parameters, such as light intensities, shutter, sensitivity and zoom, for the experiment. The predefined basic and advanced protocols are offered after a click on the "Wizard" button (Fig. 9).

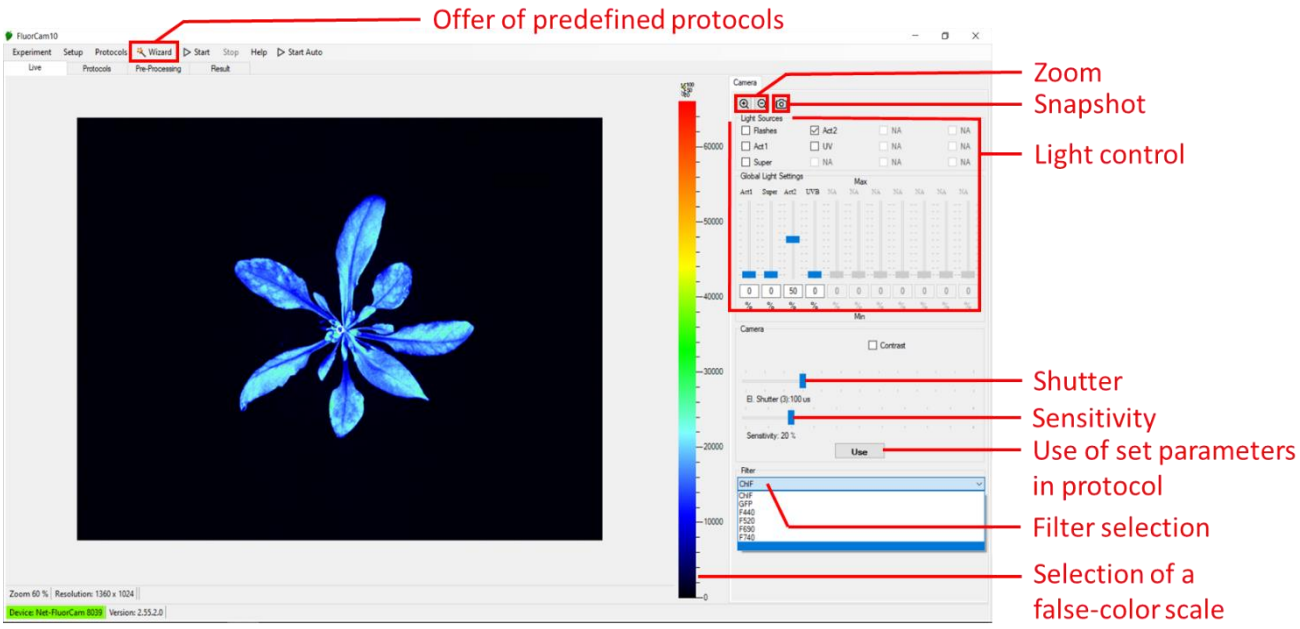

Fig. 9 The "Live" tab of the FluorCam10.

Advanced protocols, such as GFP and Multicolor, should be used in the "**Snapshot mode**". The "Live" tab can be switched from the "Video mode" into the "Snapshot mode" in the following way: Setup > Device > Mode SNAP > Set Device As > Close (Fig. 10). After setting parameters, the "**Get Frame**" button should be used to see the image.

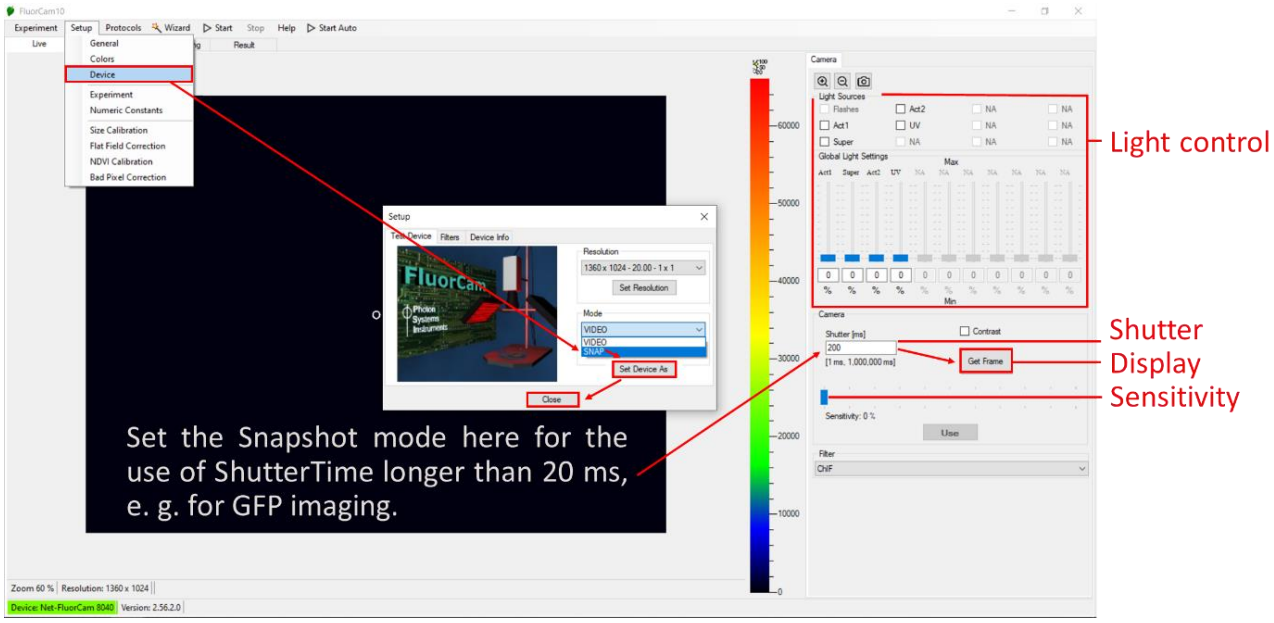

Fig. 10 The selection of the Snapshot mode in the FluorCam10.

5.4 The Protocol Tab

The "Use" button can be used for import of desired parameters into the selected protocol in the **"Protocols" tab**. The protocol is triggered by the "Start" button as shown in the Fig. 11.

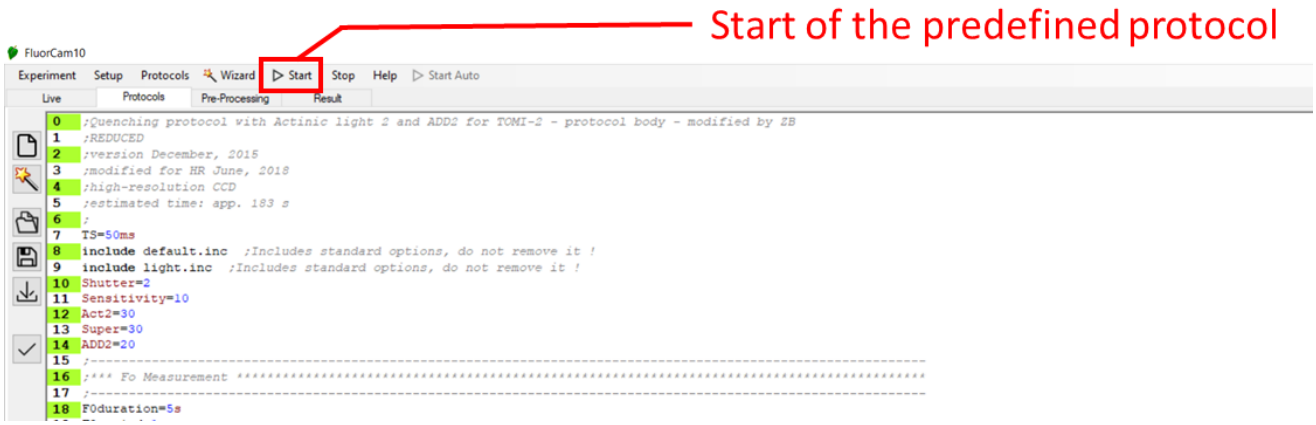

Fig. 11 The "Protocol" tab of FluorCam10.

5.5 The Pre-processing Tab

The desired mask can be selected in the **"Pre-processing" tab** (Fig. 12). The automatic mask is applied after choosing the "Background Exclusion" button. Small objects will be excluded from the analysis when the minimal object size is given. The mask can be defined manually as well. All masks can be saved and open in future experiments. After calibration of the FluorCam10 with the size calibration standard the predefined masks for Petri dishes and microtiter plates can also be applied.

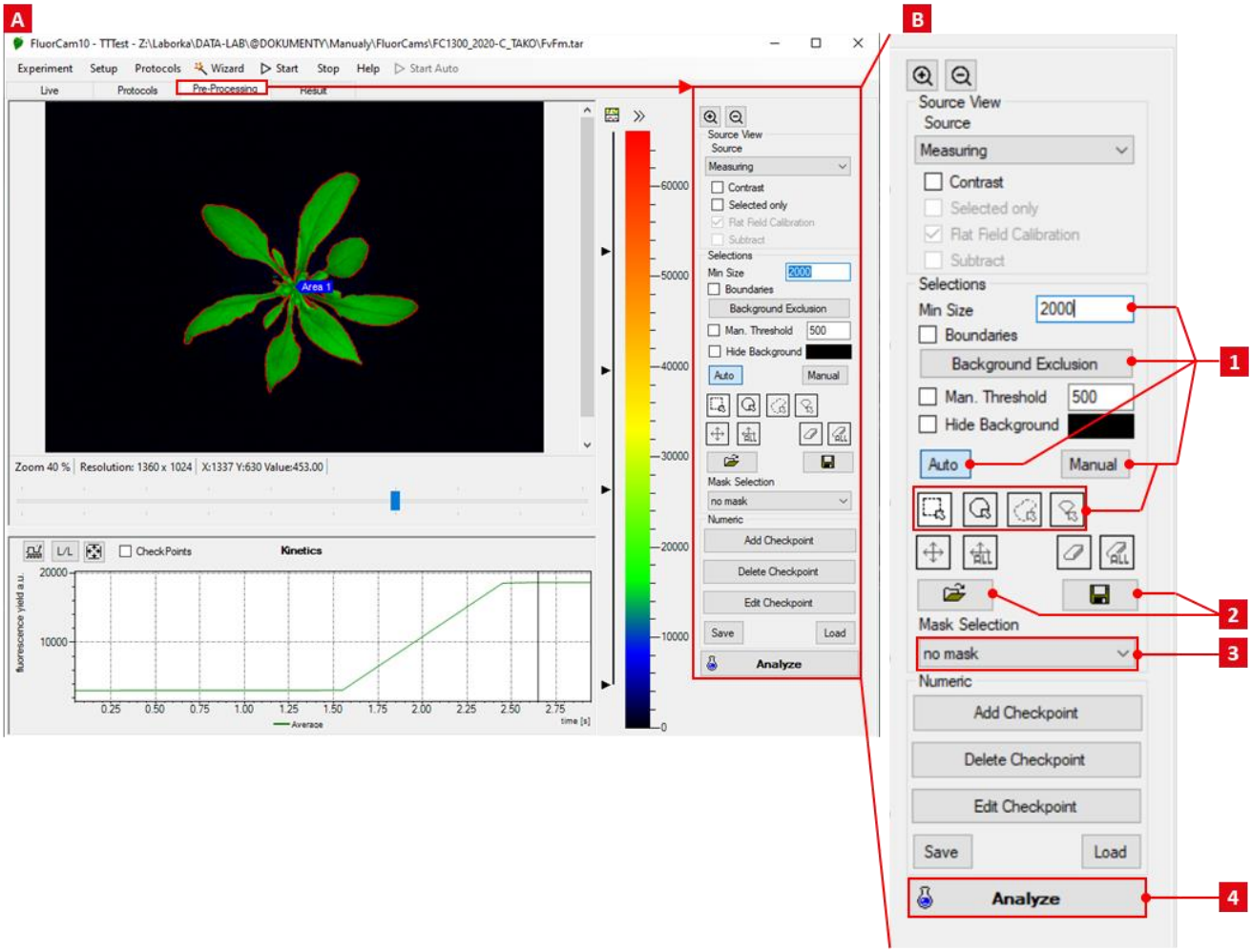

Fig. 12 The "Pre-processing" tab of FluorCam10.

(A) Preprocessing Window. (B) Individual Tools in the Preprocessing Window: 1) Definition of the mask (auto or manual), 2) Opening and saving of a mask, 3) Predefined masks (after size calibration only), 4) Start of analysis.

5.6 THE RESULT TAB

The results (curves, parameters and images) can be visualized in the **"Result" tab** and saved or exported in Experiment menu (Fig. 13).

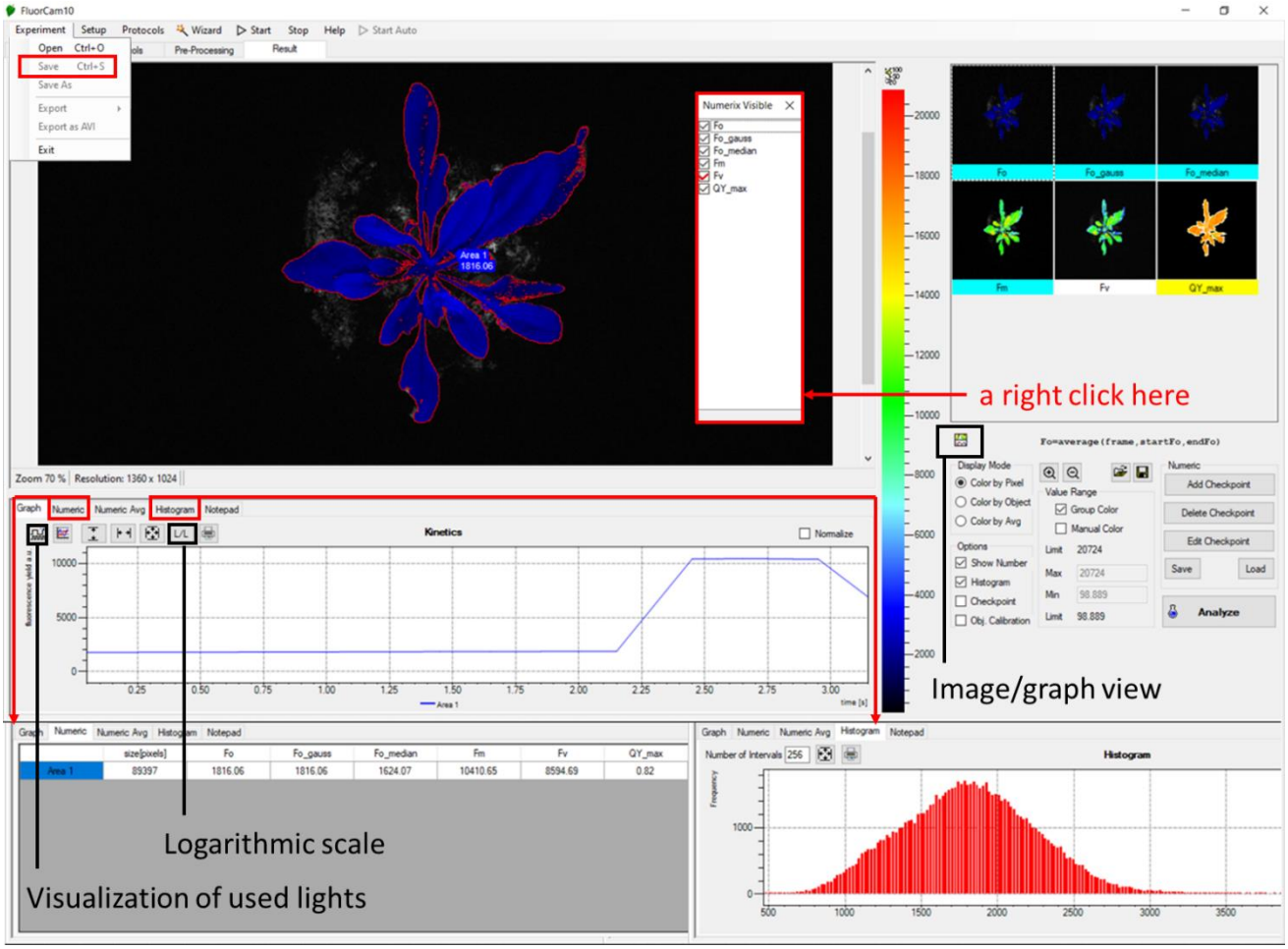

Fig. 13 The "Result" tab of FluorCam10.

6 OPERATION

6.1 PRE-DEFINED PROTOCOLS

Various basic and advanced protocols (Tab. 2 and Tab. 3) can be used for the chlorophyll fluorescence analysis. Based on the type of protocol different parameters can be measured and calculated (Tab. 3).
In the dark- adapted state a "measuring light- flashes" are used to measure minimum value of chlorophyll fluorescence, termed F_0. Measuring light is of intensity too low to induce electron transport through PSII but high enough to result in minimal level of fluorescence emitted. Application of saturating pulse to a dark-adapted leaf induces maximum level of fluorescence, termed F_M, by driving the closure of all reaction centers. At this phase in healthy plant no non-photochemical is occurring. The difference between F_M and F_0 is variable fluorescence, termed F_V. From this F_V/F_M parameter can be calculated, which is used as robust indicator of maximum quantum yield of PSII photochemistry (in healthy plants is 0.83). Most healthy plants have a very conservative value of F_V/F_M of about 0.8. With many abiotic stresses, this value declines markedly, and well before any visible indicators of stress are apparent visually. Thus, measurements of F_V/F_M can identify the onset of stress much more effectively than measuring the change in slope of a growth curve. Changes in F_V/F_M can also be used as alarms to trigger rescue protocols for stressed plants, such as irrigation of plants undergoing drought conditions.
To address the proportion of energy used for photochemical reaction and amount of energy used for non-photochemical heat dissipation, quenching protocols can be used. During quenching protocol dark adapted sample is illuminated is first illuminated with saturating pulse (for F_V/F_M calculation) and subsequently with actinic light. Initial rise in fluorescence is observed after actinic light application, which is subsequently quenched as a result of the increasing competition between photochemical and non-photochemical events. Number of saturating pulses can be applied during the actinic light exposure, which allows determine operating efficiency of PSII photochemistry (F_q'/F_M'), the level of photochemical quenching of PSII (F_q'/F_V') and the level of non-photochemical quenching (NPQ). The light curve protocol is used to quantify the rate of photosynthesis at gradually increasing photon irradiances using. The light curve protocol was proven to provide detailed information on ChlF under stress (Brestic and Zivcak, 2013). For more detail information about the fluorescence parameters please refer to FluorCam Manual or the reference list at the end of this document.
Measured and calculated parameters depend on the used protocol that can be either measured in video mode with max shutter time of 20 ms or in snapshot mode with integration time up to several seconds. Measuring time depends on the used protocol. The protocols are pre-defined in the protocol menu specifically for the given actinic light used.

List of protocols		
Protocol name	**Description**	**Mode**
Fv/Fm	Investigation of dark-adapted plant's maximum quantum yield of PSII.	VIDEO
Kautsky-Act1	Protocol for investigation of the dynamics of photosynthetic reactions during a transition from dark to light.	VIDEO
Kautsky-Act2		
Quenching-Act1	Protocol for investigation of the transitions from the dark-adapted state to light-adapted state and back.	VIDEO
Quenching-Act2		
Quenching FAR-Act1	Protocol for investigation of the transitions from the dark-adapted state to light-adapted state and back. Includes FAR illumination for measurement of F0´.	VIDEO
Quenching FAR-Act2		
Light Curve-Act1	Light Curve protocol in FC is designed to measure quenching analysis in light adapted state at different light irradiances.	VIDEO
Light Curve-Act2		

Tab. 2 List of basic protocols.

List of protocols		
Protocol name	**Description**	**Mode**
Multicolor	Protocol for measuring multicolor fluorescence (blue, green, red and far-red) caused by UV light	SNAP
GFP	Green fluorescence protein detection protocol	SNAP
YFP	Yellow fluorescence protein detection protocol	SNAP

Tab. 3 List of advanced protocols.

	Please note for the measurement of the dark-adapted F_m and F_0 values, or the fluorescent signal of proteins, the imaging must be performed in complete darkness without any ambient light interference.

Software for chlorophyll fluorescence analysis allows automated batch analysis of images for fluorescence quenching parameters including user-identified regions of interest and averaging of pixel values on background-subtracted images. Analyzed data are stored in the database with co-registration of raw image data and analyzed data. User-defined mask definition is possible in on-line automated analysis mode for each pot in the tray separately.

6.1.1 FV/FM PROTOCOL

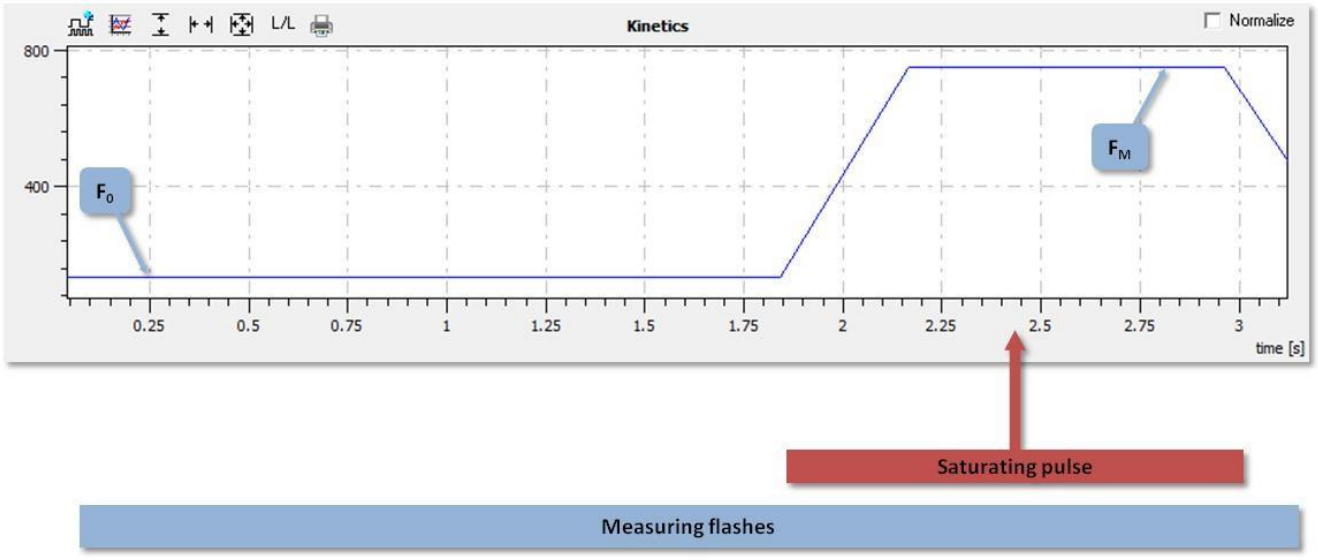

Fig. 14 Schematics of Fv/Fm protocol for dark-adapted plant samples.

Protocol duration 3-6 seconds.

F_V/F_M protocol		
F_0	Measured	Minimum fluorescence in dark-adapted state
F_M	Measured	Maximum fluorescence in dark-adapted state
F_V	$F_M - F_0$	Variable fluorescence in dark-adapted state
F_V/F_M (QY_max)	F_V / F_M	Maximum PSII quantum yield

Tab. 4 List of parameters in the F_V/F_M protocol.

6.1.2 KAUTSKY CURVE PROTOCOL

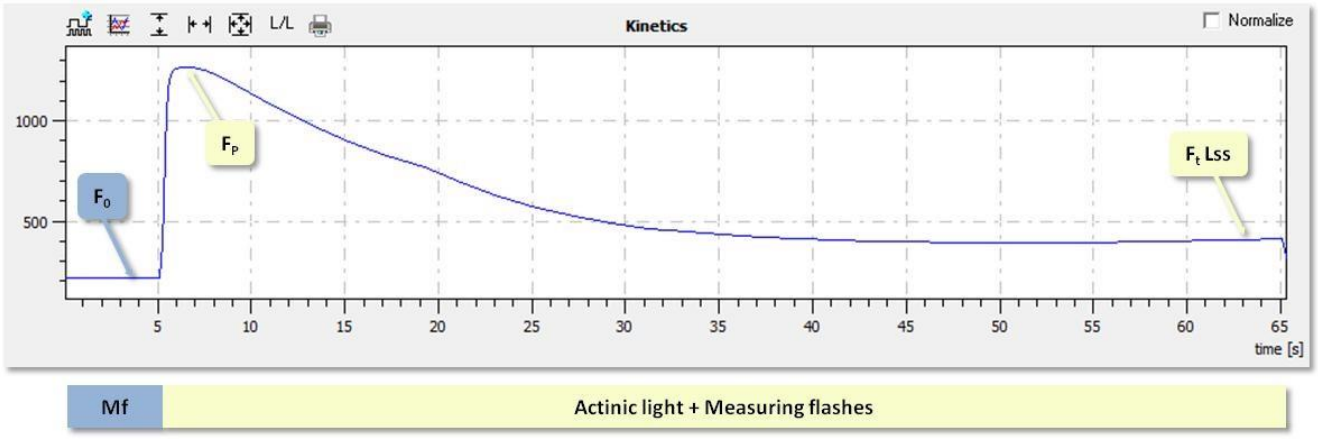

Fig. 15 Schematics of Kautsky curve protocol.

Requires dark-adaptation of plant samples. Protocol duration of 60 seconds.

Kautsky protocol		
F_0	Measured	Minimum fluorescence in dark-adapted state
F_P	Measured	Peak fluorescence during the initial phase of the Kautsky effect
F_t_Lss	Measured	Steady-state fluorescence in light
F_V/F_M (QY_max)	F_V / F_M	Maximum PSII quantum yield
Rfd_Lss	(F_P - Ft_Lss) /Ft_Lss	Fluorescence decline ratio in steady-state

Tab. 5 List of parameters in the Kautsky protocol.

6.1.3 QUENCHING PROTOCOL

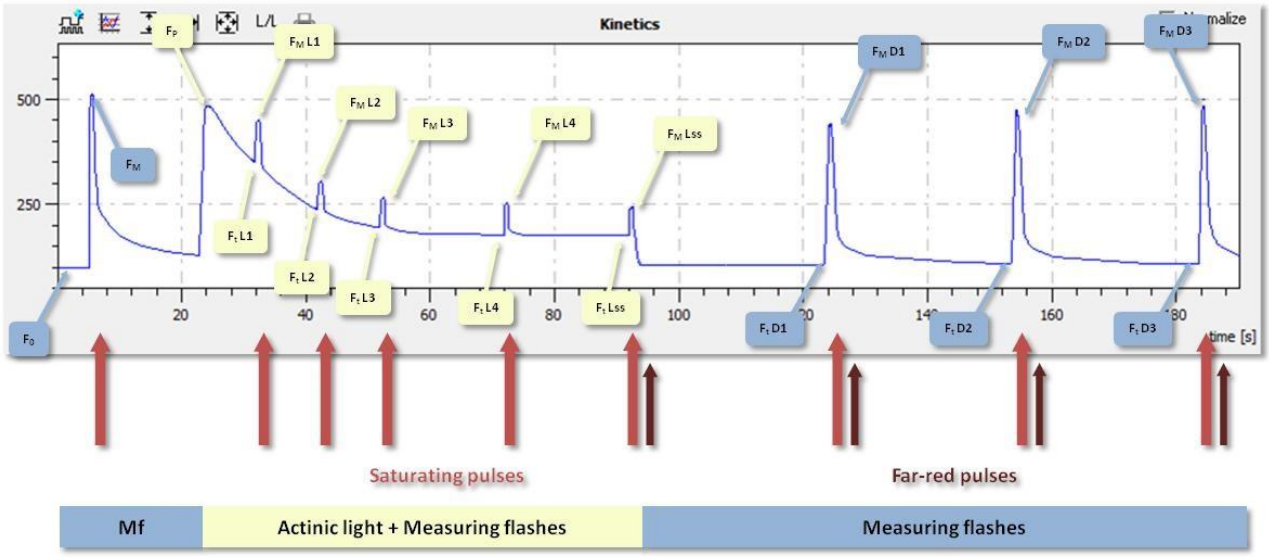

Fig. 16 Schematics of quenching analysis protocol (Quenching Act1 or Act2).

It is used to quantify photochemical and non-photochemical quenching capacity of the plant samples. Protocol duration of approx. 180 seconds.

Quenching protocol		
F_0	Measured	Minimum fluorescence in dark-adapted state
F_0_gauss		
F_0_median		
F_M	Measured	Maximum fluorescence in dark-adapted state
F_V	$F_M - F_0$	Variable fluorescence in dark-adapted state
F_P	Measured	Peak fluorescence during the initial phase of the Kautsky effect
F_P – median	Measured	
F_P – gauss	Measured	
F_P – average	Measured	
F_M_Ln	Measured	Maximum fluorescence during light adaptation
F_M_Lss	Measured	Steady-state maximum fluorescence in light
F_M_Dn	Measured	Instantaneous maximum fluorescence during dark relaxation
F_t_Ln	Measured	Instantaneous fluorescence during light adaptation
F_t_Lss	Measured	Steady-state fluorescence in light
F_t_Dn	Measured	Instantaneous fluorescence during dark relaxation
F_0_Ln	(F_0/((Fv/Fm) + (F_0/Fm_Ln)))	Minimum fluorescence during light adaptation
F_0_Lss	Quenching with FAR lights Measured	Quenching without FAR lights F_0/ ((F_V/F_M)+(F_0/F_M_Lss))
F_0_Dn	Quenching with FAR lights Measured	Quenching without FAR lights F_0/(F_v/F_M+F_0/F_M_Dn)
F_V_Ln	F_M_Ln - F_0_Ln	Variable fluorescence in light
F_V_Lss	F_M_Lss – F_0 _Lss	Variable fluorescence in light-adapted state
F_V_Dn	F_M_Dn - F_0_Dn	Variable fluorescence during dark relaxation
F_q_Ln	F_M_Ln – F_t_Ln	Difference in fluorescence between F_M_Ln and F_t_Ln in light
F_q_Lss	F_M_Lss – F_t_Lss	Difference in fluorescence between F_M_Dn and F_t_Dn in light adapted state
F_q_Dn	F_M_Dn – F_t_Dn	Difference in fluorescence between F_M_Dn and F_t_Dn during dark relaxation
F_V/F_M (QY_max)	F_V / F_M	Maximum quantum efficiency of PSII photochemistry
F_V/F_M_Ln	F_V_Ln/F_M_Ln	PSII maximum efficiency of light adapted sample
F_V/F_M_Lss	F_V_Lss/F_M_Lss	PSII maximum efficiency of light adapted sample in steady-state
F_V/F_M_Dn	F_V_Dn/F_M_Dn	PSII maximum efficiency during dark relaxation
QY_Ln	F_q_Ln/F_M_Ln	Instaneous PSII quantum yield during light adaptation
QY_Lss	F_q_Lss/ F_M_Lss	Steady-state PSII quantum yield
QY_Dn	F_q_Dn/F_M_Dn	Instaneous PSII quantum yield during dark relaxation
NPQ_Ln	(F_M-F_M_Ln)/ F_M_Ln	Instaneous non-photochemical quenching during light adaptation
NPQ_Lss	(F_M - F_M_Lss)/F_M_Lss	Steady-state non-photochemical quenching
NPQ_Dn	(F_M-F_M_Dn)/ F_M_Dn	Instantaneous non-photochemical quenching during dark relaxation
qN_Ln	(F_M-F_M_Ln)/ (F_M_- F_0_Ln)	Coefficient of non-photochemical quenching during light adaptation
qN_Lss	(F_M-F_M_Lss)/ (F_M_- F_0_Lss)	Coefficient of non-photochemical quenching in steady state
qN_Dn	(F_M-F_M_Dn)/ (F_M_- F_0_Dn)	Coefficient of non-photochemical quenching during dark relaxation
qP _Ln	F_q_Ln/F_V_Ln	Coefficient of photochemical quenching during light adaptation
qP_Lss	F_q_Lss/F_V_Lss	Coefficient of photochemical quenching in steady-state

qP_Dn	F_q_Dn/F_V_Dn	Coefficient of photochemical quenching during dark relaxation
qL_Ln	(F_q_Ln/F_V_Ln) *(F_0/F_t_Ln)	Fraction of PSII centers that are 'open' during light adaptation
qL_Lss	(F_q_Lss/F_V_Lss)* (F_0/F_t_Lss)	Fraction of PSII centers that are 'open' in steady state
qL_Dn	(F_q_Dn/F_V_Dn) / (F_0_Dn/F_t_Dn)	Fraction of PSII centers that are 'open' during dark relaxation
Rfd_Ln	(F_p-F_t_Ln) / Ft_Ln	Instantaneous fluorescence decline ratio in light
Rfd_Lss	(F_p-F_t_Lss) / Ft_Lss	Fluorescence decline ratio in steady-state

Tab. 6 List of parameters in the Quenching protocol.

6.1.4 LIGHT CURVE PROTOCOL

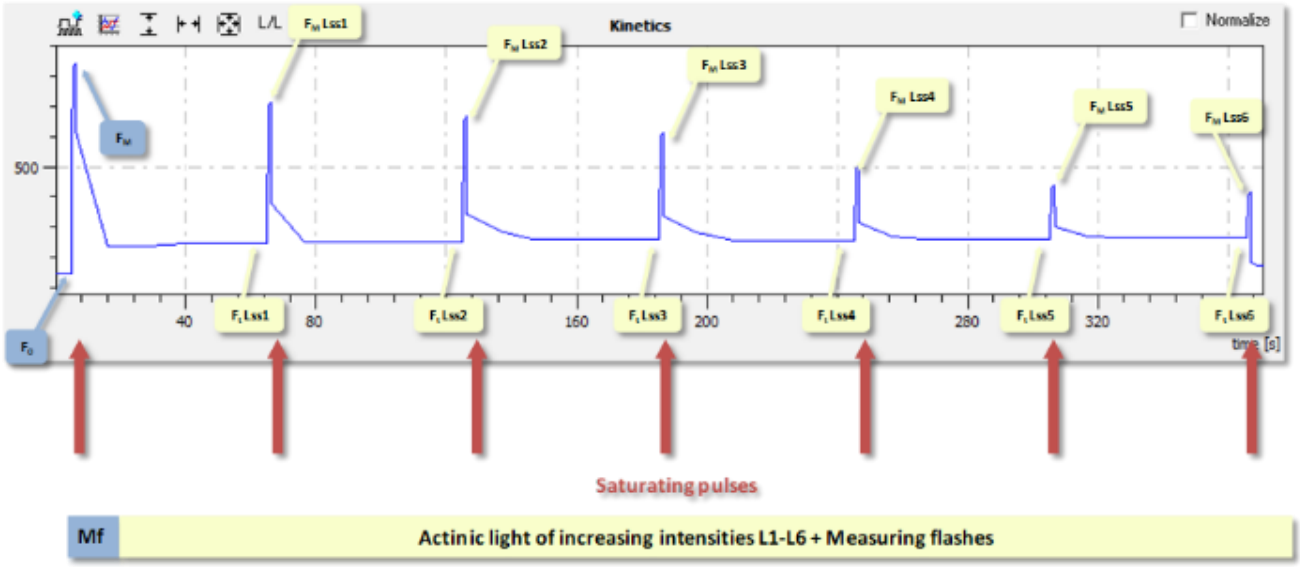

Fig. 17 Schematic of rapid light curve protocol (Light curve Act1 or Act2).

Requires dark-adaptation of plant samples. Protocol duration of approx. 360 seconds.

Light curve protocol		
F_0	Measured	Minimum fluorescence in dark-adapted state
F_M	Measured	Maximum fluorescence in dark-adapted state
F_V	F_M-F_0	Variable fluorescence in dark-adapted state
F_M_Lss	Measured	Steady-state maximum fluorescence in light
F_t_Lss	Measured	Steady-state fluorescence in light
F_0_Lss	F_0/ ((F_V/F_M)+(F_0/F_M_Lss))	Steady-state minimum fluorescence in light
F_v_Lss	F_M_Lss-F_0_Lss	Variable fluorescence in light-adapted state
F_q_Lss	F_M_Lss-F_t_Lss	Difference in fluorescence between F_M_Dn and F_t_Dn in light adapted state
F_V/F_M (QY_max)	F_V / F_M	Maximum PSII quantum yield
F_V/F_M_Lss	F_V_Lss/F_M_Lss	PSII quantum yield of light adapted sample in steady-state
QY_Lss	F_q_Lss/ F_M_Lss	Steady-state PSII quantum yield
NPQ_Lss	(F_M - F_M_Lss)/F_M_Lss	steady-state non-photochemical quenching
qN_Lss	(F_M-F_M_Lss)/(F_M-F_0_Lss)	Coefficient of non-photochemical quenching in steady state
qP _Lss	F_q_Lss/F_V_Lss	Coefficient of photochemical quenching in steady-state

qL_Lss	(F_q_Lss/F_V_Lss) / (F_0_Lss/F_t_Lss)	Fraction of PSII centers that are 'open' in steady state
PAR*	LightA * LightIntesity + LightB	Photosynthetically active radiation calculated from light calibration curves
ETR_Lss	0.8 * 0.5 * QY_Lss * PAR	Calculated electron transport rate in steady-state

Tab. 7 List of parameters for the light curve protocol

* For PAR calculation, please add the values for Coefficient A and Coefficient B from Light Intensity Calibrations sheet into the protocol body row __LightA and __LightB. Use the coefficient for the given actinic light used in the light curve protocol.

6.1.5 Multicolor Fluorescence Wizard

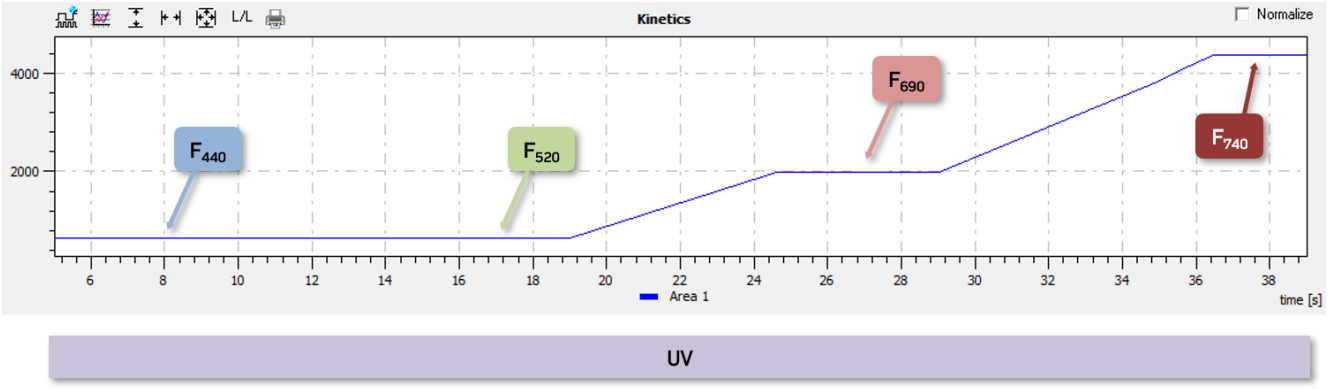

Fig. 18 Schematics of Multicolor fluorescence wizard (Multicolor).

Multicolor protocol		
F440	Measured	Blue autofluorescence value
F520	Measured	Green autofluorescence value
F690	Measured	Chlorophyll fluorescence in red region
F740	Measured	Chlorophyll fluorescence in IR region

Tab. 8 List of parameters for multispectral fluorescence measurement.

6.1.6 Fluorescence Proteins Detection Protocols

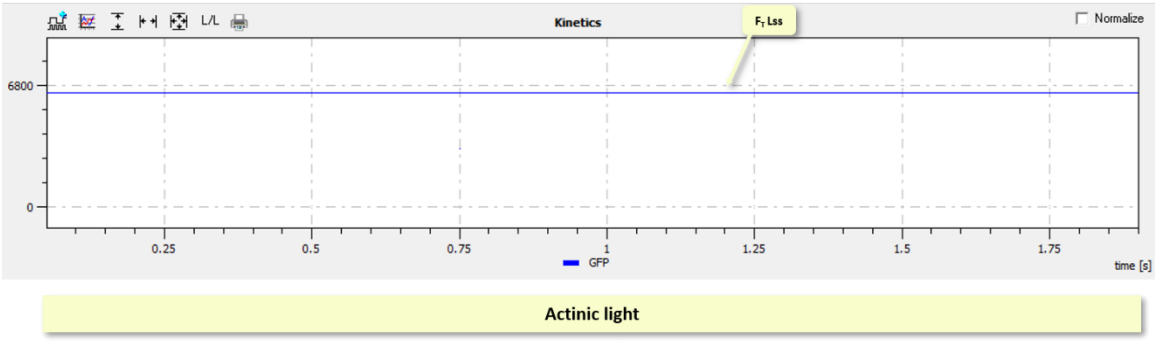

Fig. 19 Schematics of protocol for fluorescence proteins detection protocol (GFP, YFP, etc.).

FPs protocols		
F_t_Lss	Measured	Steady-state fluorescence in light

Tab. 9 List of parameters for fluorescence proteins detection protocol.

6.2 How to Start

There are few simple steps the user should follow before starting fluorescence measurement with FluorCam. The steps are briefly described in the following section (a detailed description of the individual steps is in the next sections).

1. Define the FluorCam protocol or Wizard used for the measurement. Select the protocols from the Wizard Protocol Menu or open previously used and/or modified protocol
2. Optimize the measurement settings. Adjust the camera settings, such as electronical shutter and sensitivity and set the light intensity used for the measurement (actinic light intensity or intensity of some of the additional lights).
3. Perform the test measurement with the optimized protocol and defined settings.
4. Start the protocol with optimized settings. Analyze and store obtained results.

6.2.1 Protocol Selection and Initial Set-up

Before starting a measurement, the user needs to decide on the type of measuring protocol to be used. After selecting the protocol, a number of settings needs to be optimized, such as light intensity, sensitivity and shutter duration. In the steps below, it is described how to find correct settings for light and camera before starting a real experiment.

CAMERA MODE (ONLY IF YOU USE A MULTICOLOR PROTOCOL, or for imaging of fluorescent proteins): Switch the camera from video mode to snapshot mode, since during measurement of the fluorescence proteins or of the multicolor protocol it is necessary to use longer integration time. Go to the upper ribbon menu Setup/Device and select Snapshot mode.

PROTOCOL: Open the protocol Menu and choose the measuring protocol. There is a set of predefined protocols or user alone can modify the protocols (e.g., duration of the light phase) by using Wizard function.

The predefined protocol F_V/F_M is a simple protocol determining F_0, F_M and F_V/F_M. This can be used to check if Saturating pulse is strong enough before running any other more complicated protocol.

When selecting the Multicolor wizard:

When the window Select Menu Protocol or Wizard Type appears, in the right part of the Wizard type select Multicolor.
The table Multicolor is divided into two parts – Light and Parameters, where the illumination and camera settings are set for the current experiment:

- UV for F440, F520 [%]: UV light intensity, which will be used during measuring with blue and green filters.
- UV for F690, F740 [%]: UV light intensity, which will be used during measuring with red and IR filters.
- Illumination [s]: length of UV illumination in seconds.
- Shutter [ms]: integration time of camera in milliseconds.
- CCD Sensitivity [%]: camera CCD chip sensitivity.

There are two restrictions:
1. The UV light intensity value cannot be lower than 5 %.
2. The shutter cannot be higher than 1000 ms.

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 (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 2000-2500 digits for (dark blue or blue color). For getting signal with less noise, keep the Sensitivity at lower level if possible (up to 20 – 30 %, 40 % max).

LIGHT SETTINGS – ACTINIC LIGHT OR ADDITIONAL LIGHT: Choose the intensity of Actinic light (Act1 or Act2) or Additional light (UV, royal blue, blue, green, amber, red): (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.

Light calibration curves for all lights available in the FC panel are part of the documentation provided and allow the conversion of the light intensity recorded in % to µmol.m^{-2}.s^{-1}.

Let measuring flashes switched on and adjust Actinic light intensity by trucking the bar in Global light settings table in LIVE window. An intensity between 20 – 50 % is usually strong enough for plants grown in chambers.

After choosing the Actinic light intensity, test the image quality: turn on the measuring pulses and the desired actinic light and check if the signal is not too low or saturating. If the fluorescence signal is too low, increase the Sensitivity to use a best adynamic scale. TOMI-2 CCD camera A/D converter is 16-bit, therefore the signal can be acquired on the scale 0 – 65 536. If the signal is saturated, an error message will appear. In this case, it is necessary to decrease Sensitivity or both Sensitivity + El. Shutter.

LIGHT SETTINGS – SATURATING PULSE INTENSITY: Truck the Super bar in Global light settings table in LIVE window to set the proper intensity of saturating pulse and then mark check box Super in Light Sources panel. 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 + Actinic light Act + Super) switched on as described above.

IMPORTING SETTINGS TO PROTOCOL: Click button Use in the bottom of LIVE window to import camera and light settings to the protocol.

TEST THE SETTINGS: Start Experiment in the top panel to launch the measurement. In the PRE-PROCESSING window select areas of interest, press button Analyze and observe the results (kinetics and parameters) in RESULT window for estimation of FC settings correctness. If the settings are correct (e.g., healthy green plant has QY_{max} value of 0.83), proceed with next step, if not, repeat the entire procedure.

SAVING RESULTS: Apply the protocol with correct settings. Analyze the results using the manual or automatic mask and save the experiment.

	For a more detailed description of the FC measurement protocol set-up, please also refer to FluorCam SW Manual.

6.2.2 FC Imaging System Corrections and Calibrations

Fish Eye Correction

Lens, which are used in the large FluorCam devices, always have some percentage of the barrel distortion, which can be corrected by software. It is necessary to obtain right values for constants *barrel* (adjustment of distortion), *k1* a *k2* (intensities in the corners of scene), which ensure the proper straightening of the scene. The Fish eye correction is usually done by PSI personnel or under the guidance of PSI personnel.

	Do not perform or change the fish eye correction process or constants by yourself! Incorrect settings can influence the results negatively.

Flat Field Correction

Flat Field correction is widely used for overcoming image artefacts, which can influence the resulting image negatively. Often these artefacts would be caused with non-homogeneous illumination across the imaged area, lens imperfections, etc. The correction consists in comparing the pixel values from the edges to the pixel values from the center of the image, in order to obtain homogeneous pixel values across the whole imaged area.

The flat field correction is available in all FluorCam devices and the procedure is done as follows:

1. The square area of the pixels with size ¼ is selected in the center of the image.
2. The weighted average value from the square is calculated.
3. Edge pixel values are corrected to the average value from the center square of the image.
4. The result provides a homogeneous distribution of the pixel values in the image and thus suppression of the artefacts.

For an effective flat field correction, please follow the next steps:

1. Insert the PSI Fluorescence standard (pink laminated sheet) under the FluorCam camera. The standard must face the pink side towards the camera.
2. Go to the upper ribbon menu Setup/Flat Field Correction (Fig. 20).

	Please note, that the correction can be done just for one light at time; therefore, select the light that will be used in the next measurement. If another light will be used in the next experiment, the calibration must be done again (from step 1) for this particular light.

3. Select the type of light (depends on FluorCam configuration - Act1, Act2, UV) for which the correction should be done.
4. The flat field correction information window appears, wait 30 seconds and confirm it with button OK. The application adjusts the ideal light intensity and camera settings automatically.
5. Launch the measurement by hitting the Start Experiment button in the upper ribbon menu (► Start).
6. After the measurement, select Flat Field Calibration in the right panel menu of Pre-processing widow to enable the flat field correction (Fig. 20B). Another option for enabling flat field correction is to go to upper ribbon menu Setup/Experiment and select Flat Field Calibration.
7. The measured images are corrected now.

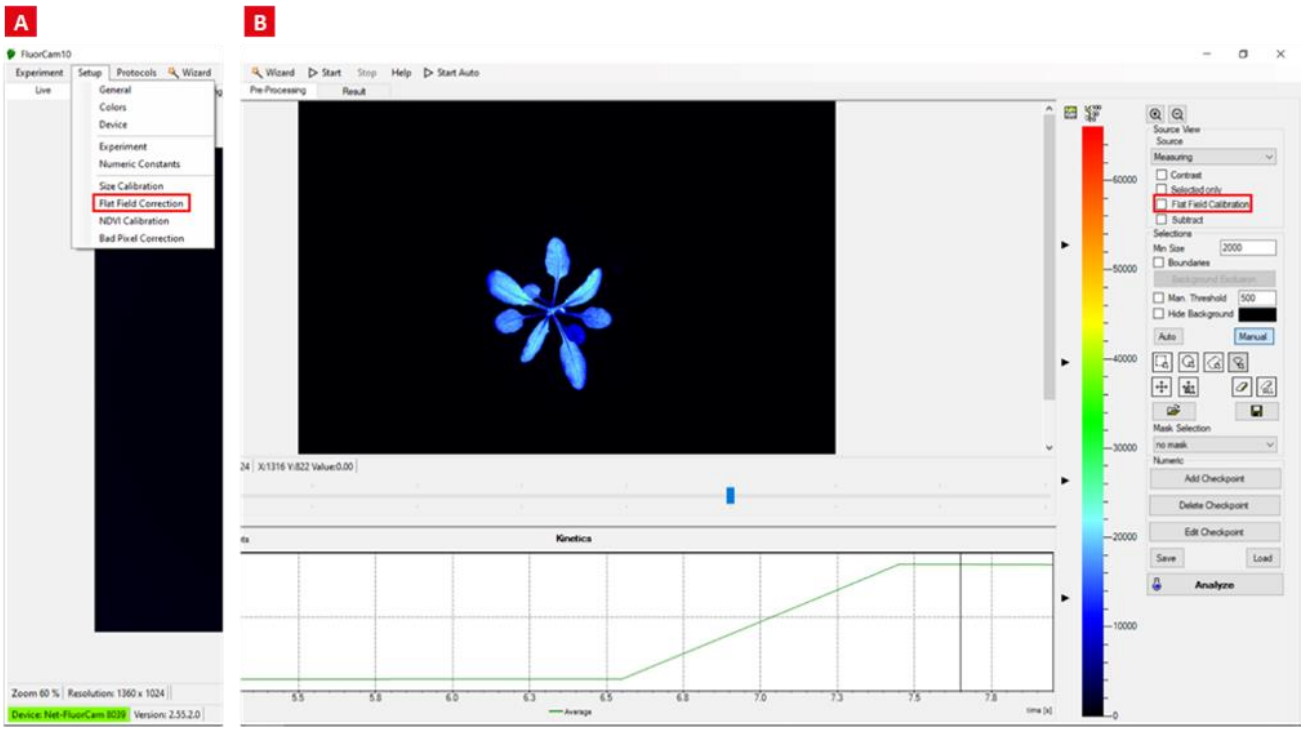

Fig. 20 Flat Field Correction setup.

Size Calibration

The image recorded by a FluorCam system can be calibrated from pixels to real size units such as millimeters (mm^2) or inches. One of the applications, inevitably coupled with this function, is an employment of pre-defined masks for Petri dishes or micro-titrate plates for automated image analysis in Pre-processing window of FluorCam10 application.
The size calibration is a simple algorithm based on automated recognition of fluorescent object of known dimension/area. The Size calibration standard (Fig. 21) is supplied with each FluorCam system for optimal calibration. This procedure is optimized for chlorophyll filter. Please note, that calibration standards would have different dimensions according to the type of your device. The size of the standard is always listed directly on it – on the back or front side (see size of calibration standard on Fig. 21– 400 mm^2).

1. Put calibration standard under FluorCam camera approximately to the same position (distance) as your experimental material (leaf or plant) would be placed – usually on the top shelf. Adjust focus (Fig. 22A)

2. In top panel menu in FluorCam10 application, select Setup (Fig. 22B) and then Size Calibration. Once the message INSERT CALIBRATION PLATE appears, the excitation light is turned on and software starts to adjust optimal exposure settings automatically.
3. Place fluorescent area of the calibration standard to the center of the field of view, wait until the image is stable and confirm it by button OK.
4. The new pop-up window invites you to enter the size of identified area in mm^2. The correct value is typically written on the bottom of the calibration standard (Fig. 21).

Fig. 21 Fluorescence standard.

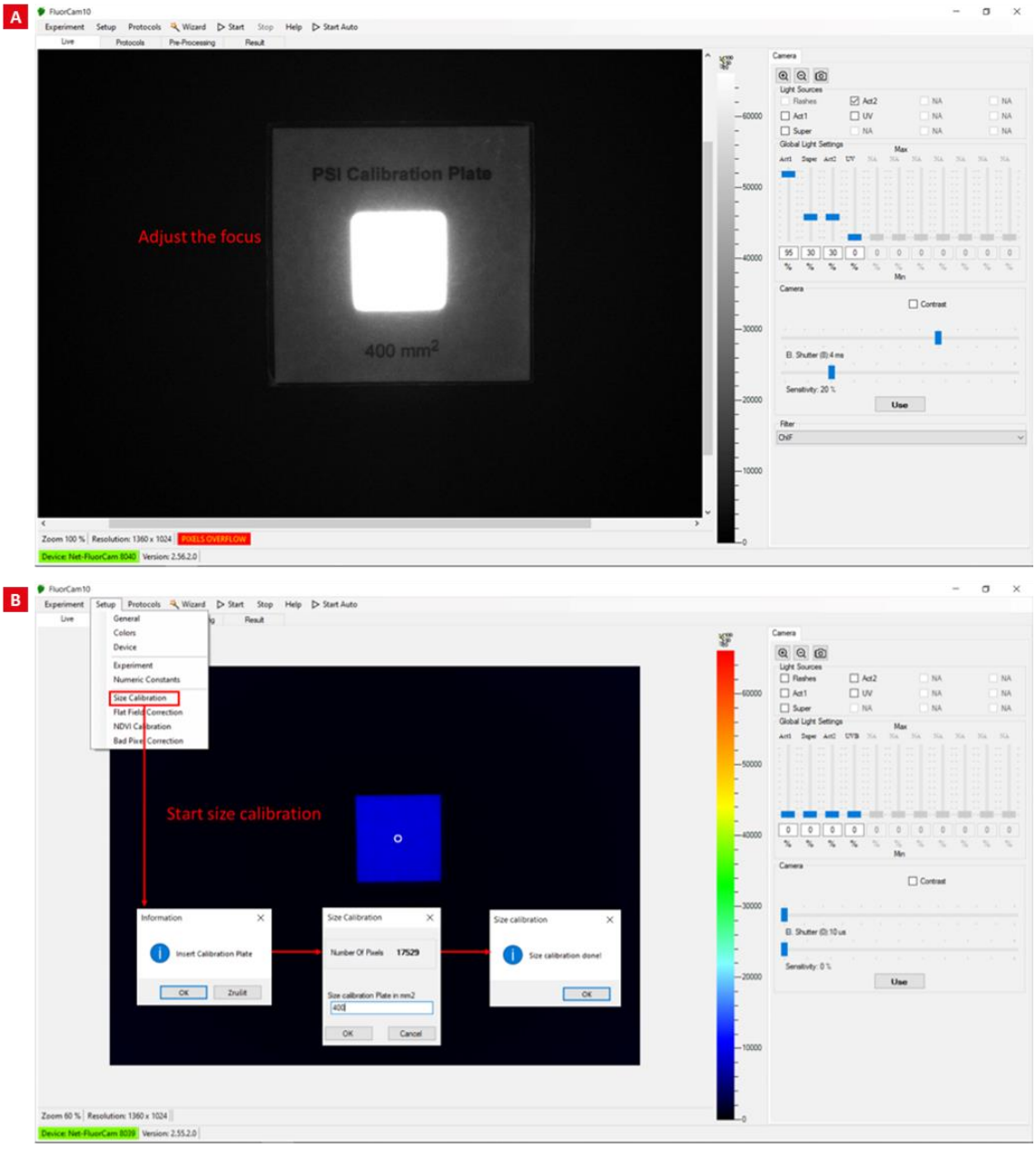

Fig. 22 Size calibration setup.

	Please note: The system must be re-calibrated whenever the distance between experimental object and camera is changed (e.g.: selecting different shelf position in Closed FC version). Once the system is calibrated, this information is saved together with measured experiment. Thus, the Size calibration must be done in advance, before the experiment is measured.

After successful Size calibration, the Area of analyzed object in Result window is displayed in mm^2 or inches instead of number of pixels.

Bad Pixel Correction

The appearance of bad pixels in the image can be fixed by the Bad Pixel Correction:

1. Cover the lens with a lens cap.
2. In top panel menu in FluorCam10 application, select Setup (Fig. 23) and then Bad Pixel Correction. Once the message COVER OBJECTIVE appears, the software starts to detect bad pixels automatically.
3. Wait until the calibration is done and confirm it by button OK.
4. The application of the Bad pixel correction is enabled in the Setup/Experiment menu after selection of the Bad Pixel Correction.

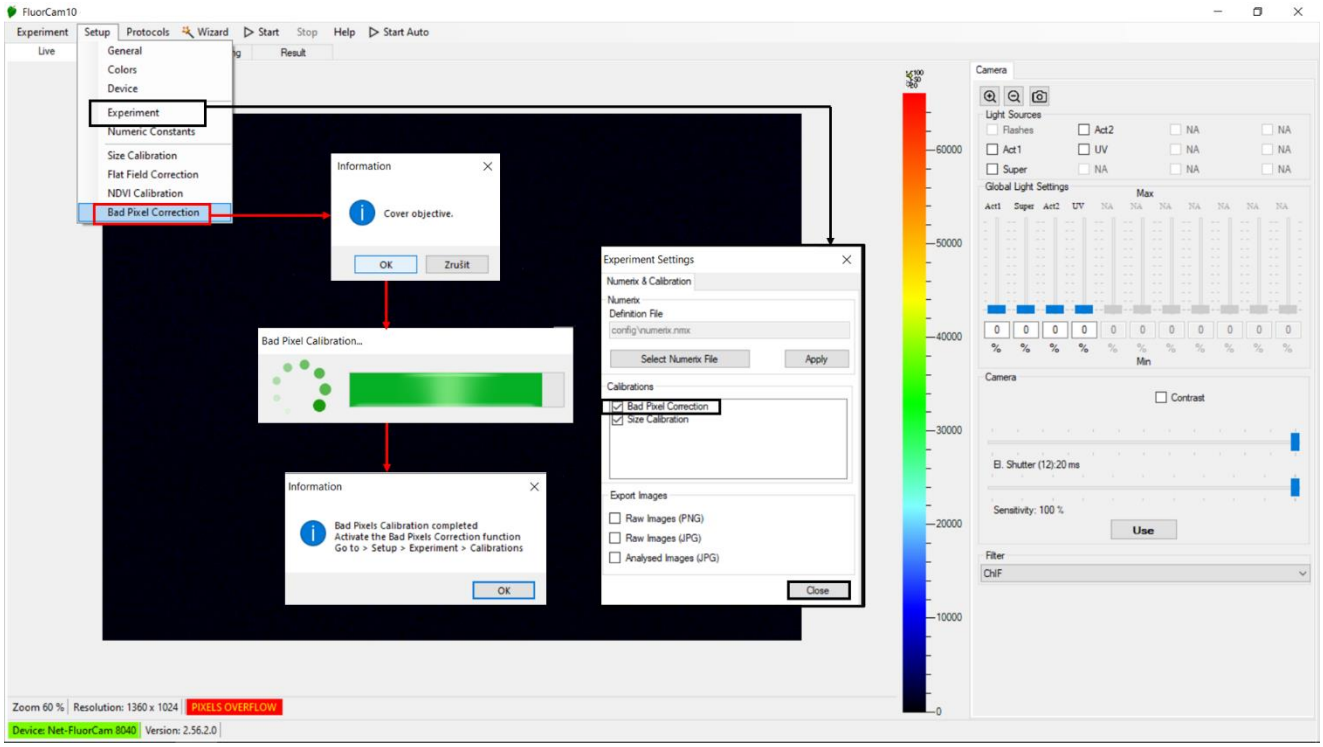

Fig. 23 Bad pixel correction setup.

7 FLUORCAM KEYWORDS

Flashes – for accurate measurement of minimal fluorescence (F0) determination. The duration of flashes is controlled by shutter time. Try to keep 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.

Actinic light 1 – (usually red-orange light) corresponds to the ambient light in which the plants are growing.
Actinic light 2 – (usually cool white) corresponds to the ambient light in which the plants are growing.
Saturating pulse – Short and strong pulse of light used for maximal fluorescence (FM value) determination.
FAR – far-red light for determination of parameter F0.
TOMI-2 – High resolution CCD camera.
Binning - the procedure of combining a cluster of pixels into a single pixel.
Shutter (time) – controls the exposure time. It is the length of time during which light is admitted by the image sensor of the camera. Controls also duration of the measuring flashes. User can usually choose shutters 0 – 12 representing values in range 0 us – 20 ms.
Sensitivity – The sensitivity of the camera sensor to light.
Protocol – predefined set of instructions for the FluorCam.
Video mode – automated measurement defined by the protocol. Video mode is used for measurement of chlorophyll fluorescence protocols. If the flashes are not used in the measurement, maximal shutter time is set to 20 ms.
Snap mode – capturing the single images with longer shutter times (up to 100 seconds). This mode is useful for measurement of fluorescent proteins, where long integration time is necessary. Measuring in snap mode can be also automated using the protocol.
Fisheye correction – used for correction of the fisheye distortions.
Flat Field correction - is widely used for overcoming image artefacts caused with non-homogeneous illumination across the imaged area, lens imperfections, etc. Easily said, the correction is evaluating the pixel values from the edges regarding to the pixel values from the center of the image to obtain homogeneous pixel values across whole imaged area.
Size calibration – for image calibration from pixels to real size units such as millimeters (mm2) or inches.
Background exclusion – button used for excluding background and the objects (mold, algae, …), which also reflects the light, from analyzed area.
Emission filters - transmit a wavelength range that corresponds to the respective fluorophore emission spectrum, while blocking out unwanted wavelengths.
Filter wheel – If there is more than one (usually chlorophyll) filter, filter wheel is used to position a selected filter in the imaging path quickly and accurately.
Fluorescence standard – is made of pink fluorescence material, which reacts very strongly to excitation illumination and is used for different system calibrations (size calibration, focusing etc.).
Teflon plate - used for the different types of the calibrations, where the light reflection is essential, such as NDVI calibration.
Wizard menu – contains Protocol menu with the list of predefined protocols and Wizard type window where user can modify settings of the given protocols.
Multicolor module – option for measuring multicolor fluorescence caused by UV light. For this measurement FluorCam device must be equipped with 4 UV LEDs 380 nm, four filters - BLUE (FF01-440/40), GREEN (FF02-520/28), RED (FF01-690/8), FAR-RED (FF01-747/33).
Fluorescence protein measurement - detection and imaging of different fluorescent proteins, mostly of green fluorescent protein (GFP), red-shifted GFP (EGFP). The FluorCam device must be then equipped with additional LED panels with appropriate wavelength for selected fluorescent protein excitation and emission filter for correct fluorescence detection.

8 WARRANTY TERMS AND CONDITIONS

1. This Limited Warranty applies only to the FC1300-C/3535 device. It is valid for one year from the date of shipment.
2. If at any time within this warranty period the instrument does not function as warranted, return it and the manufacturer will repair or replace it at no charge. The customer is responsible for shipping and insurance charges (for the full product value) to PSI. The manufacturer is responsible for shipping and insurance on return of the instrument to the customer.
3. No warranty will apply to any instrument that has been (i) modified, altered, or repaired by persons unauthorized by the manufacturer; (ii) subjected to misuse, negligence, or accident; (iii) connected, installed, adjusted, or used otherwise than in accordance with the instructions supplied by the manufacturer.
4. The warranty is return-to-base only and does not include on-site repair charges such as labor, travel, or other expenses associated with the repair or installation of replacement parts at the customer's site.
5. The manufacturer repairs or replaces faulty instruments as quickly as possible; the maximum time is one month.
6. The manufacturer will keep spare parts or their adequate substitutes for a period of at least five years.
7. Returned instruments must be packaged sufficiently so as not to assume any transit damage. If damage is caused due to insufficient packaging, the instrument will be treated as an out-of-warranty repair and charged as such.
8. PSI also offers out-of-warranty repairs. These are usually returned to the customer on a cash-on-delivery basis.
9. Wear & Tear Items (such as sealing, tubing, padding, etc.) are excluded from this warranty. The term Wear & Tear denotes the damage that naturally and inevitably occurs as a result of normal use or aging even when an item is used competently and with care and proper maintenance.