IMAGING-PAM
M-Series
Chlorophyll Fluorometer

Instrument Description
and
Information for Users

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1 Safety instructions

1.1 General safety instructions

1. Read the safety instructions and the operating instructions first.
2. Pay attention to all the safety warnings.
3. Keep the device away from water or high moisture areas.
4. Keep the device away from dust, sand and dirt.
5. Always ensure there is sufficient ventilation.
6. Do not put the device anywhere near sources of heat.
7. Connect the device only to the power source indicated in the operating instructions or on the device.
8. This product can be damaged by some volatile cleaning agents. Clean the device only according to the manufacturer's recommendations.
9. If the device is not in use, remove the mains plug from the socket.
10. Ensure that no liquids or other foreign bodies can find their way inside the device.
11. Keep shipping material – poor packaging of the instrument may cause damage during shipping
12. The device should only be repaired by qualified personnel.

1.2 Special safety instructions

The IMAGING-PAM is a highly sensitive research instrument which should be used only for research purposes, as specified in this manual. Please follow the instructions of this manual in order to avoid potential harm to the user and damage to the instrument.

Never use the *Multi Control Unit* IMAG-CG with more than one *Measuring Head* plugged in at the same time.

The IMAGING-PAM employs strong blue light for excitation of chlorophyll fluorescence, for driving photosynthetic electron transport and for transient saturation of photosynthetic energy conversion (Saturation Pulse method). To prevent damage to the eyes, avoid looking directly into the light sources. This is especially true during saturating flashes.

2 Introduction

The IMAGING PAM Chlorophyll Fluorometer is designed for analyzing spatial heterogeneities in PSII activity. The Imaging PAM M series supports a broad range of applications, from large samples exceeding multiwell plate dimensions to microscopic specimens at the single-cell level. The IMAG-3D version further enables three-dimensional sample scanning.

All versions—**IMAG-3D, MAXI, MINI, and MICROSCOPY**—are operated via the common **Multi Control Unit IMAG-CG**. As with all PAM fluorometers, the instruments employ pulse-amplitude-modulated (PAM) measuring light to quantify chlorophyll fluorescence yield. The same LEDs provide measuring light, actinic illumination to drive photosynthesis, and Saturation Pulses, which transiently close PSII reaction centers. This non-invasive **Saturation Pulse method** enables precise assessment of photosynthetic performance by determining the quantum yield of PSII energy conversion, which is influenced by physiological state, light environment, and stress factors.

Since the introduction of PAM fluorometry in 1985, extensive literature has demonstrated its value across plant sciences. With the IMAGING PAM M series, characteristic fluorescence parameters (Fo, Fm, Fm′) can be measured directly, allowing calculation of quenching coefficients, PSII quantum yield (Fv/Fm or ΔF/Fm′), as well as Induction Curves (IC) and Rapid Light Curves (RLC) for quenching analysis.

The key advance of **fluorescence imaging** lies in its ability to detect lateral heterogeneities in fluorescence parameters, which mirror physiological variation. Even healthy leaves exhibit patchiness in stomatal conductance, and stress-induced limitations—often preceding irreversible damage—are distributed unevenly across the

leaf surface. Fluorescence imaging thus provides a sensitive tool for the **early detection of stress-related damage**, with major applications in **plant stress physiology** and **plant pathology**.

A distinctive strength of the **Imaging PAM** compared to conventional PAM fluorometers is the **parallel analysis of multiple samples under identical conditions**. The **MAXI-version** is particularly suited for such applications, including mutant screening in molecular biology or high-throughput analysis of multiwell plates (up to 96 wells) in ecotoxicological studies. The **MINI-version**, by contrast, is compact and easy to handle, making it ideal for field applications. Both MINI and MAXI systems can be integrated with the **GFS 3000 Gas Exchange System**, enabling simultaneous measurements of CO_2 assimilation, transpiration, and additional physiological parameters.

Furthermore, Imaging PAM instruments can be coupled with the **Walz ULM-500 Universal Light Meter** and an appropriate cosine-corrected PAR sensor (e.g. LS-C), allowing automated calibration of the measuring light or operation of MAXI and MINI versions under ambient light conditions. The **IMAG-3D version** extends the MAXI imaging head with a 3D scanner that generates detailed point clouds of plant surfaces, which can be further processed into quantitative morphological datasets.

The MICROSCOPY-version provides the imaging of heterogeneities at the level of single cells (e.g. guard-cells or algae cells). Using the RGB-Head it is even possible to differentiate between different algae groups like diatoms, chlorophytes and cyanobacteria.

The MAXI-, MINI- and 3D-versions not only measure fluorescence yield but also provide estimates of incident light absorptivity (parameter *Abs.*; not available for the MICROSCOPY version). This capability is particularly relevant for detecting lateral heterogeneities in chlorophyll content, which often accompany

stress- or pathogen-induced damage. Absorptivity of incident photosynthetically active radiation (PAR) is determined by irradiating the sample with diffuse, balanced near-infrared (NIR) and red light; the reflected portions are recorded using the same camera employed for fluorescence imaging.

Because the different Imaging PAM M series versions are optimized for distinct sample sizes, they employ different LED configurations, while applying the same measurement principles. Power requirements for measuring, actinic, and saturation pulse illumination vary substantially. The **MAXI IMAGING PAM** features a high-performance array of 44 Cree 3 W LEDs, arranged in four groups for exceptional illumination homogeneity. The **MINI IMAGING PAM** uses 12 of the same LEDs, while the **MICROSCOPY IMAGING PAM** employs either a single LED coupled to the excitation port of Zeiss AxioScope 5 Epifluorescence Microscopes or an optional RGB head, offering three excitation channels that can also be combined to "white." All measuring heads are operated via the common **IMAG-CG Power and Control Unit**.

Depending on application requirements, different camera systems are available. Historically, highly sensitive CCD cameras such as the **IMAG-K6** were employed; however, since such models are no longer manufactured, the range has been expanded to include a **new CMOS-based system**. The **IMAG-K9 CMOS camera** sets new standards for sensitivity and flexibility, featuring a large detector chip and optional 2×2 pixel binning. In the medium term, CMOS technology will fully replace the previous CCD systems.

For high-sensitivity applications, such as phytotoxicity bioassays with dilute algal suspensions in 96-well plates, the **2/3″ CCD camera IMAG-K6** (1392 × 1040 pixels, used in binned mode) remains the preferred option and is also employed in the MICROSCOPY versions. For routine applications involving samples

with relatively high chlorophyll content, an economical **1/2″ CCD camera** (640 × 480 pixels) is available, particularly in combination with a powerful zoom objective lens.

This manual provides essential information on the components of the various Imaging PAM M series versions and on **ImagingWin software**. While the software applies in principle to all models, certain features are version-specific—for example, RGB-Fit imaging in the MICROSCOPY/RGB version or morphological analysis with the 3D version. As the most versatile configuration, the **MAXI version** is emphasized throughout this manual.

3 Components of the IMAGING-PAM MAXI-version

In the following chapters the three different versions: the MAXI-, MINI- and Microscopy-version of the Imaging-PAM M-series are described. As the MAXI-version is most frequently used, those components that are common to all versions are dealt with in this chapter on the MAXI-version.

The basic measuring system of the MAXI-IMAGING-PAM consists of:

1) Control Unit IMAG-CG with Battery Charger 2120-N and External 300 W Power Supply

2) LED-Array Illumination Units IMAG-MAX/L (blue) or IMAG-MAX/LR (red). A useful accessory for measurements with IMAG-MAX/L investigating mirroring samples (like 96-well plates or petri dishes) is the Filter Plate IMAG-MAX/F, which absorbs the small fraction of red light contaminating in the blue LED light.

3) CCD Cameras IMAG-K7 or IMAG-K6 with accessories and mounting sets (please inquire for further options)

4) Mounting Stand with Eye Protection IMAG-MAX/GS, laboratory stand ST-101, additional filter plate IMAG-MAX/F or Leaf Distance Holder IMAG-MAX/B

5) PC with ImagingWin-software (recent versions available on Walz homepage)

Combining imaging and gas exchange measurements, the adapter IMAG-MAX/GWK for gas exchange chamber GWK1 is available.

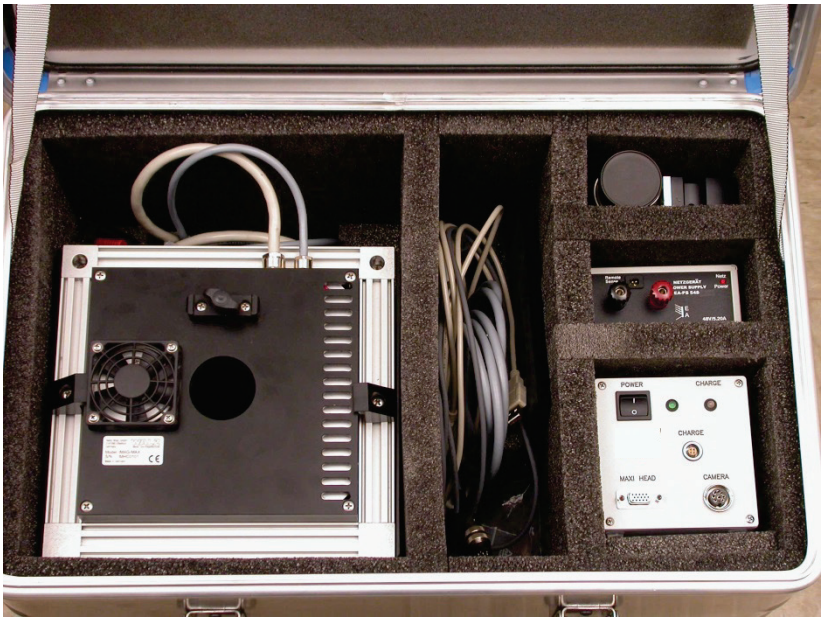


Fig. 1 Transport Box IMAG MAX/T provided with each of the basic Imaging systems (except 3D-version) offers enough space for all instrument components.

For transport of components 1 - 4, as well as of all essential cables, the sturdy transport box IMAG-MAX/T is provided.

In the LED-Array Illumination Unit an Adapter Ring may be inserted (depending on the lens used). To avoid damage to the instrument, please do not take the instrument out of the box by using this hole as a handle – LEDs might get damaged.

3.1 Control Unit IMAG-CG

The Control Unit IMAG-CG contains a rechargeable Li-ion battery (14.4 V/6 Ah). The main printed circuit board of the IMAG-CG contains a RISC microcontroller processor, the power supply for the CCD camera and LED drivers for the Maxi-LED-Arrays. The same LED drivers also serve for the alternative MINI- or MICROSCOPY-Heads (MINI- and RGB-sockets at the backside of the instrument). Control Unit IMAG-CG provides the power for driving the LED-Arrays of all members of the M-Series systems except the MAXI-IMAGING-PAM, which is driven by an external 300 W Power Supply.

Never use Control Unit IMAG-CG with more than one Measuring Head connected at the same time.

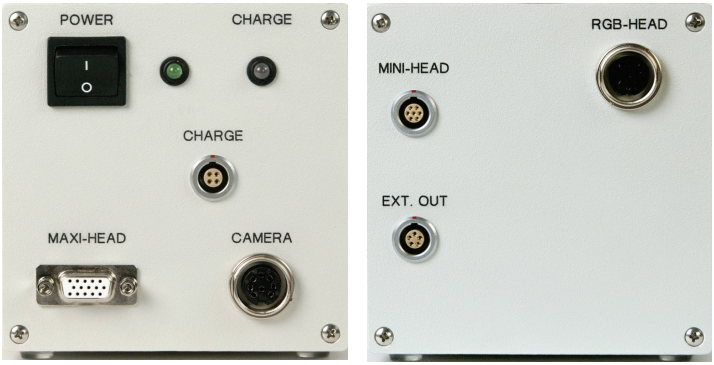


Fig. 2: Front and rear side views of the Control Unit IMAG-CG Control units compatible with the new IMAG-K9 will have a different Camera connector than the older versions (Serial numbers will end with a “C” (like IKEC0…C)

The functional elements at the front side of the instrument are:

POWER Power on/off switch; when switched on, the green status LED at the right-hand side of the switch lights up.

CHARGE-LED lights up red while battery is charged (only use standard battery charger 2120-N). When battery is fully charged, the LED lights up green as long as charger is connected. Battery lifespan can be prolonged when not permanently charged.

CHARGE socket Only connect battery charger 2120-N. An external 12 V battery cannot recharge the internal 14.4 V Li-ion battery.

Avoid charging the internal Li-ion battery while IMAGING-PAM is switched on.

MAXI-HEAD Socket for connecting LED-Array Illumination Unit IMAG-MAX/L (MAXI-Head).

CAMERA Socket for camera cable via which trigger signals and power is transferred to the CCD-camera of the MAXI-Head or MICROSCOPY-IMAGING-PAM.

Please pay attention to the fact that older central units IMAG-CG may have a slightly different Camera Socket that does not fit to the IMAG-K9 I/O cable (number of pins differ)

Functional elements at the rear side of the housing:

The Control Unit features three sockets, which apply for use of alternative Measuring-Heads:

MINI-HEAD Socket for connecting MINI-Imaging heads as well as the IMAG-L470M5 or IMAG-L625M5 LED lamps of the MICROSCOPY-version

RGB-HEAD Socket for connecting the optional Red/Green/ Blue-Head of the MICROSCOPY-version

Ext. out Socket for connecting an optional external light source.

When using a MINI-version, the measuring head can directly be mounted on the top of the control unit housing IMAG-CG facing upwards. For this purpose, a wing-screw is provided.

3.2 LED-Array Illumination Unit IMAG-MAX/L and IMAG-MAX/LR

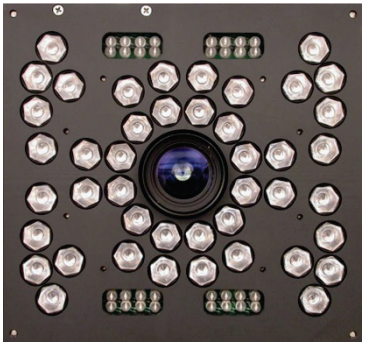


Fig. 3 Front view of LED array IMAG MAX/L with objective lens of CCD-camera protruding through central opening

The LED-Array Illumination Unit IMAG-MAX/L is equipped with 44 high-power royal-blue (450 nm) Cree LEDs with collimating optics, arranged to ensure maximal intensity and homogeneity at a working distance of 17–20 cm from the object plane. These LEDs provide the pulse-modulated blue excitation light and simultaneously serve for actinic illumination and Saturation Pulses.

In addition, four groups of eight LEDs deliver the pulse-modulated light required for PAR absorptivity assessment (see 10.1.1.10). Each pair within these groups consists of one red (660 nm) and one near-infrared (780 nm) LED. To achieve homogeneous sample illumination, the lenses of these LEDs are

milled away. Although only a relatively small fraction of this light is remitted from the sample to the CCD camera, the signal quality is sufficient because both wavelengths pass through the red long-pass filter in front of the CCD chip—unlike the blue excitation light.

Since 2018, individually cut diffuser films have been mounted in front of the IMAG-MAX/L LED array to further improve illumination homogeneity at the sample plane.

Owners of current LED panels can skip this section, as the individual diffuser film described here is no longer necessary due to a realignment of the LEDs. These newer, blue LED panels can be identified by their serial number. Serial numbers will end with a “B” (like LRLB0…C). This does not apply to red LED panels because a different type of LED is used there.

It is essential that the diffusor films used in the older versions of the blue LED panels, once removed, are reinstalled in the correct orientation. Depending on the instrument configuration, up to three film layers may be held in place either by the IMAG-MAX/F filter plate or by a separate diffuser holder (part no. 000244625113).

If the blue/green filter plate IMAG-MAX/F is already installed, the separate diffuser holder is supplied as an accessory in the instrument box. It can be mounted instead of the IMAG-MAX/F BG filters when no additional emission filters are required for a given measurement. For mounting diffusor films, it must be checked that the film

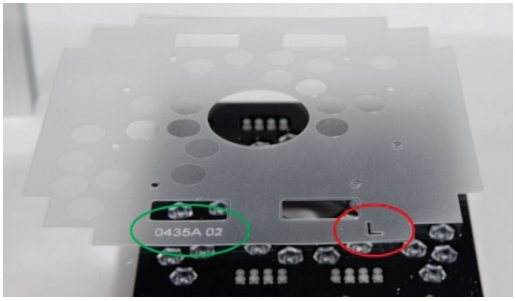


Fig. 4 diffusor film provided has an individual design – check for correct orientation

layers are having the correct orientation. The correct order is not important.

Start by detaching the LED array from the rest of the Imaging system, place the IMAG-MAX/L upside down onto the work bench and make sure that cables are not hanging down the table to avoid dropping of the part.

Each diffusor film carries the serial number of the LED array IMAG-MAX/L for which it has been adjusted (marked green, Fig. 4).

On the right lower side of each diffusor film an L-shaped orientation mark can be found which has to be aligned with the cables entering of the LED array IMAG-MAX/L (marked red).

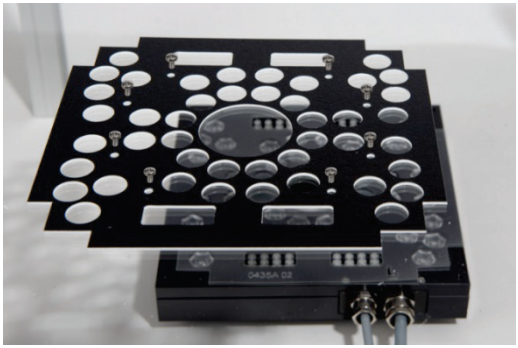


Fig. 5 If no filter plate is mounted a plastic holder keeps diffusor film in place

To keep the diffusor layers in place the blue-green filter plate IMAG-MAX/F or the alternative diffusor film holder 000244625113 can be used. Please note that in the latter case the white side of the plastic plate shall point towards the LED array as shown in the image (Fig. 5). As mounting material 8 screws are used.

A useful accessory for measurements with reflective samples (like 96-well plates filled with algae suspensions or Petri dishes) is the filter plate IMAG-MAX/F (Fig. 6) that absorbs the small fraction of red light still contained in the blue LED light. This plate can be mounted with 4-8 screws at the front side of the Illumination Unit and would substitute for the plastic diffusor film holder described

above. IMAG-MAX/F will hold the plastic film in place in the same way.

Unavoidably, the effective PAR-values are lowered by about 15% by the filtering and thus a change in configuration will make a recalibration of the internal light list necessary.

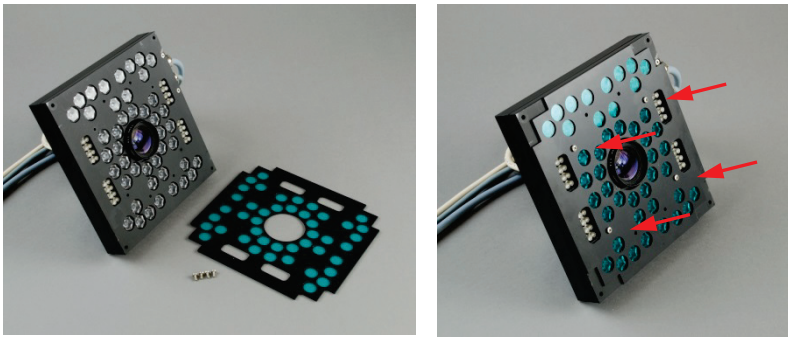


Fig. 6: Mounting of IMAG-MAX/F in front of blue LED array IMAG-MAX/L. Four screws are provided with the filter plate. The filter plate shown in the image is only compatible with the optional blue version. The IMAG-MAX/LR red excitation light version includes a different filter plate that is mounted by default.

The LED-Array Illumination Unit IMAG-MAX/LR has a similar organization as IMAG-MAX/L but features 44 red (650 nm) high-power LED-lamps and the four groups of red (660 nm) and a near-infrared (780 nm) LEDs. **The IMAG-MAX/LR includes the filter plate IMAG-MAX/FR which must stay mounted permanently**.

Both illumination units feature two cables, which connect to the "MAXI-HEAD" socket at the front side of control unit IMAG-CG and to the external grey 300 W power supply.

Please note that due to changes of LEDs during the last years also the voltage of the grey power supply (only MAXI version) had to be changed. Each power supply has a serial number that indicates the

IMAG-MAX/L LED array that it has been adjusted to. Interchanging these power supplies may damage the LED array.

If such information got lost, please us at info@walz.com and send the serial number of your LED array.

This information does not apply to chargers for central control unit IMAG-CG or IMAG-CM

Fig. 7: IMAG-MAX/L with CCD camera IMAG-MAX/K6 on top of IMAG-MAX/GS

On the top side of the Illumination Units a fan is located that serves for cooling the aluminum plate on which the slugs of the high-power LEDs are mounted. The adapter, on which the CCD-camera is mounted, at the rear side features an adapter hole for installation of a 15 mm ∅ metal bar (provided with the system), which can be used for secured fastening of the measuring head independently of its mounting stand with eye protection (IMAG-MAX/GS) (shown in Fig. 8). The rod is fixed with a small grub screw for which a suitable Allen key is supplied.

Please note that the screw must press against the flattened side of the rod to ensure secure fixing.

Fig. 8: Measuring Head consisting of LED-array illumination unit and CCD-camera mounted via 15 mm Ø metal rod on optional stand (stand with base plate ST 101)

If the MAXI measuring head is operated without eye protection, the user is obliged to ensure that no one can accidentally look into the LEDs while a measurement is in progress.

In these cases, the user is strongly recommended to provide an alternative eye protection or cover that effectively protects the eyes in applications where the standard IMAG-MAX/GS cannot be used.

If the LED Array is mounted independently of the mounting stand with eye protection (IMAG MAX/GS, see 3.5), the working distance of the LED Array can be adjusted between 14.5 and 22.5 cm, resulting in image areas between 7.5 x 10 and 11 x 15 cm. Optimum light field homogeneity is achieved at the standard distance of 18.5 cm (max. +/- 7% deviation of the intensity from the average value).

A fixed standard distance of 18.5 cm is provided when the LED array IMAG-MAX/L is mounted on the IMAG-MAX/GS (see 3.5) or leaf clip adapter IMAG-MAX/B (see 3.6 for details). Since LED intensities are controlled by ImagingWin software, the photon flux

density (PAR) at this standard distance is well defined and reproducible if the measurement configuration is maintained.

Small deviations in the PAR distribution as well as the unavoidable vignette effect of the camera objective lens can be measured and corrected by software (see Image Correction in section 10.5.5).

Three individual image corrections can be recorded by the ImagingWin software: Type 1, Type 2 and MAXI (chapter 10.5.5). For repeated use of defined measuring configurations, it saves time to switch to a previously recorded image correction.

An adapter ring can be inserted into the LED array illumination unit (depending on the configuration for small diameter lenses), this is not screwed on. To avoid damaging the instrument, please do not remove it from the packaging by using this hole as a handle - the adapter ring could slip out or damage the LEDs.

3.3 CMOS Camera IMAG-K9 and lenses K9-MAX and K9-MAX/Z

Fig. 9: CMOS Camera IMAG-K9 without detection filter

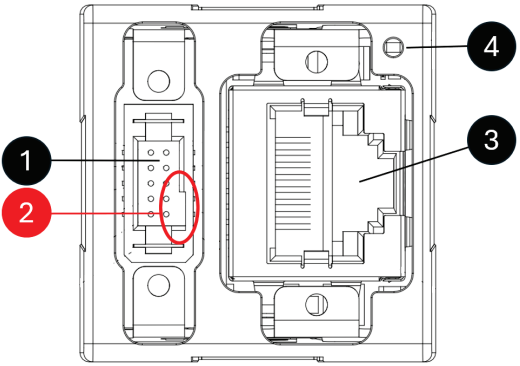


Fig. 10: The back panel of CMOS Camera IMAG-K9: 1. I/O connector, 2. the 10-pin TFM I/O port connector has a small notch that can easily be overseen. Please make sure that the plug is inserted in the correct direction, 3. Ethernet connector (RJ-45), 4. status LED

LED codes	Behavior	Status
	Continuously active	Camera is initializing
	1 flash per second	Camera is operational
	Continuously active	Error state

Fig. 11: The IMAG-K9 camera has an LED to signal its status in yellow, green, or red color

The CMOS camera IMAG-K9 features a 2/3" sensor chip that is typically used in 2x2 binning mode, resulting in a resolution of 640 x 480 pixels across the measuring field. The software also offers the option of using the IMAG-K9 camera in unbinned mode. This function acts as a kind of digital zoom, automatically setting the camera's internal signal amplification to a higher value so that the user can continue to use the previously selected settings.

The recorded images are digitized by the camera and transmitted via ethernet interface (GigE-Vision®) to the PC. The sockets for connecting the GigE-cable and the I/O cable located on the back of the camera (top side, when the camera is mounted).

For applications that need a lot of sensitivity it is recommended to use the K9-MAX lens we provide for the MAXI IMAGING-PAM. The camera achieves outstanding imaging performance and sensitivity through a combination of 2x2 binning and a large lens aperture.

The aperture of the IMAG-K9 lenses can be closed to increase the depth of field. This necessarily reduces the signal intensity.

For plane objects, it is recommended that aperture remains fully open. In case of IMAG-3D aperture must not be changed!

In the complete system (IMAGING-PAM MAXI version), the IMAG-K9 camera is connected to the IMAG-MAX/L lighting unit via a metal rail (IMAG-MAX/M).

The installation of an IMAG-K9 camera for use with the MINI version is described in the relevant chapters. The camera can be used for various measurement setups. In this case, it may be necessary to also change filters and/or spacer rings between the lens and camera. Both must be used in a defined combination to obtain a sharp image and good signal quality.

A Kowa lens with 16 mm focal length (F=1.4; K9-MAX) is used in conjunction with the LED-Array Illumination Unit IMAG-MAX/L or IMAG-MAX/LR. An RG 665 (Schott) color glass filter serves as long-pass filter for protecting the CMOS-chip from blue excitation light, while passing the red fluorescence as well as the 660 and 780 nm measuring beams for PAR-Absorptivity (see 10.1.1.10 - 10.1.1.14). A short-pass filter ($\lambda < 790$ nm), mounted in a threaded metal ring, protects the detector against excess near-infrared radiation contained in ambient daylight. For the optical properties of the camera it is essential that this filter as well as the RG 665 are placed between CCD-chip and objective lens (increase of effective focal length by plane-parallel glass plates). For other M-Series instruments like the GFP MINI-Version this filter must be removed before mounting the camera (with the GFP measuring head an exchange filter is provided.

Fig. 12 Kowa prime lens with 16 mm focal length, short pass filter and mounting device (left), distance ring (middle) used with IMAG-K9 CMOS camera on MAXI heads. The distance ring must be placed between lens and camera to enable correct focusing at short distances

Detection filters as shown in Fig. 12 are matched to a specific excitation light color by the LED panel used. For this reason, they must not be interchanged, e.g., when the LED panel in the measuring head is replaced. The corresponding detection filters are either color-

coded or labeled when measuring heads with the interchangeable option or several LED panels are supplied.

Please note that due to the significant differences between the IMAG-K7 and IMAG-K6 CCD cameras and the IMAG-K9 CMOS camera, the I/O cables of the two systems have different connectors to prevent confusion. For this reason, the Central Units cannot be exchanged between the camera systems. The corresponding IMAG-CG central unit also has different connections, depending on the type of camera for which it was built.

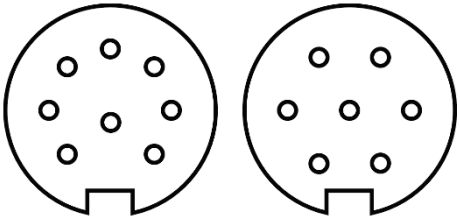


Fig. 13 IMAG-K9 camera with Kowa 16 mm prime lens mounted. In a new set, the cameras are already mounted on the mounting set. If a lens is to be changed, the correct camera position must be observed. To prevent ambient light from entering the housing, the adapter ring supplied can be used Fig. 16.

Fig. 14 IMAG-K9 camera with Ricoh zoom lens mounted. Due to their different lengths, the lenses are attached to different positions on the mounting set. The plastic block on the rear is used to hold the cable. It moves to the vacant position during conversion. To use the zoom lens with a MAXI imaging system, the LED array adapter ring must be removed before installation (Fig. 16).

The IMAG-K9 camera has a very handy digital zoom option. However, a continuous zoom lens is also available for its 2/3" CMOS chip Fig. 14 the K9-MAX/Z. This is a Ricoh lens with a variable focal length of 12.5 to 75 mm. With an aperture of F1.8, the zoom lens is

significantly less light-sensitive than the recommended fixed focal length, but offers the possibility of very flexible framing for a wide variety of sample sizes. It should be noted that Kowa's fixed focal length offers far better image quality and that digital zoom is also available via the software.

Installation on the most commonly used IMAG-MAX/GS privacy screen frame is described in detail in the following chapters and does not differ from the installation of the old camera/lens combination.

3.4 CCD Camera IMAG-K7 and objective lenses K7-MAX/Z and K7-MAX/S (old)

Fig. 15 IMAG-K7 camera with zoom lens K7-MAX/Z and mounting set IMAG-MAX/M.

IMAG-K7 (Allied Vision Technologies) features a 1/2" chip with 640 x 480 pixel resolution. The data are digitized within the camera and transferred via Ethernet interface (GigE-Vision®) to the PC. Analog to camera IMAG-K6 the sockets for connecting the Ethernet cable and camera-power-supply cable (round-shaped connector) are located at the rear side of the camera. (Fig. 10). The camera needs an additional trigger cable that provides power and control signals from ImagingWin software via central control IMAG-CG.

The IMAG-K7 camera can be equipped either with a **standard objective** prime lens featuring 12 mm focal length (F=1.2 / f=12mm - K7-MAX/S, not shown) or a more flexible **zoom objective lens**

with 8 – 48 mm variable focal length (F=1.0 - K7-MAX/Z, Fig. 15) which provides a larger range of magnifications. Different mounting positions on IMAG-MAX/M are used for the two objective lenses provided. In case the MAXI imaging system shall be used in a setup in which the configuration must be kept constant e.g. phenotyping setups it is recommended to use a prime lens rather than a zoom objective lens (or lock the zoom ring).

The black adapter ring in the LED-Array Illumination Unit (IMAG-MAX/L – shown left) must be detached for using the Zoom objective.

Fig. 16 Adapter ring of the LED-array illumination unit IMAG-MAX/L

In between objective lens and camera CCD chip a 3 mm RG 645 (Schott) color glass filter serves as long-pass filter for protecting the CCD-chip from blue excitation light, while passing the red fluorescence as well as the 660 and 780 nm measuring beams for PAR-Absorptivity (see 10.1.1.10). A short-pass filter ($\lambda < 790$ nm), mounted in a threaded metal ring, protects the CCD-detector against excess near-infrared radiation contained in ambient daylight. For the optical properties of the camera it is essential that this filter as well as the RG 645 is placed between CCD-chip and objective lens (increase of effective focal length by plane-parallel glass plates).

Using the IMAG-K7 camera fluorescence image intensity is about 50 % of that obtained with the IMAG-K6 camera. While on one hand it does not allow 4-pixel binning (it is used in its native

resolution), on the other hand the applied objective lenses display a slightly lower aperture compared to the ones available for IMAG-K6.

Please note that with the zoom objective lens the aperture and consequently signal intensity drop at focal lengths exceeding 30 mm. Minor focus adjustments may be required when changing the focal length.

3.5 Mounting Stand with Eye Protection IMAG-MAX/GS

The IMAG MAX/GS Mounting Stand provides a standard means of mounting the powerful LED array illumination unit at a defined distance from the object, ensuring full eye protection for the user when closed. It features a red Perspex hood that absorbs the strong blue light emitted by the LED array while allowing the user to view the red chlorophyll fluorescence of the sample with the naked eye.

The measuring head, consisting of the LED array illumination unit and the camera, is mounted on the top of the IMAG-MAX/GS mounting stand with the help of two black clamps (see Fig. 14, foreground).

Using this mounting stand, the illumination unit and the camera are coupled at a fixed working distance of 18.5 cm between the LED array and the object plane. This results in homogeneous illumination of an imaged area of 10 x 13 cm.

A defined working distance is important for proper Image Correction (see 10.5.5), which corrects for unavoidable inhomogeneities in measuring light intensity and camera sensitivity over the imaged area.

Fig. 17 when setting up the MAXI imaging version it is recommended to mount the LED array on top of IMAG-MAX/GS with the clamps provided (a) and for continuity of measuring configuration also use the wing nut screw to fix the camera

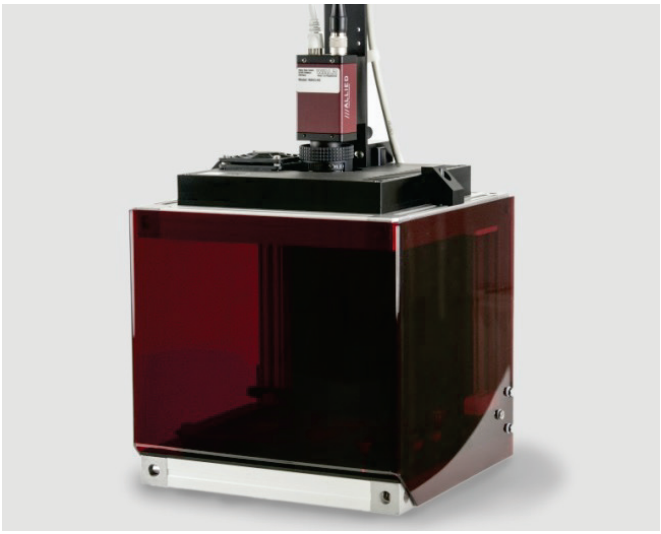


Fig. 18 IMAG-MAX/GS with mounted LED-Array Illumination Unit IMAG-MAX/L and CCD-Camera IMAG-K6

In principle, it is also possible to vary the working distance, when the LED-Array Illumination Unit is mounted independently from the IMAG-MAX/GS (see Fig. 8). However, in this case, the homogeneity of the light field may be suboptimal. Therefore, the user must take care to protect their eyes against excessive light, particularly during saturation pulses.

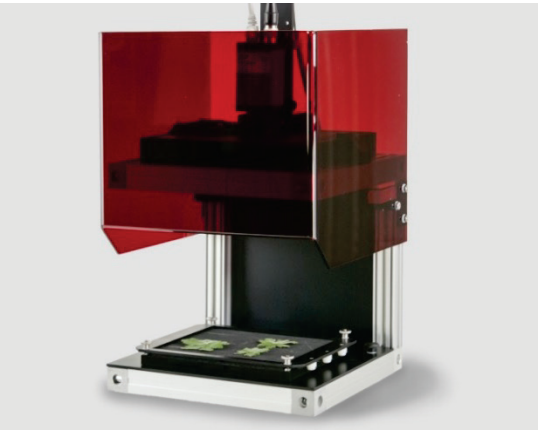


Fig. 19: IMAG MAX/GS with lifted red-Perspex hood showing detached leaf samples resting on x-y stage

In standard applications, the sample is resting on an x-y stage covered with non-fluorescent and non-reflecting black foam-rubber. The sample compartment becomes accessible after sliding the red Perspex hood upwards, using two flat hands gently pressing against the two sides. In its fully lifted position, the hood is held by two magnets.

The x-y stage allows to move the sample by maximally 25 and 19 mm in x- and y-directions, respectively. The force stabilizing its position on the bottom of the Mounting Stand is increased by magnets mounted at its reverse side.

During transportation the x-y stage plate and other unattached parts must be removed from the sample compartment of IMAG-MAX/GS and packed separately within the Transport Box IMAG-MAX/T to avoid damages.

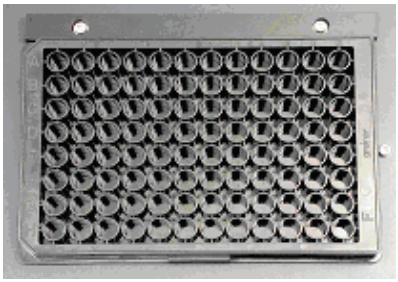


Fig. 20: Bottom plate of IMAG MAX/GS with black 96-well multiwell plate in defined, centered sample position

After removing the x-y stage plate, alternatively a multiwell plate can be placed into the sample plane. A correctly centered position is defined by two positioning elements (horizontal Perspex bar and metal screw defining right limit). It is recommended to use black non-fluorescent multiwell plates (e.g. Sigma-Aldrich article no. M9685). At high sensitivity measurements (high ML intensity, high Gain), in this application a mirror image of the LED-lamps may be superimposed on the fluorescence image of the samples contained in the wells. This is because the blue LEDs emit some red light, which can be reflected from the multiwell plate and/or the surface of the suspension samples' water via the camera objective lens onto the CCD chip. If this causes a problem, the red emission can be removed using the optional IMAG MAX/F filter plate, which can be mounted in front of the LED array illumination unit. Clear multiwell plates can now also be used, but please note that strong fluorescence signals may crosstalk to neighbouring wells.

Fluorescence imaging of algae suspensions in 96-well plates is a powerful tool in ecotoxicology and plant molecular biology (e.g. screening for mutants).

Fig. 21: The mounting stand (IMAG-MAX/GS) is configured for potted plants. The base plate has been removed, and the mounting stand has been jacked up using the additional legs provided with the IMAG-MAX/GS article.

For the measurement of larger or potted plants, it is also possible to remove the bottom plate of the IMAG-MAX/GS.

The Mounting Stand can be readily jacked up with the help of four profile-metal legs with mounting-angles, which can be screwed to the bottom corners of the Mounting Stand (see Fig. 21).

To achieve this, the Mounting Stand must first be turned upside down and the four nuts placed in the grooves at the bottom corners. The instrument is delivered with standard legs measuring 20 cm (including mounting screws and nuts). The increased distance to the

bottom makes it advantageous to use a screw jack to move a sample up and down.

The operator can then adjust the focus distance and check the light calibration (changing the working distance affects the PAR calibration of the imaging system). To ensure homogeneous illumination, the working distance should remain within the range of 18.5 cm ± 2 cm. PAR readings remain unchanged when the lab jack is used to bring the sample into focus from now on.

3.6 Leaf Distance Holder IMAG-MAX/B

Fig. 22: Leaf Distance Holder IMAG-MAX/B with IMAG-MAX/L and IMAG-K6

The illumination unit with CCD camera is mounted at the standard working distance of 18.5 cm using the Leaf Distance Holder IMAG-MAX/B, resulting in homogeneous illumination of the 10 x 13 cm imaged area of the X-Y stage plate.

Assembly of this lightweight device is straightforward. The four black arms are screwed into the screw holes provided at the bottom corners of the IMAG-MAX/L lighting unit.

The base plate of the IMAG-MAX/B sheet holder can then be screwed onto the four mounted arms. For convenient sample preparation, the sample hold-down device can be removed from the base by unscrewing the knurled head screws.

IMAG-MAX/B does not provide eye protection! The user must not look directly into the LED-Array Illumination unit while the device is taking measurements.

3.7 Notebook PC IMAG-PC

Operation of the IMAGING-PAM requires a PC with the following minimum requirements:

- Intel processor like Core i5 or better
- RAM memory min 8 GB
- built-in Ethernet interface GigE-Vision®
- operating system Win10 or 11 (64-Bit OS)

Please avoid the usage of port replicators working via USB-C connection since these adapters tend to produce communication errors often resulting in missing images.

The **Notebook PC IMAG-PC** ordered from Walz is delivered with fully tested and installed software. Depending on the market situation, the most affordable brand-name devices that have proven themselves for use in conjunction with IMAGING PAM are selected.

If the device was purchased without a PC, the user must first install the software as described in chapter 7.2.

3.8 Adapter IMAG-MAX/GWK

Using IMAGING-PAM alongside the GFS-3000 gas exchange meter provides a range of comprehensive analysis options. For instance, physiological heterogeneities or genotype differences can be visualized in fluorescence images under CO_2, O_2 or temperature conditions specified by the gas exchange system. The IMAG-MAX/GWK adapter positions the MAXI-IMAGING PAM on top of the large 3010-GWK1 gas exchange cuvette, protecting the eyes during the combination of imaging and gas exchange analysis across a 10 x 13 cm measurement range.

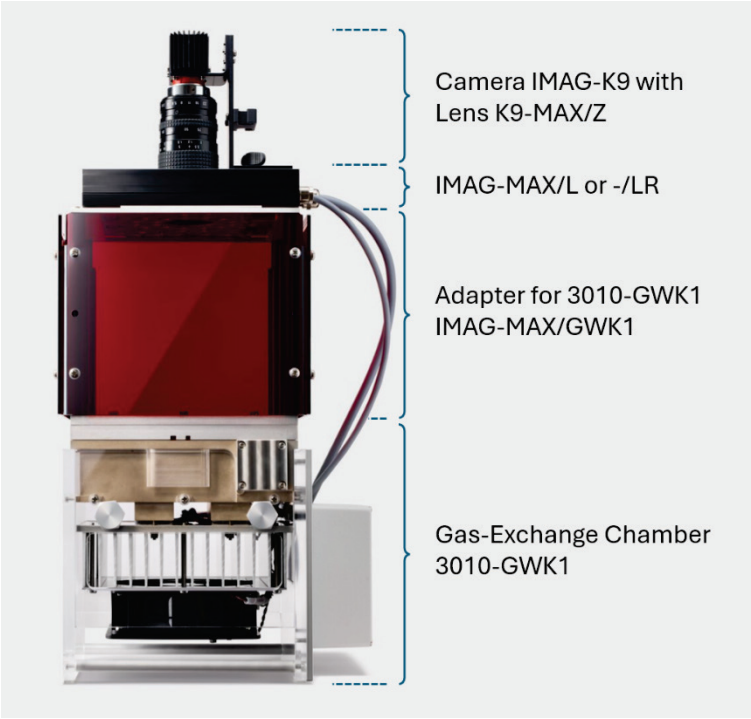


Fig. 23: IMAG-MAX/GWK on top of 3010-GWK1 gas exchange cuvette, equipped with Imaging head MAXI version.

4 Components of the IMAGING-PAM MINI-version

The MINI-version of the IMAGING-PAM consists of:

1) Control Unit IMAG-CG with Battery Charger 2120-N
2) MINI-Head (blue) IMAG-MIN/B or

   MINI-Head (red) IMAG-MIN/R or

   MINI-Head (GFP) IMAG-MIN/GFP
3) CCD Cameras IMAG-K7 or IMAG-K6 with camera objectives K7- or K6-MIN and mounting sets K7- or K6-MIN/M and K6-MIN/FS for use with IMAG-MIN/GFP
4) PC with ImagingWin-software

The Leaf Holder IMAG-MIN/BK is a useful accessory for leaf measurements. For simultaneous gas exchange measurements with the GFS-3000, the IMAG-MIN/GFS adapter is available. Convenient laboratory stands, a tripod and a fine drive tripod adapter for outdoor applications are also available.

4.1 Multi Control Unit IMAG-CG

The same Multi Control Unit (IMAG-CG) is used for all versions of the IMAGING-PAM M-Series. It was previously described in Section 3.1.1 in conjunction with the Maxi version. The LED array of the mini head is plugged into the mini head connector on the rear of the IMAG-CG unit. The camera cable connector is located on the front of the IMAG-CG unit.

The MINI-Head with mounted camera can be attached directly to the IMAG-CG Control Unit housing. A wing screw is provided for this

purpose. Alternatively, it can be mounted on a tripod or held by hand using a special grip (see 4.5).

4.2 MINI-Head LED-Array IMAG-MIN/B and IMAG-MIN/R

Fig. 24: Top view of MINI-Head IMAG-MIN/B LED-array without aluminum rods holding the sample-platform

The blue and red versions of the MINI-Head (IMAG-MIN/B and IMAG-MIN/R) are identical in all respects except for the color of the LEDs and filters. As blue and red LEDs have different intensity-current relationships, different PAR lists apply. These are incorporated into two different versions of the ImagingWin software. (see chapter 11).

The MINI-Head's LED array comprises 12 high-power LEDs, each fitted with collimating optics, arranged in four groups of three. A short-pass filter is attached to each group to eliminate red light, which would otherwise pass through the long-pass filter in front of the CCD camera and overlap with the chlorophyll fluorescence. The blue version (IMAG-MIN/B, with an emission peak of 460 nm) uses a blue-green glass filter (Schott BG39). The red version (IMAG-

MIN/R, with an emission peak of 620 nm) uses a special short-pass interference filter (<645 nm). The LEDs are mounted at an angle optimised to produce a homogeneous light field at the given working distance.

These LEDs provide pulse-modulated excitation light and simultaneously serve for actinic illumination and saturation pulses. In addition, four further groups of LEDs (2x6 and 2x4) provide the pulse-modulated light for assessing PAR absorptivity. (see 5.4.1.10 - 10.1.1.14). These LEDs are arranged in pairs, with each pair featuring a red (660 nm) and a near-infrared (780 nm) LED. While only a relatively small amount of this light is remitted from the sample to the camera, this is sufficient to give good signals, as both wavelengths can pass the red long-pass filter in front of the detector chip, in contrast to the filtered excitation light.

The LED-array cable connects to the MINI-HEAD socket at the rear side of the IMAG-CG Control Unit.

To prevent eye damage, please avoid looking directly into the LED array illumination unit!

The MINI-Head is designed for a fixed working distance of 7 cm between the camera and the sample, which is defined by four aluminum rods with a sample platform on top of the LED array. At the standard working distance and using the 16 mm lens, an area measuring 24 x 32 mm is imaged.

All MINI-Head LED-Arrays can be extended with a Leaf Clip and a handle (see chapter 4.5).

The MINI-Head LED-Array IMAG-MIN/GFP is described in chapter 0.

4.3 CCD Camera IMAG-K6 or IMAG-K7 (old)

The CCD cameras IMAG-K6 or IMAG-K7 are described in chapter 0 for the imaging MAXI-version. For the recent CMOS camera IMAG-K9 a description can be found in section 3.3. All cameras can also be used in the imaging MINI-version in combination with IMAG-MIN/B and IMAG-MIN/R. In combination with IMAG-MIN/GFP only IMAG-K6 can be used (see chapter0.)

For usage in the Imaging MINI-version these cameras need to be equipped with the objective K6-MIN for IMAG-K6 (F1.4/f = 25 mm) and a 7.2 mm distance ring or K7-MIN for IMAG-K7 (F1.4/f = 16 mm) with a 4.2 mm distance ring. Imaging systems using IMAG-K9 camera are equipped with a 25 mm Tamron lens with very little diffraction. For all configurations a 3 mm RG 645 (Schott) color glass filter serves as long-pass filter for protecting the CCD-chip from blue excitation light, while passing the red fluorescence as well as the 660 and 780 nm measuring beams for PAR-Absorptivity (see 10.1.1.10). A short-pass filter ($\lambda < 770$ nm), mounted in a threaded metal ring, protects the detector against excess near-infrared radiation contained in ambient daylight. For the optical properties of the camera, it is essential that this filter as well as the RG 645 is placed between CCD-chip and objective lens (increase of effective focal length by plane-parallel glass plates).

Adjust the objective aperture! Closing the aperture increases focal depth and decreases light delivery to the camera (to avoid signal saturation).

The cameras are mounted to the metal holder preinstalled on top of the MINI-Head LED-Array. The camera side with four screw holes and the label points to the same direction as the label of the LED-Array, the camera side with seven screw holes points to the same direction as the backside of the LED-Array, where the cables are located, and the metal angle bar of the mounting set is mounted.

Fig. 26 displays the mounting positions and required screws for mounting the camera and the metal angle bar.

Fig. 25: Camera side with 4 screw holes and label (left) - facing the labelled LED-Array side, camera side with 7 screw holes (right) facing the LED-Array backside and the metal angle bar (Fig. 26 A)

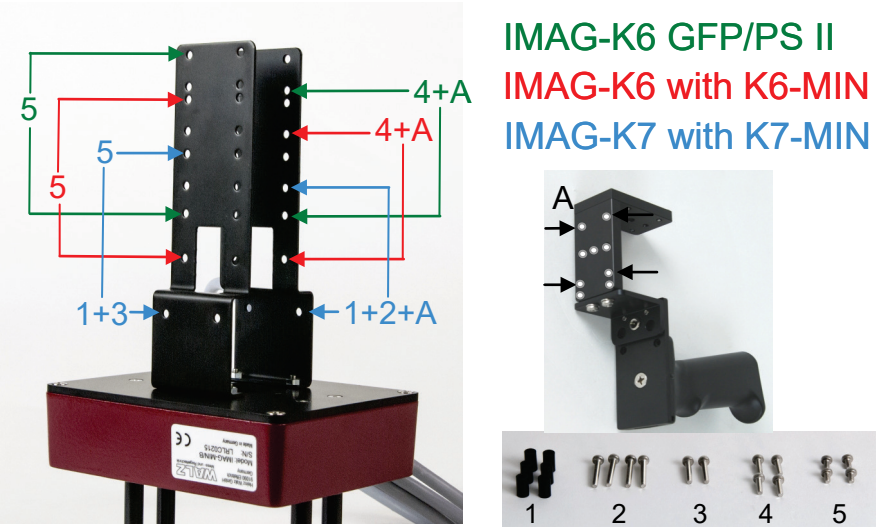


Fig. 26: Screw indication and camera mounting positions, metal angle bar including grip of Leaf Holder IMAG-MIN/BK, screw annotation

The metal angle bar of the mounting set K6 and K7-MIN/M (Fig. 26 A) serves for mounting the MINI-Head with camera onto the IMAG-CG Control Unit, to the handle of the Leaf Holder IMAG-MIN/BK or to a tripod. Furthermore, the mounting set K6 and K7-MIN/M contains a Perspex device to unplug the GigE cable from a mounted CCD camera (see Fig. 27)

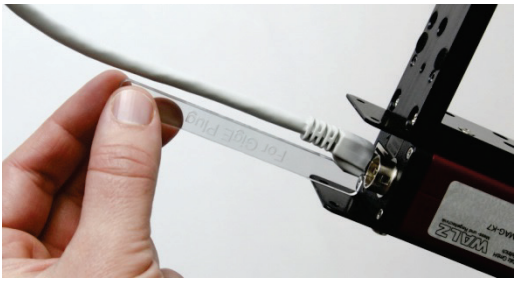


Fig. 27: Perspex device to unplug GigE cable from a mounted camera

4.4 CMOS Camera IMAG-K9

The new CMOS camera IMAG-K9, the successor to the IMAG-K6 and -K7 cameras, described in the previous chapter, has a significantly different size. For this reason, the mounting components also had to be adapted. The functions have remained the same, so users of the old system will certainly quickly find their way around the new one.

There are various ways to use the MINI imaging measuring head. In the laboratory on a table with flat samples, it works very well with our ST-101 stand and the IMAG-MIN/ST fine drive tripod adapter. The latter can also be mounted on a camera tripod, allowing precise positioning of the measuring head via a ball head. The measuring head can then either be moved freely towards the sample or held in place using the leaf holder IMAG-MIN/BK.

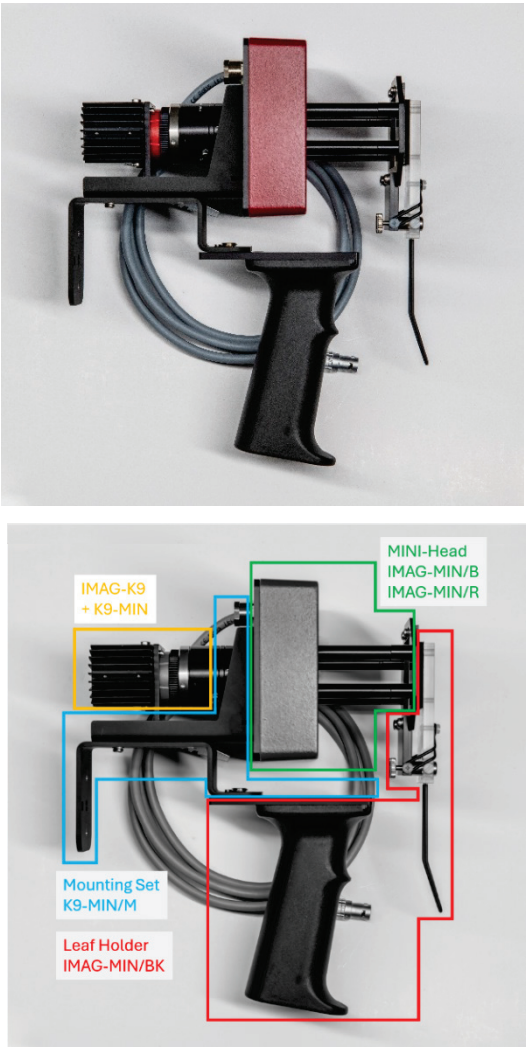


Fig. 28: Typical MINI Imaging head as it would be used in the blue or red excitation light version and leaf holder IMAG-MIN/BK mounted. With the handle mounted it can be used for freehand work, with detached handle it can be mounted on a foto tripod or the fine drive tripod adapter IMAG-MIN/ST (see Fig. 29)

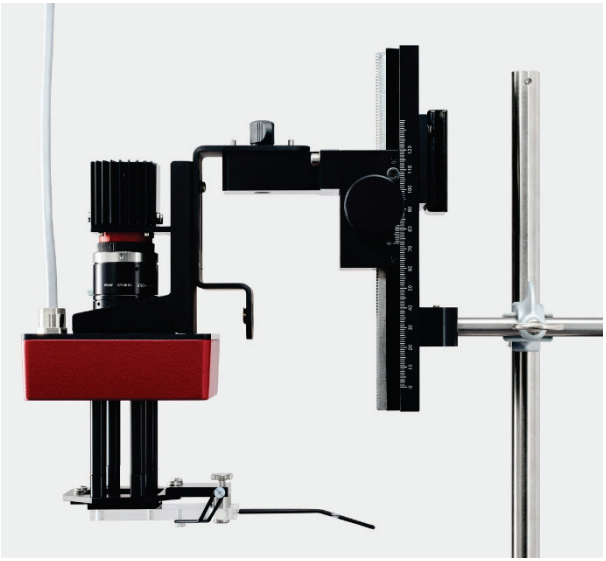


Fig. 29 MINI Imaging head mounted via IMAG-MIN/ST fine drive tripod adapter on a laboratory stand ST-101

The camera with comes fully mounted. However, if the lens or measuring configuration is changed, it is important to maintain the same optical setup to ensure the image remains in focus.

For the intended working distance of the MINI head the 5mm distance ring is essential. If not used the recommended lens would only have an m.o.d. of 30 cm.

When changing the lens, please bear in mind that an internal PSII detection filter is required. This filter, together with the working

distance, determines the required thickness of the distance ring. To exchange the internal detection filter, use the provided filter tool (see image below). Screw the detection filter in until it reaches the end of the C-mount thread. Do not apply force at this point.

Fig. 30 IMAG-K9 camera, 5 mm distance ring, PSII detection filter with filter tool in front, 12 mm F/1.4 lens for MINI head (K9-MIN)

4.5 Leaf Holder IMAG-MIN/BK with Handle

Fig. 31: MINI-Head with CCD camera and handle with IMAG-K7 camera. The optional leaf holder is also available for the recent IMAG-K9 based MINI head (leaf clip of IMAG-MIN/BK shown in next image)

When the Leaf Holder is delivered together with the MINI-version of the Imaging-PAM, it is already mounted on the MINI-Head. A grip holder is provided, which can be fixed to the metal angle on the camera. Using this

handle, the MINI-Head can be carried with one hand, and the lever of the leaf clip can be moved up with the same hand (see Fig. 32).

Fig. 32: Mini-Head with grip holder IMAG-MIN/BK used in field

This is particularly useful in field applications. The MINI-Head may either be carried separately or together with the IMAG-CG, on which it may be mounted with the help of a wing screw. Alternatively, the Mini-Head may also be mounted on a tripod using the same metal angle to which the carrying grip can be connected (see Fig. 33). The IMAG-MOBILE is an even more flexible version of the MINI-HEAD. It has been specially optimized for ground-level work and portability. A rugged outdoor tablet computer can replace the laptop to enable long periods of work in the field under outdoor lighting conditions.

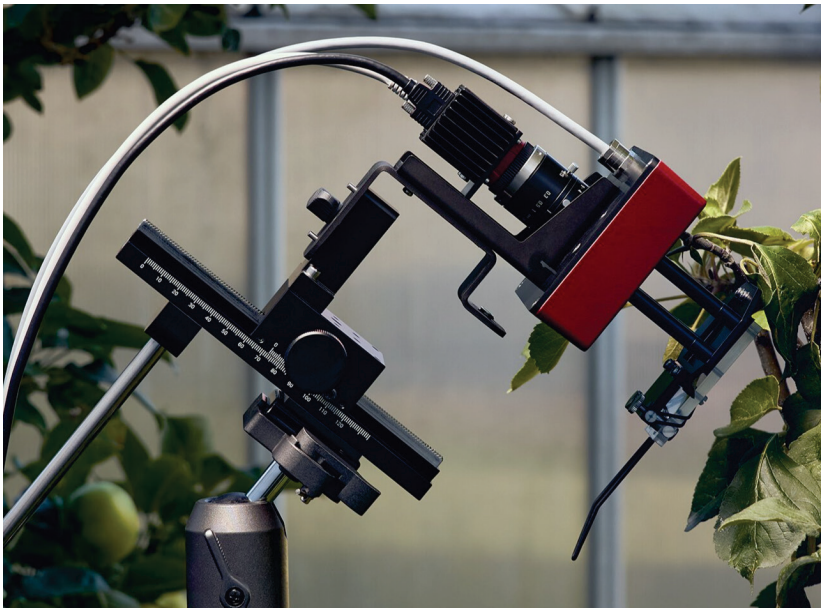


Fig. 33: Mini-Head with Leaf Holder IMAG-MIN/BK and IMAG-MIN/ST mounted on a tripod

When the Leaf Holder is ordered separately from the MINI-Head, it must be assembled and mounted by the user. Fig. 34 shows the components of the Leaf Holder. The following figures Fig. 34 - Fig. 37 on the following pages will help to put the various parts together.

Fig. 34: Components of the Leaf Holder

First the frame (1) is mounted with two screws (2) on the sample platform of the MINI-Head. Then the clip (3) is fixed with two screws (4) to the sample platform. While Fig. 35 shows the mounted clip from the bottom (camera) side, Fig. 36 shows it from the top side.

On the Perspex top side of clip the nylon screw (5) is fixed which holds the two O-rings (6) which function as a spring forcing together the two parts of the clip (see Fig. 35 and Fig. 36). The O-rings may age and then must be replaced.

For this purpose, the spare O-rings (7) are provided. The grip (8) is fixed with the two screws (9) via a metal angle to the camera, as shown in Fig. 37.

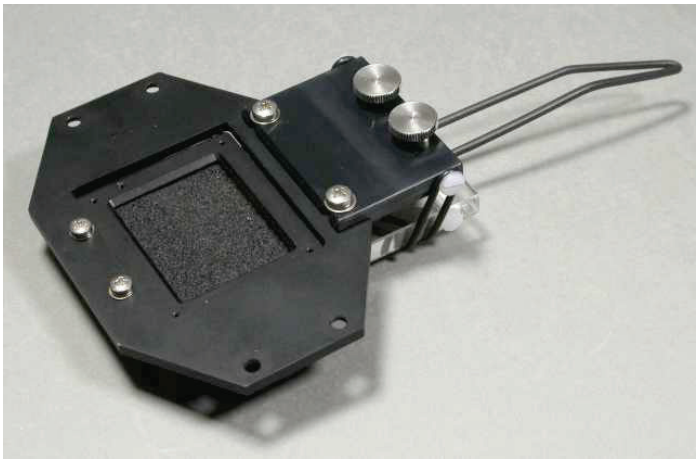


Fig. 35: Leaf clip mounted on sample platform of MINI-Head viewed from camera side

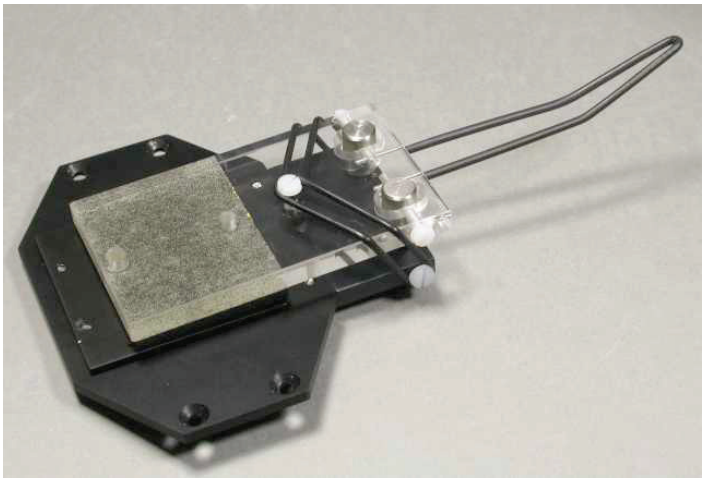


Fig. 36: Leaf clip mounted on sample platform of MINI-Head viewed from top side

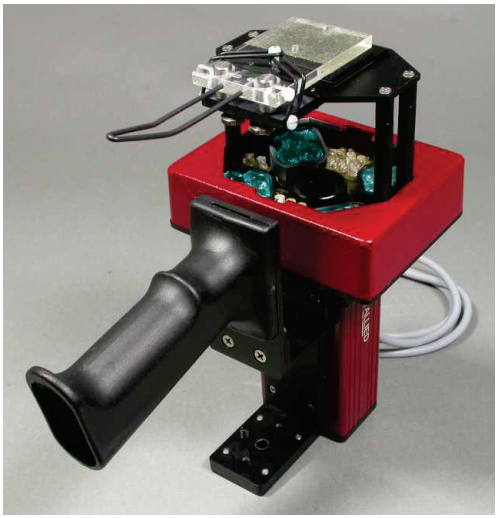


Fig. 37: Leaf clip and grip holder mounted on MINI-Head

4.6 IMAG-MIN/GFP with IMAG-K6

For GFP measurements using the MINI-version the IMAG-MIN/GFP LED array is needed in combination with the filter slide IMAG-K6/FS, the 2/3" CCD camera IMAG-K6, the K6-MIN objective and the K6-MIN/M montage set.

As the LED-array of the other MINI-Heads IMAG-MIN/GFP features 12 high-power LEDs (emission peak 470 nm) arranged in 4 groups of 3 LEDs and equipped with a short-pass filter (< 500 nm). These LEDs provide the pulse-modulated GFP excitation light and at the same time serve for actinic illumination and Saturation Pulses. In addition, there are four groups of LEDs featuring red (660 nm) and a near-infrared (780 nm) LED pairs providing the pulse modulated light for assessment of PAR-Absorptivity (see 5.4.1.10 - 10.1.1.14).

The LED-array cable connects to the MINI-HEAD socket at the rear side of the IMAG-CG Control Unit.

The detector filter slide K6-MIN/FS is mounted in front of the 25 mm camera objective K6-MIN as shown in Fig. 38 .

Fig. 38: Standard lens for IMAG-MIN/GFP (focus ring = f, aperture = a), filter slider in GFP position

Before mounting the objective with filter slide to the IMAG-K6 CCD camera a 5 mm distance ring needs to be mounted and if IMAG-K6 has been used in another IMAGING-PAM configuration additional short pass filters between camera and objective as well as other distance rings need to be removed. Afterwards the IMAG-K6 CCD camera with distance ring, objective and filter slide is attached to the IMAG-MIN/GFP head using the topmost position of the metal holder (see Fig. 26).

For special applications it is also possible to use a lens with a wider angle (16 mm). In this case the camera has to be mounted in one of the two lower positions (not shown) with the filter slider mounted on the camera lens subsequently.

With the help of this filter slider the detection filter in front of the 25 mm lens can be exchanged from PSII measurement to GFP measurement and vice versa.

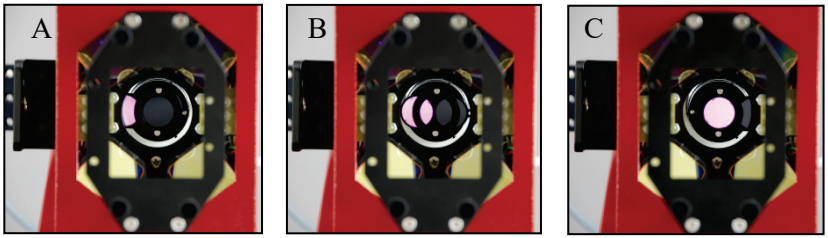


Fig. 39: switching of the filter slider between two positions (A – PSII, C-GFP position)

The view into the GFP Mini-Head from the sample side (Fig. 39) explains the two positions of the filter slider.

The filter slider carries two detection filters. The dark red one (RG665) enables the camera to detect PSII-fluorescence and a special interference filter for the detection of wavelengths between 500 and 600 nm is used for GFP detection.

The filter slider can be pushed from left to right (Fig. 39 A to C), to change the detection filter from PSII-fluorescence to GFP. For lowering background effects, the GFP Head is shipped with a sample of a low fluorescent black adhesive film which can be used as a background layer.

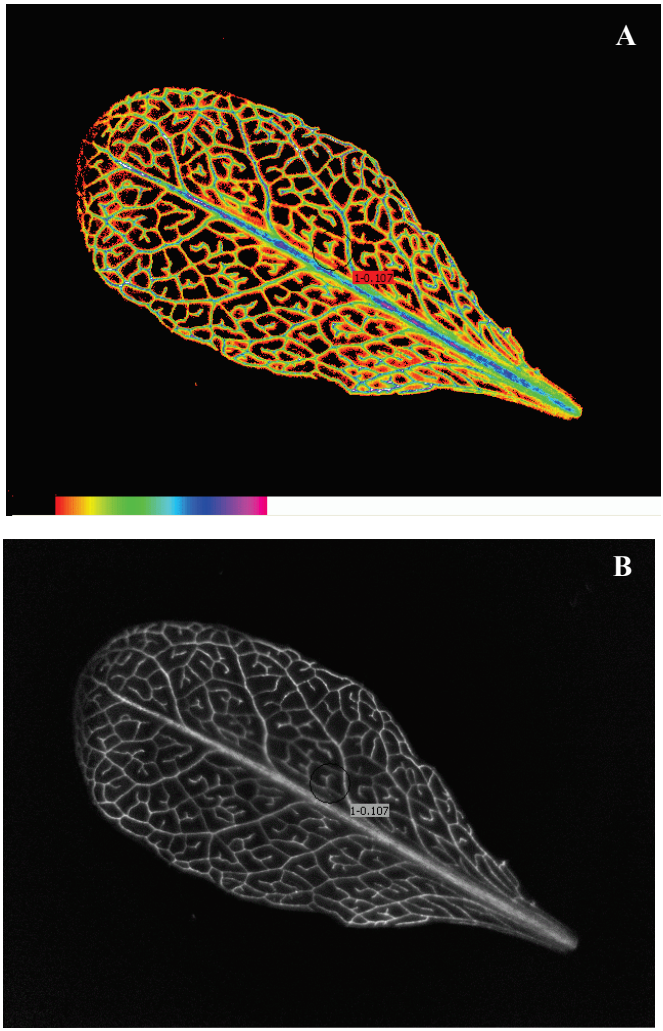


Fig. 40: possible color modes for the GFP images

There are different colour modes reasonable for the GFP measurements. Fig. 40 A shows an image in the false colour scale mode of the “Analysis” function (see also chapter 10.1.2.7). Another option is the black and white mode as in Fig. 40 B. The Description of the Display Parameters can be found under chapter 10.5.8 (display parameters) of the manual. Pictures can be exported via the export function described in chapter 9.2.

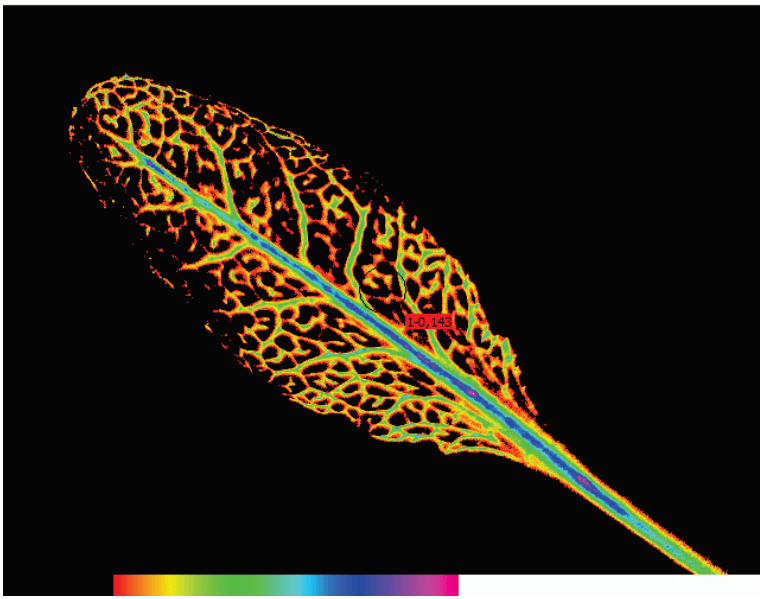


Fig. 41: “Expanded Color” setting for enhancing GFP images

This example picture (*Arabidopsis thaliana* with promSUC2 GFP) has been measured with the following settings:

- Filter slider in the GFP position
- MF 8, ML max, Damping 4, Gain on 8

In contrast to the PSII fluorescence, for GFP measurement some settings must be very high. For this reason, long exposure to these

settings may lead to the bleaching of the sample. Using the „Analysis“ sliders, the inevitable background can be suppressed.

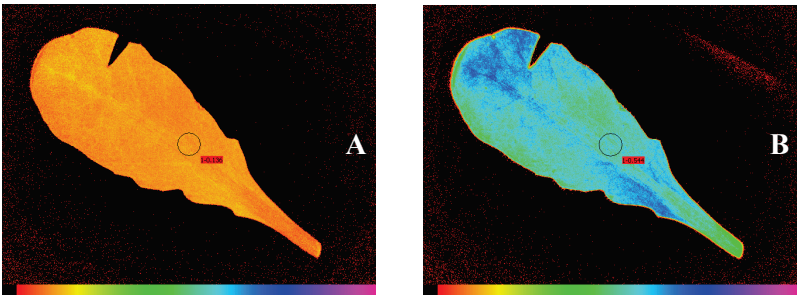


Fig. 42: Fo image in the PSII mode (A) vs. Fm image (B)

Since the camera used for the combined GFP-Head is highly sensitive, settings have to be changed between GFP and PSII measurement:

- PSII fluorescence – ML=1, Gain=2, Damp=1

The basic image fluorescence now should have a value of nearly 0.15, to prevent overflow during the saturating flash. If still overflow appears, please also use the aperture of the camera lens to lower the fluorescence signal. (The benefit of doing this is that the *depth of focus* is increased which may help to get also bent leaves in focus without cutting.)

For Absorptivity measurements in the PSII mode no starting values for **red** and **NIR** can be given because these values depend on the aperture of the used lens. For details read more under chapter 5.8.3 and 5.4.2.1 of this manual.

As for the other MINI-head LED arrays the metal angle bar of the mounting set K6-MIN/M serves for mounting the MINI-Head with camera onto the IMAG-CG Control Unit, to the Leaf Holder IMAG-MIN/BK or to a tripod and can be extended with a Leaf Clip and a Grip Holder.

4.7 Adapter for GFS-3000 (IMAG-MIN/GFS)

The MINI-version of the IMAGING-PAM M-Series can be applied for simultaneous measurements of CO_2 gas exchange and chlorophyll fluorescence. For this purpose, the adapter IMAG-MIN/GFS is available which replaces the sample-platform of the standard version. The adapter features a frame with two ball-pins that allows you to click the MINI-Head onto the Measuring Head of the GFS-3000. The distance rods carrying this frame are 11 mm shorter than in the standard version in order to assure identical working distance from the camera to the leaf sample within the gas exchange cuvette.

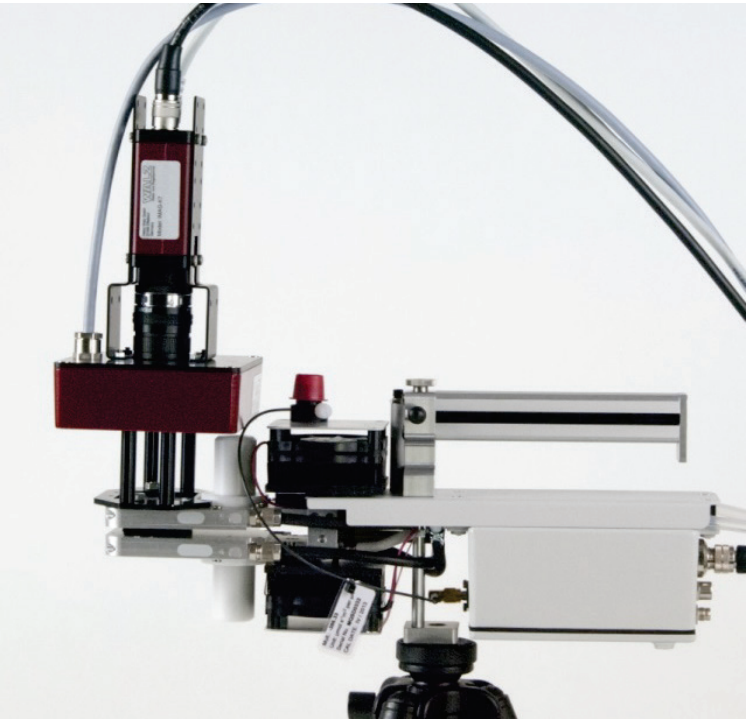


Fig. 43: MINI-Head mounted on Standard Measuring Head of the GFS-3000 Gas Exchange Fluorescence System

When the MINI-Head is used in combination with the gas exchange measuring system, it is controlled by the combined ImagingWin and GFSWin programs running synchronously on the same PC. The synchronous operation allows an accurate time assignment of gas exchange and fluorescence data in a common Report file. While the actinic illumination for driving photosynthetic electron transport is provided by the LED-array of the MINI-Head, the switching on/off of actinic light is controlled via the GFSWin software. Further peculiarities that are important for the combined operation of MINI-Head and GFS-3000 under GFSWin and ImagingWin are described in a separate brochure.

4.8 ImagingWin software versions for various types of MINI-Version

Upon start of the ImagingWin program the user may choose between different software versions for the various types of MINI-Heads. While these versions are practically identical, they feature different **PAR-Lists** (found under „Options“ in the software or chapter 11 in the manual).

Particular **Image Corrections** (found under „Settings“ or 10.5.5) apply to different MINI-Heads.

When the program is started for the first time after installation of the software, it comes up with a warning that the file for Image Correction is not found. It is recommended that the user determines the Image Correction for his particular MINI-Head before starting serious measurements (see chapter 10.5.5).

With every Measuring Head of the three different correction images can be stored: **Type 1, Type 2 and Maxi** or **Mini** or **IMAG L450** and **RGB**.

For **measuring Image Correction** please proceed as follows:

- set the **optical conditions** under which the actual measurements are going to be done (working distance, focusing position, see above)
- select Type 1, Type 2 or Maxi/Mini/Micro/IMAG L450/RGB (under Settings/Image Correction)
- in the case of MAXI- and MINI- versions place at least two layers of white paper (e.g. folded DIN-A4) into sample plane; in the case of the MICROSCOPY-version the plastic fluorescence standard
- put the image somewhat **out of focus** to avoid imaging fine structures of the white paper tissue or dust etc. on the surface of the fluorescence standard
- **press the measure button** (under Settings/Image Correction)

The measured correction image will be saved until it is overwritten by a new measurement. The correction images will remain valid as long as the same optical parameters apply (LED Illumination Unit, working distance, focusing position, camera objective lens, microscope objective lens).

5 Components of the IMAGING-PAM 3D version

The IMAG-3D consists of the MAXI Imaging head (described in the prior chapter and an additional projector that functions as 3D scanner together with the camera of the MAXI-IMAGING-PAM.

The functional principle is the triangulation of surface areas determined by projection of a structured light pattern using straight lines which are deformed by the surface of the subject. Depending on the distance between light source and the surface of the object, a triangulation angle can be calculated since the angle between camera and projector is fixed. Such a system has to be calibrated.

From this scan a point cloud and finally a polygon mesh can be produced that represents the simplified topology of the visible sample surface.

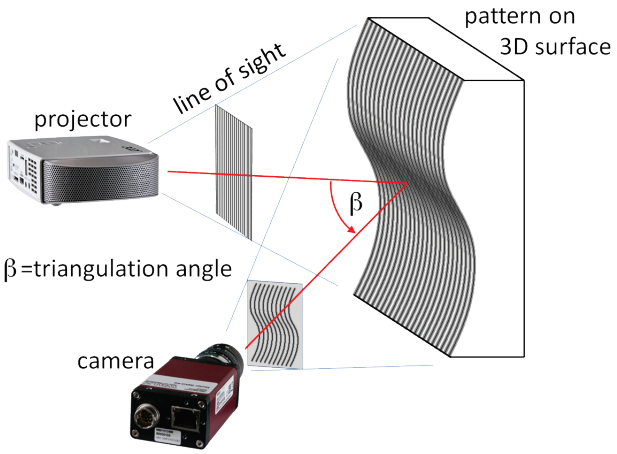


Fig. 44: Operation principle of a fringe light scanner

From this polygon mesh basic information like surface angle (normal), distance to neighbor edges so that the surface area and its properties can be calculated.

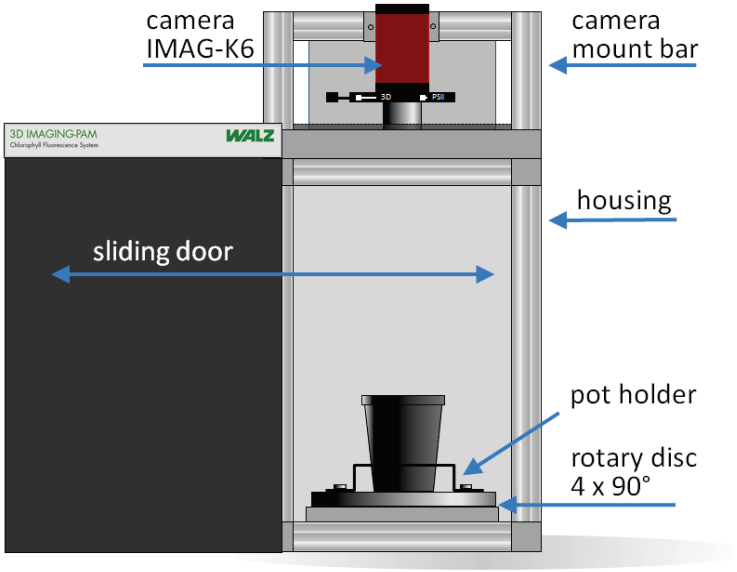


Fig. 45: Front view of IMAG-3D

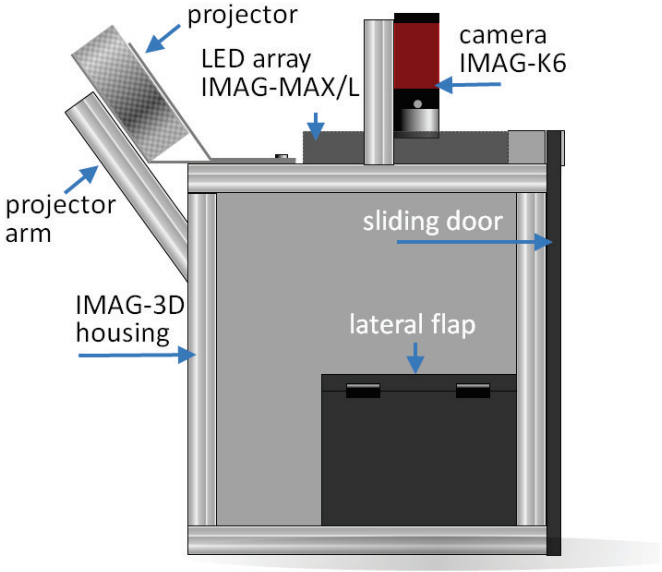


Fig. 46: Side face of IMAG-3D

Main difference between IMAG-3D and the other members of M-Series family is its integrated design. Except the central control unit IMAG-CG no other parts can easily be detached or exchanged without influencing the 3D calibration of the system.

On the left side the core system is shown with its functional parts depicted. Starting from top there is a camera (IMAG-K6) mounted on a mount bar.

Please do not use the camera mount bar as handle since this can influence the calibration of the 3D scanner part and lead to bad fusions of measured views.

IMAG-3D is equipped with a sliding door that opens to the left side. It consists of grey translucent Perspex as eye protection and for shading the sample during 3D and PSII measurements.

Inside the housing of IMAG-3D is a motorized rotary table which offers highly reproducible positions (4 x 90° used) to project structured light patterns from four sides onto sample for 3D measurements and from one side for RGB images.

On top of rotary table a pot holder can be mounted, for initial calibration of the system, calibration standard shall be used.

We recommend to use spray casted flower pots from "Göttinger" (www.goettinger.de). The ones our pot holder is designed for is the 7x7x8 mould cast pot. On the rear side of the housing an LED projector is mounted. Its control buttons are covered to avoid an accidental change of settings. Only the power button is accessible Prior to any measurement the projector has to be switched on.

The position of the projector is essential for an accurate calibration. In case the instrument has been transported under rough conditions it might be necessary to check the calibration (chapter 5.3.1).

On both sides of the IMAG-3D housing flaps can be found which offer easy access to the inside of the without opening the front sliding door.

Further separate components needed for IMAG-3D system:

IMAG-CG central and control unit (chapter 3.1)

IMAG-3D/PC - Notebook computer with high processing power – especially for the GPU (graphics processing unit) an NVIDIA system is recommended to reduce processing time after 3D-calibration (chapter …) or –measurement (chapter …). In case the computer is purchased together with a 3D unit, the ImagingWin software has already been installed

5.1 Setting up IMAG-3D

The basic component of IMAG-3D is almost completely set up when it arrives. All that has to be done is to unpack the components and connect the cables. Most of the cables already connected on the IMAG-3D bundled as thick cable harness so that only the integration of the Notebook computer, power supply of LED array (IMAG-MAX/L) and the projector is needed.

Fig. 47: connectors in cable harness coming from IMAG-3D.

USB-A, RJ45 and HDMI have to be connected to control computer; the +/- cable from LED array go to “RGB head” port and “MAXI head” cable need to be connected with the LED-array-power-supply on central control unit IMAG-CG (please pay attention to different number of pins in Hirose and RGB head cable.

On the instrument side of the cable harness two cables to the camera may have to be connected (RL45 and Hirose I/O are normally detached when the instrument is shipped). Please also check if projector power supply and HDMI cable are plugged in (see image below).

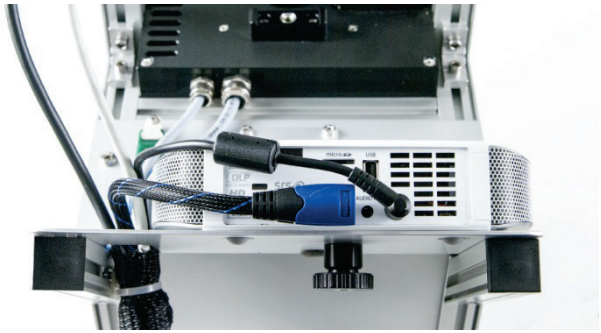


Fig. 48: Rear view of the projector with correct connections for power and HDMI (cable for filter slider actuator is not visible here)

A blue USB-A cable in the cable harness is needed for operating the ImagingWin software (chapter 7.3). It connects a hard lock with the computer. To prevent loss, we mounted the hard lock on to of IMAG-3D housing. If necessary, it can also be detached to connect it to a computer directly.

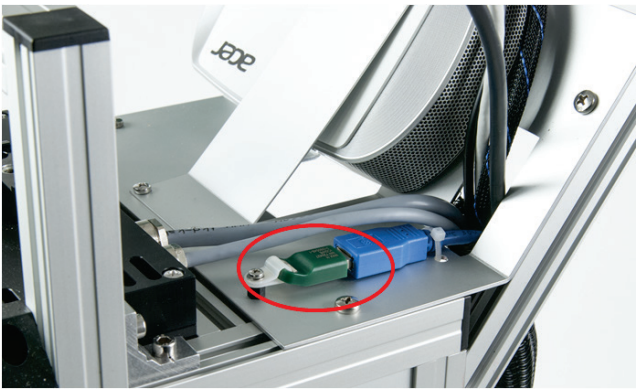


Fig. 49: USB hardlock (IMAG-3D/ANA). It is needed to unlock the 3D functionality of ImagingWin. One dongle is shipped with the basic instrument; more can be purchased for additional work stations.

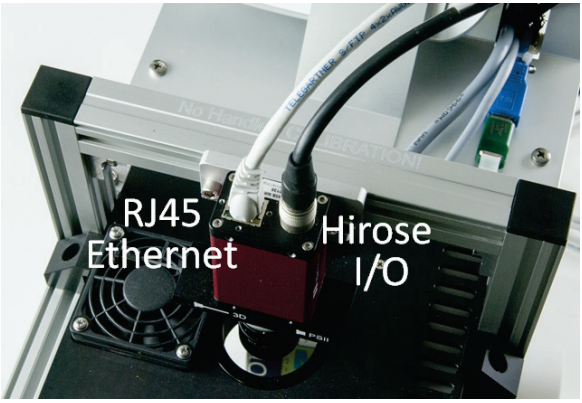


Fig. 50: Connections of camera

Furthermore, some power supplies for the components must be connected:

Projector power supply

Rotary table power supply (via interface unit additionally connected via USB with the computer)

Charger for central control unit (IMAG-CG)

As last component the interface for turntable control must be connected:

Fig. 51 interface for turntable control

USB cable connects to the control computer; the D-Sub connector is plugged into the rear panel of interface and controls the turntable. With the help of green buttons, the turntable can also be moved manually to reach the rearward mounting screws for potholder or calibration stand. It will automatically return to the initial position when software is started.

After IMAGING-3D has been wired correctly, it can be switched on before ImagingWin software is started:

- Projector
- Central control unit IMAG-CG
- Power supply of LED array and turntable
- Computer

When all connections are checked, and the components are switched on, ImagingWin software can be started. If this is done for the first time or IMAG-3D has been transported, the user should assure that 3D calibration is still valid or just perform a new calibration with the help of the calibration phantom shipped together with the instrument (chapter 5.3.1)

5.2 Installation of ImagingWin 3D software

Installing the ImagingWin 3D software is very similar to installing other software. Please check the Walz homepage (www.walz.com) from time to time for updates.

5.3 3D-Survey of a sample

The pot holder is designed for rectangular pots (type: see beginning of chapter 5) With these pots a stable and reproducible sample position can be guaranteed. It is recommended to use a lid of a petri dish underneath the pots to prevent dirt or water from getting into the mechanics of the turntable.

The IMAG-3D is normally set to a focal plane slightly higher than the upper edge of the recommended flowerpots, so that the user can start measuring immediately when a plant grows or has been moved or replanted in such a pot. On request we can also supply holding frames for special pots (please inquire at info@walz.com).

The IMAG-3D should be wired according to the previous chapters and ImagingWin3D should be started. The hard lock (mounted on the IMAG-3D) is also required for subsequent evaluation of the data, otherwise the ImagingWin3D software will refuse to provide 3D functionality. The resulting meshes from previous measurements and the corresponding PSII data are also available without the hardlock.

First measurement of a plant:

Make sure that all necessary components are switched on

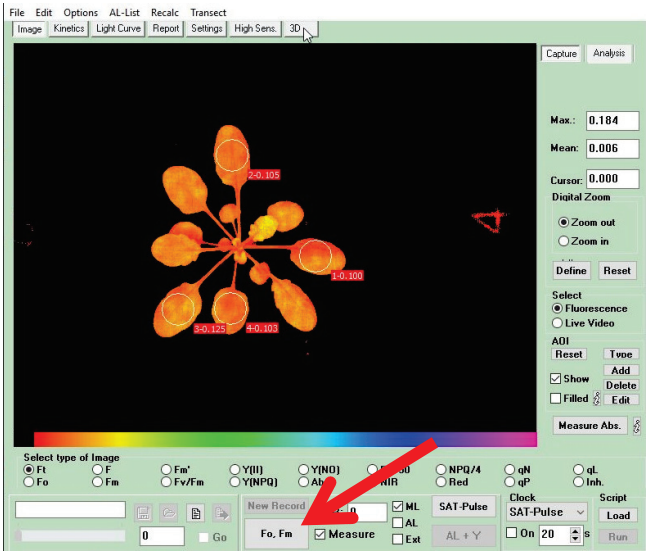
- Projector
- Central control unit IMAG-CG
- Power supply of LED array
- Turntable controller
- Computer

The filter slider already moves to its initial position when IMAG-CG central control unit is switched on.

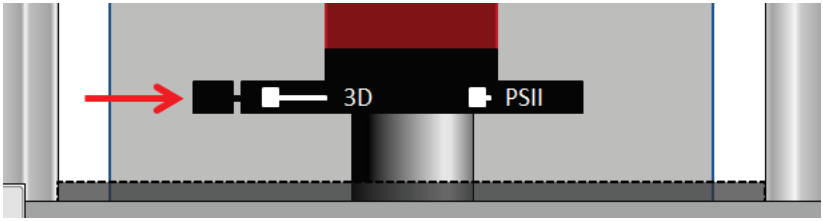
Start ImagingWin3D software and observe if the filter slider responsible for detector filter changes is moved to its initial position once again (PSII filter). Additionally, turntable will move to its initial position when 3D measurement is initiated (position 1).

Place a sample into potholder frame and make sure that it cannot move during the measurement. To prevent dirt or moisture reaching the rotary table mechanics it is recommended to you a Petri dish or similar underneath flowerpots during the measurement.

The ImagingWin3D software needs a mask to distinguish between sample and background. This mask is determined from an image showing fluorescence. Hence before opening “3D” tab, the “Fo, Fm” button must be clicked, even if no further PSII measurement shall be performed.



For recording such an Fo Fm image or perform previous PAM fluorescence measurements the filter slider in front of the camera has to be in “PSII” position. This should be done automatically by the actuator mounted behind the camera.



5.3.1 Calibration of the IMAG-3D scanner

To train the IMAG-3D for 3D reconstruction of a sample calibration is necessary. This calibration produces a folder in "C:\ImagingPam3D \Calibration_3D" in which further subfolders can be found:

 CalibrationMeasurements (contains 102 image files)

 FusionMeasurements (contains 89 image files)

 Calfile (grey value information / gamma correction)

 Calibration.calib

 default_Calfile (similar to Calfile)

 Inifile (configuration file for point cloud determination)

When later on a combined PSII and 3D measurement has been performed, a copy of these files will be transferred into a data folder together with the fluorescence data so that later data can be fused again even though the instrument configuration may have changed.

For calibrating IMAG-3D the calibration phantom with two different dot patterns is necessary

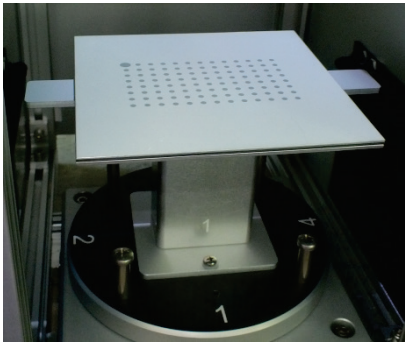
To keep the scanning cooperativity of the calibration pattern if possible, do not touch or scratch the surface. Clean only with water and a soft cloth if necessary.

The calibration stand (phantom) is used in three different configurations (horizontal, tilted 30° to the right or tilted 10° to the left) with two different calibration patterns. For a successful calibration it is important that the acquired images are within the contrast range of the camera and that all points of the calibration patterns are visible (for the camera) during the whole procedure. This defines the configuration of the setup. If some of the dots are

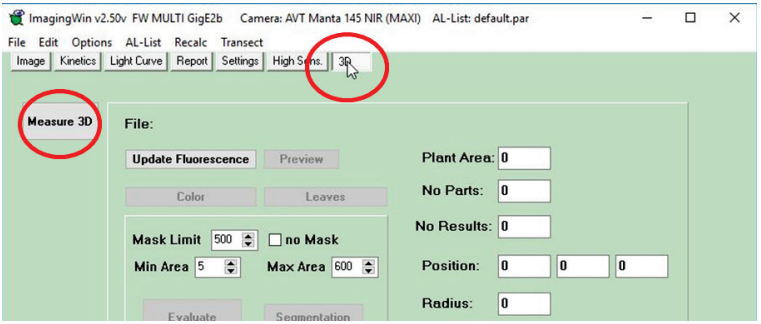
invisible to the camera in one or more images, a message ("no pattern found") will appear and the calibration must be repeated. The error can be caused by a shifted or incorrect calibration pattern or by excess light falling on the calibration pattern.

A sequence of images is displayed by the projector in each position of the calibration phantom. Each individual image is important and opening the door during the calibration sequence can cause pattern recognition errors, resulting in an error message.

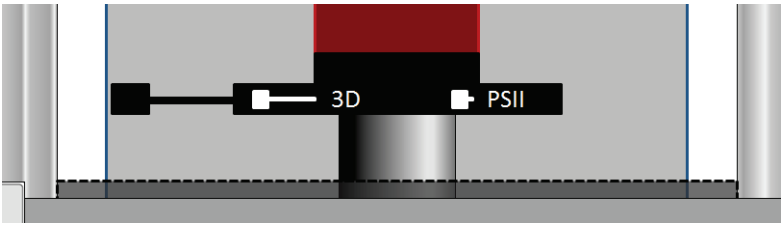
1. Mount the calibration phantom with large calibration pattern so that the “1” on its foot aligns with position “1” of the rotary disk. Use the large calibration pattern (make sure that the larger dot is located at the rear left edge). Orientation is horizontally in the beginning (as shown).



2. Switch to “3D” and afterwards activate “Measure 3D”



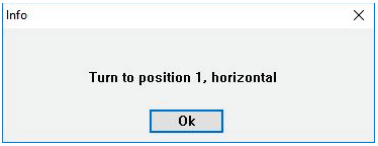
The position of the camera filter slider must be ”3D” also. In this position a neutral density filter is used and thus no Chlorophyll fluorescence measurements can be taken.



3. Now click “Calibrate” in the newly appearing window (upper button next to “Measure”, afterwards start calibration by clicking the lower “Calibrate” button. A

A message appears:

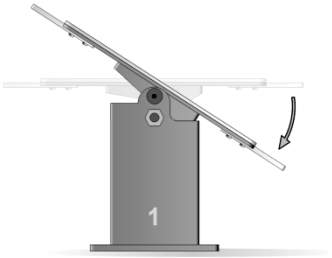
4. Check if the table with large calibration pattern is correctly placed and in horizontal position, then resume with O.K.



Before clicking “OK” the slider door shall be closed to avoid interfering light on the calibration pattern.

5. A sequence of images is projected onto the calibration pattern. They can be judged in the software preview window

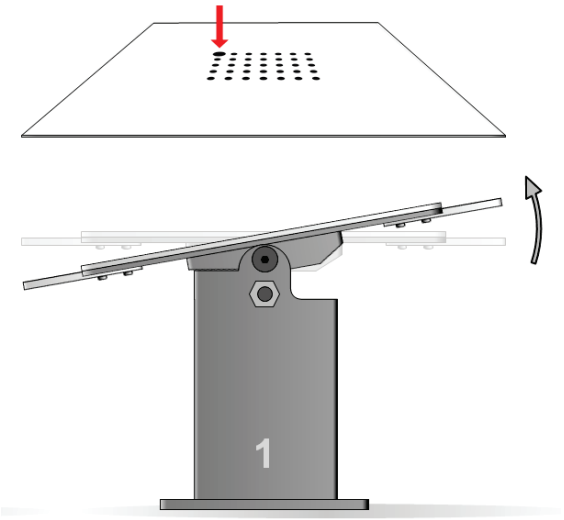
6. For the next sequence the table has to be tilted to the right (30°)



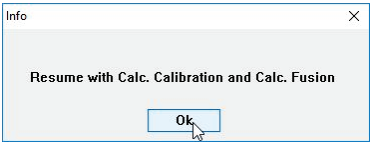
and click OK

The after the first sequence of images the table automatically turns by 180° (from position 1 directly to position 3 and back to position 1).

7. The following calibration step is the “Fusion”. Now the small calibration pattern must be used. Detach the large patter and mount the small pattern. Orientation must be similar to the large one (make sure that the larger spot is located in the rear left edge). “1” on calibration phantom foot aligns with position “1” of the rotary disk facing to the user. See image below.
Tilt left from the beginning on! (10° in contrast to horizontal or 30° as before).



8. Follow the instructions of the software. The rotary table will turn four times 90° and ends up in position 1 again. At the end the following message appears:



9. After pressing OK, click the “Calc. Calibration” button. The Calibration takes some time after which the “Calc. Fusion” button must be pressed.
   Wait until the calculation has been finished. That may take some minutes during which the computer might not be responsive. Working time depends on the computer performance.

At the end of the procedure the 3D-viewer window is automatically opened, and the fusion result can be examined. The calibration phantom is shown as surface rectangle (sometimes with the dots visible as negative). If the rectangle is incomplete or undulating, the

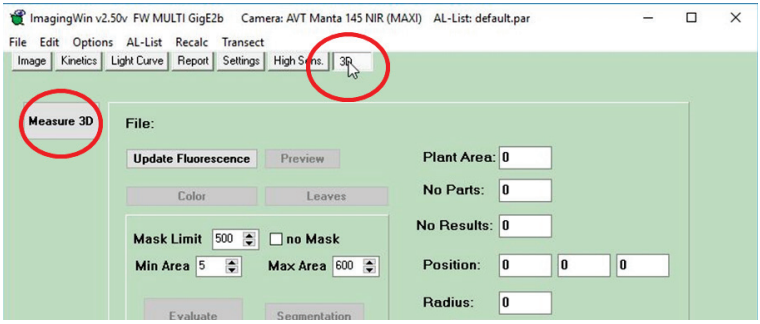
calibration procedure may have to be repeated. Check for interfering light sources or if all the dots are visible in recorded images.

After successful calibration of the system, calibration phantom can be detached and potholder can be mounted again.

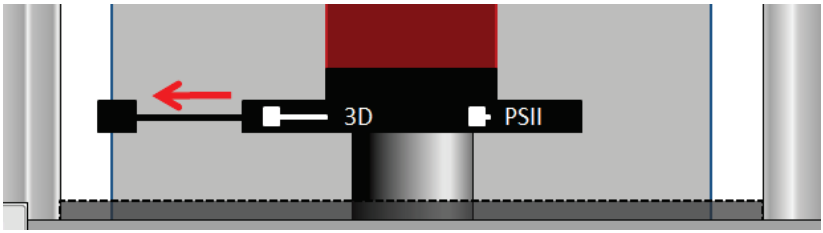
Viewer commands. Please refer to chapter 5.3.2.1 for details about the 3D viewer

5.3.2 3D measuring workflow with IMAG-3D

For the exact differentiation between background and plant sample, the ImagingWin-3D software requires a fluorescence image. This means that an Fo Fm measurement must be performed before each 3D scan (pressing the "Measure 3D" button). This is independent of whether chlorophyll fluorescence is to be used later or not.
For the first steps it should be resumed as described for 3D calibration on the pages before.

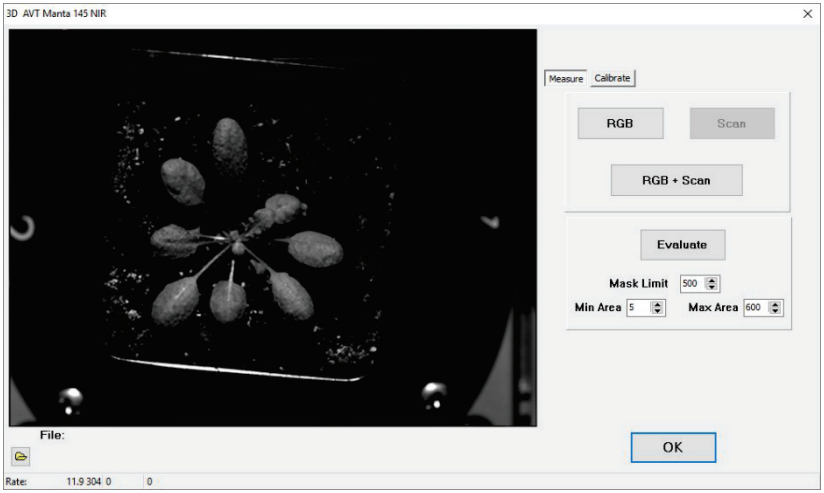


The following steps need a neutral grey filter replacing the PSII detector filter used in the prior steps. This is done by the software automatically if you are using an automated version and in the manual version the filter slider must be pulled into “3D” position manually (image below).

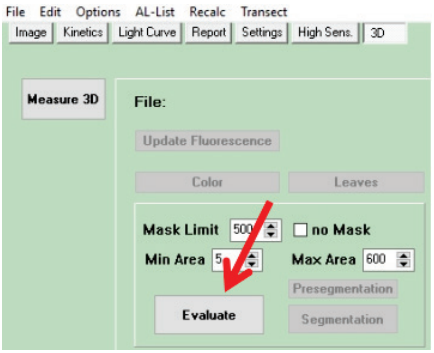


As next step the 3D tab can be clicked in ImagingWin and afterwards the button “Measure 3D”. While being in 3D mode ImagingPAM measuring light is switched off.

Please make sure that the detector filter in front of the camera is set to “3D” position. After clicking the “RGB” button, the projector displays the three colors so that a reflection image of each can be recorded which is then combined into a real color image.



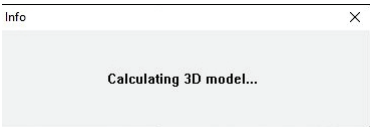
Every 3D scan starts with an RGB image so that the upper right button “Scan” is inactive initially. Alternatively, also the complete scan including 3D scan can be chosen “RGB + Scan”. The resulting RGB image will later be used as texture for the merged surface meshes.



After completion of the RGB image and Scan the extra window shown above closes and brings you back to the 3D tab of ImagingWin software.

Here you can resume by pressing the “Evaluate” button to start the calculation of point clouds and fusion of surface mesh. As next step after successful calculation a viewer window opens in which you can already see the result and zoom (mouse wheel), turn (pull with pressed mouse button).

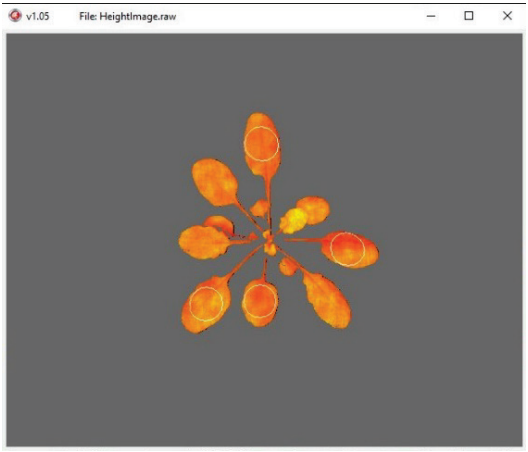
Scanning of a sample is done from four directions whereas in each position a sequence of structured light patterns is displayed and images are taken with each pattern.



At the end some computing time is needed to finish a 3D model. During this time the computer might not be responsive since its CPU is completely busy.

In case the 3D model is defective due to many overlapping leaves or sizes, measuring the sample can be optimized by changing mask limit (smaller for very small leaves) and also “Min Area” and “Max Area” might help to predefine the leaf size ImagingWin 3D module deals with.

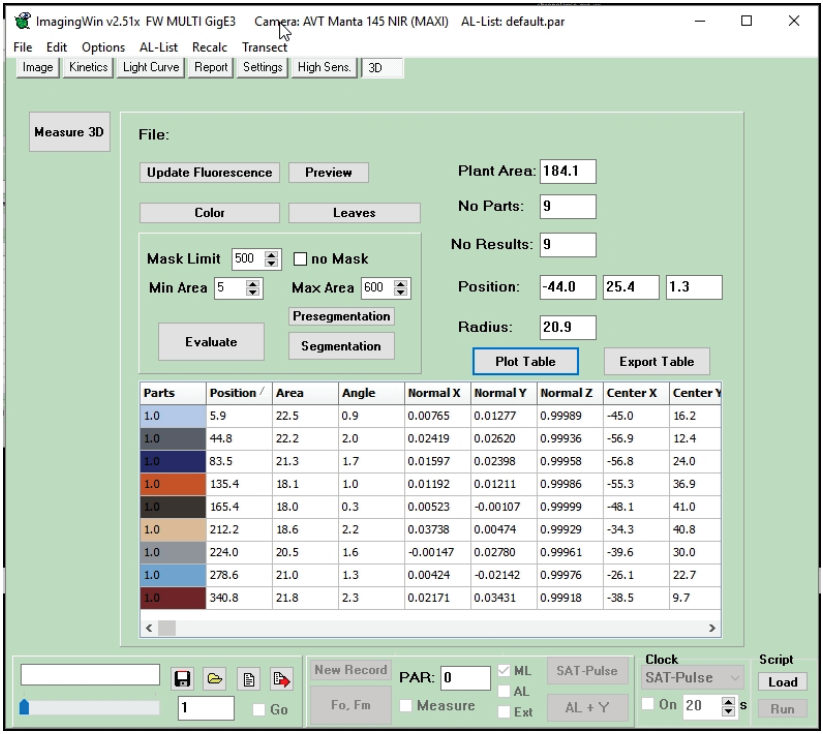
When the calculation came to an end a 3D viewer window opens:



5.3.2.1 3D Viewer Commands

The image represents the position of the sample in which it has been imaged firstly (Position 1). After a right mouse click into the viewer window, the image starts to turn slowly to give a stereoscopic impression. By pulling the imaged sample with the cursor (hold it with left click), the viewing angle can be changed.
Sample magnification can be changed with the help of "up" and "down" keys or the scroll-wheel of the computer mouse.
Right click stops the rotation.
A double left-click resets the image to starting position and magnification.
Closing this window leads back to 3D tab of ImagingWin software.

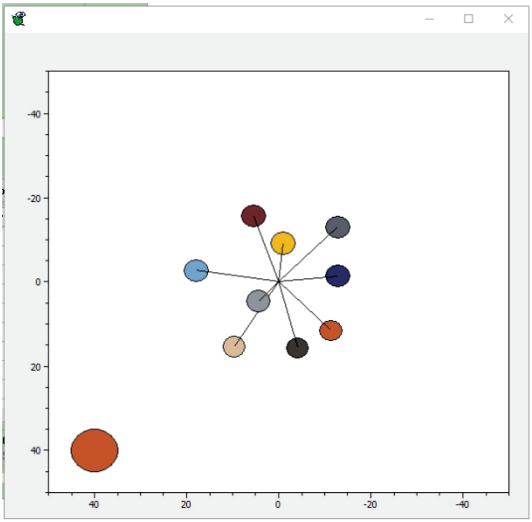
5.3.2.2 ImagingWin 3D evaluations



After performing a 3D measurement and closing of viewer windows further 3D options and evaluations on the basis of the previously measured sample are shown.

On the upper right quadrant of the 3D tab parameters like “plant area” [mm^2], initial number of segments and the resulting number of segments that have been found are listed. “Position” gives the coordinates of sample balance point under the assumption that the sample is a single plant and the sample radius of the smallest circle that can be drawn around the rosette.

opens a window in which a scheme of the sample is shown:



The colors and sizes of circles in the graphic represent the part number color and area of parts found during segmentation of mesh data. This graphic can help to align and find leaves of a sample. Orientation is always starting direction of sample (position 1).

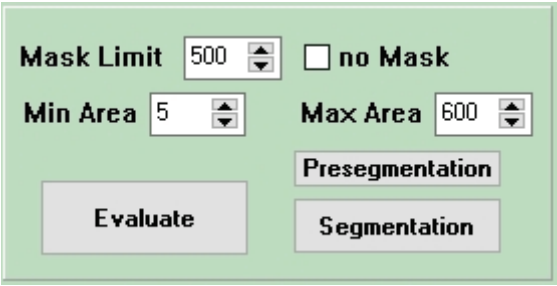
Exports evaluated morphological data, shown as table in the 3D tab, into a CSV file that is saved. Additionally, a tiff file is stored containing a “realcolor” image taken from the sample.

Exported files are always stored in a 3D folder that has been created in 3D data folder: C:\ImagingPam3D\Data_3D_[NAME].

Denotation of exported files is done automatically as follows: both carry the name of measurement so that the real color image is denoted as “realcolor_ Data_3D_[NAME]” and the CSV file is denoted as “Leaves_Data_3D_[NAME]”. Please note that exporting of data several times leads to overwriting of the previously written file as longs as it has not been renamed before clicking “Export Table” anew.

In left upper quadrant can be used on currently loaded or freshly measured data. They refresh features like segmentation (Leaves) or

the real color measurement of a sample (Color). “Update Fluorescence” will lead to a reevaluation of loaded data and thus will take some computing time.



Mask values can be changed if leaves of a sample are not recognized correctly. Using “Pre-segmentation” function is indicated if a sample has many leaves which are close together and overlapping. This function can help to increase the image contrast and thus leads to a better likelihood for a correct segmentation.

Afterwards it is necessary that the “Evaluate” button is clicked.

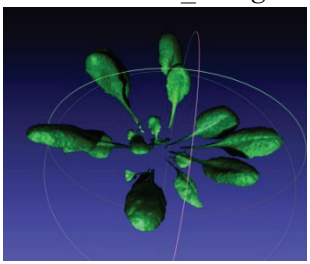
If after a measurement a dataset is saved. PSII data (as PIM file) and 3D data are written into a folder with the name of the experiment. The software automatically offers the creation of a TXT file in which comments can be pasted. This comment files will also be stored into the experiment folder (C:\ImagingPam3D\Data_3D_[NAME]).

Further content is the complete set of images that have been taken for 3D evaluation and the calibration folder of the instrument that has been used for the experiment. The dataset itself is quite large since it contains all the calibration data also but it is recommended to keep them, since evaluation depends of the current 3D calibration so that it would not be possible to reevaluate data without the knowledge of the instrument calibration at the time the experiment was done.

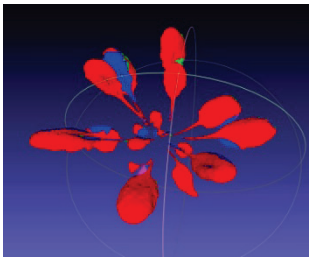
When opening a data_3D folder, stored images appear to be just black. The reason is the color value of the jpgs and the fact that a computer only shows previews of 8-bit images – 3D images are

stored as 12 bit and can be opened in normal imaging processing software.

Most of 3D related files need a hard lock to be used in ImagingWin3D, but some of the 3D files are stored in PLY format so that they can be opened in 3D dedicated software like e.g. MeshLab (which is a quite powerful freeware). These PLY files have always the same name (like “Reconstruction_triangulation_color.ply” distinguishing can only be done by their creation date or the folder in which they are stored.



Reconstruction_triangulation_color.ply and *Reconstruction_triangulation_realcolor.ply* contain similar data as backup. The mesh data are overlaid with RGB image information.



*Reconstruction_triangulation_fluorescence.*ply carries information about mesh segments that have been used to complete the resulting merged mesh. Red colored surface mesh is related to the first viewing angle, blue is a further contribution of position 2, mesh data used from position 3 are shown in green and pink data have been derived from position four.

Reconstruction_triangulation_segmented.ply shows the fusion sample mesh with an overlay of the segmentation image. It can be useful to analyze problems with the segmentation algorithm.

5.3.2.3 Opening of a 3D dataset in ImagingWin3D

The data structure of the stored data was discussed briefly in the previous chapter. Unlike a single PSII measurement, which is stored as a PIM file in the ImagingWin data folder, a lot more data has to be stored in a folder named after the experiment in a 3D system. This folder contains both PIM and 3D data, so opening an experiment in ImagingWin3D involves selecting a folder containing all the necessary information, rather than opening a single file.

As previously mentioned, PIM and PLY files can be opened in ImagingWin or 3D software without a hard lock. However, a dongle is necessary to open a 3D folder with full data access in ImagingWin3D and to use 3D software modules programmed by Fraunhofer IIS (Fürth, Germany).

Additional license dongles can be purchased if an additional workplace for data evaluation is required.

Generally, 3D data are opened by clicking the folder button in ImagingWin3D. A window then pops up in which a folder can be selected. The files contained are not shown! Simply select a folder and click 'Open'. The image window will open in ImagingWin3D as it would in the 2D version of the software, but the 3D viewer can be started by double-clicking on the fluorescence image.

You can also scroll through an experiment and select another image with the desired parameters. After double-clicking on the image window, the viewer will open with the PSII/parameter image as an overlay on the merged mesh. AOI borders are also shown if selected.

After closing the viewer window, you can re-evaluate the data via the PSII functions in ImagingWin or the 3D tab in ImagingWin3D.

6 Components of the IMAGING-PAM MICROSCOPY-version

The MICROSCOPY-version of the IMAGING-PAM consists of:

1) Control Unit IMAG-CG with Battery Charger 2120-N
2) CCD Camera IMAG-K6 (2/3")
3) Modified Zeiss AxioScope 5
4) LED Modules as:

   Microscopy LED Lamp (blue) IMAG-L470M5 or

   Microscopy LED Lamp (red-orange) IMAG-L625M5 or

   Microscopy LED Lamp (UV-A) IMAG-L365M or

   Red-Green-Blue Microscopy LED Lamp IMAG-RGB
5) PC with ImagingWin-software

Operation of the MICROSCOPY-IMAGING-PAM requires an epifluorescence microscope. For this purpose, relatively simple microscopes with short excitation pathways are suited. Most essential components for optimal image qualities are high aperture objectives and a suitable video adapter. While the IMAG-K6 camera features a 2/3" CCD chip, it is recommended to use a 0.5x video adapter for 1/2" chips, in order to obtain a more intense fluorescence image.

The Zeiss AxioScope.A1 microscope may be particularly recommended for use in conjunction with the MICROSCOPY-version.

The features of these components will be described briefly in the following subsections.

6.1 Multi Control Unit IMAG-CG

The same Multi Control Unit IMAG-CG is used for all versions of the IMAGING-PAM M-Series. It was already described in section 3.1 in conjunction with the MAXI-version. The cable of the MICROSCOPY LED Lamps IMAG-L470M5 or IMAG-L625M5 is connected to the MINI-Head socket at the rear side of the control unit. The cable of the Red-Green-Blue Microscopy LED Lamp IMAG-RGB is connected to the RGB-Head socket at the rear side of the control unit. The rear side also features a CAMERA-socket to which the Camera cable has to be connected. This cable is used to for trigger signal and to power the IMAG-K6 camera.

6.2 CCD Camera IMAG-K6

The CCD Camera IMAG-K6 as described in chapter 3.3 features a 2/3" chip with 1392 x 1040 pixels and 4-pixel-binning, resulting in fourfold image intensity for the 640 x 480 pixel displayed on the monitor screen.

If the IMAG-K6 has been used in another IMAGING-M application. Please take off the objective, filters and the distance ring. The IMAG-K6 camera can now be connected on top of the phototube of the Axio Scope.A1 via video adapter. 0.5x adapter for 1/2" CCD cameras (Zeiss; 416112-0000-000, Fig. 52).

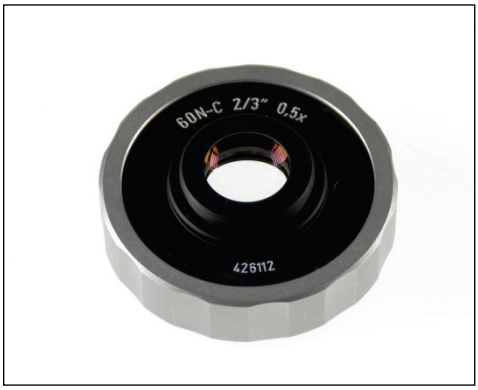


Fig. 52: Camera adapter for connecting AxioScope.A1 with IMAG K6

Fig. 53: Zeiss camera adapter mounted on Axio Scope.A1

The camera IMAG-K6 (not shown) can be screwed onto the C-mount adapter shown in Fig. 53. Afterwards the camera needs to be connected to the camera port of the IMAG-CG multi control unit as well as to the computer via Gigabit Ethernet.

The user should get familiar with the switch which allows switching between binocular or phototube / photo-multiplier pathways (see Fig. 54).

Furthermore, the halogen transmitted light lamp should be switched off during epifluorescence measurements to avoid actinic illumination of the sample.

Fig. 54: Optical pathway switch

6.3 AxioScopeA1 Epifluorescence Microscope

Fig. 55: MICROSCOPY-IMAGING-PAM based on Zeiss ScopeA.1 Epifluorescence Microscope

The MICROSCOPY-IMAGING-PAM is readily adapted to the **Zeiss AxioScopeA.1** microscope giving excellent fluorescence

images. If a complete IMAGING-PAM MICROSCOPY Version has been purchased, some parts are already mounted to make the first setup a bit easier. For safe shipping purposes some components had to be detached.

Existing Zeiss AxioScopeA.1 microscopes can be adjusted to the MICROSCOPY-IMAGING-PAM on request.

Please note that openings in the microscope parts are sealed by stickers or caps that have to be removed before mounting the parts

The AxioScope.A1 has a special port that can carry up to four LED modules which are available in ten different wavelengths starting with 380 nm up to 625 nm. For the standard PAM application, we are recommending the 625 nm red LED module or the 470 nm blue light version (not suitable for the measurement of cyanobacteria). More Information on the Zeiss LED modules can be found in chapter 3.4.9 of the AxioScope.A1 manual or in the internet on www.Zeiss.de. The LED modules offer very homogeneous illumination of the measured area and are modified for PAM applications with additional filters by Walz.

When other wavelengths, than the recommended ones, shall be applied for special measurements, please contact Walz for recommendations on appropriate filter combinations.

Important parts required for optimal performance are the microscope **objective lenses**. High aperture lenses like the Zeiss Fluar 10x/0.5, Fluar 20x/0.75 and Fluar 40x/1.30 Oil are recommended. In this context, it has to be considered that a high aperture enhances image intensity two-fold by increasing excitation intensity as well as fluorescence collection. The image intensity with a Zeiss Fluar

20x/0.75, for example, is 6.6 times higher than with a Zeiss Apoplan 20x/0.45. Since image intensity is the limiting factor in MICROSCOPY-PAM applications, an investment in **high aperture lenses** should have high priority.

6.3.1 Reflector Modules

In complete IMAGING-PAM MICROSCOPY instruments necessary filters of the reflector modules are already mounted.

In case the reflector module is purchased as a separate part, please follow the instructions below. The correct orientation of the beam splitter filter is essential for the correct functioning of the IMAGING-PAM system.

It is required to mount the reflector modules into the turret of the Zeiss AxioScope.A1 as described in chapter 6.3.3. When using more than one LED module, please note that each LED module needs its own reflector module (chapter 3.1.6 Fig 3-9 and 3.4.6 Zeiss manual).

6.3.2 Assembling of beam splitter and filters

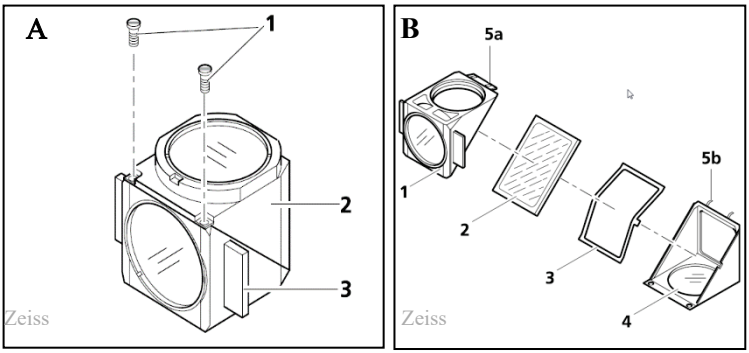


Fig. 56: The reflector module – mounting the beam splitter

A) For opening the reflector module, loosen two screws (A/1). The emission part (A/2) can now be detached from the excitation part (A/3) by a turn around the lower angle. B) Tilt the excitation part on top and lift the emission part out of the holding fixtures. A spring box (B/3) holds the beam splitter filter. Take care that the mirrored side of the beam splitter is pointing upwards.

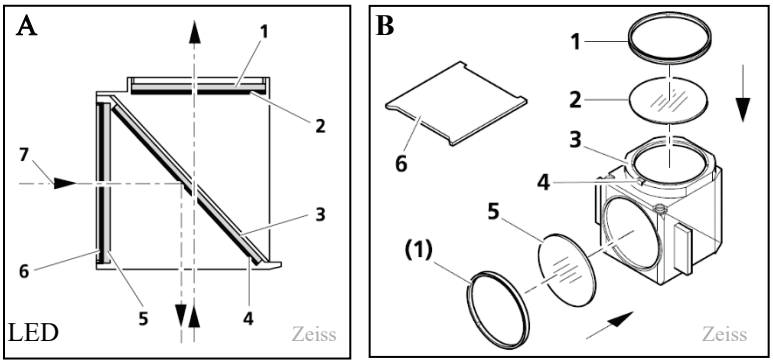
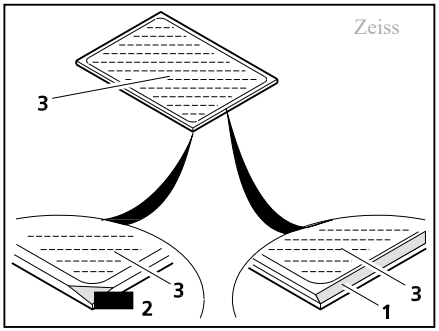


Fig. 57: Reflector module with filter configuration

A) The arrows in Fig. 57/A1 mark the path of the illumination beam or the imaging beam seen from the side. B) lists the different filters and parts that may be needed. The detector filter B /2 (RG665), a filter for the excitation light source (B /5 - normally not needed). B /6 is the mounting tool for the adapter rings (B /1). The beam splitter filter (A /3) is already mounted in this figure (see Fig. 57/B).



Note:

the reflecting (coated) side of the color splitter has a tapered edge or corner.

Fig. 58: Labelling the color splitter

When purchasing the filters separately, independently of the original Zeiss reflector module frame (424931-0000-000), they have to be mounted into the reflector module FL according to the description in this chapter. The red filter RG665 (25 mm in diameter) is inserted on top of the reflector module and held by the adapter ring 1 (Fig. 57/B), so that the filter will show towards the camera of the IMAGING version.

The rectangular beam splitter filter is mounted in a 45° angle (Fig. 57/A3). The mirrored side has to show towards the LED light source. The coating faces outward (in relation to the reflector module) in the direction of the excitation filter (Fig. 57/B5 – not used in the standard setup).

6.3.3 Mounting of the reflector module (AxioScope.A1)

The mounting of the reflector module into the Zeiss Axio Scope.A1 is described in the following Fig. 59.

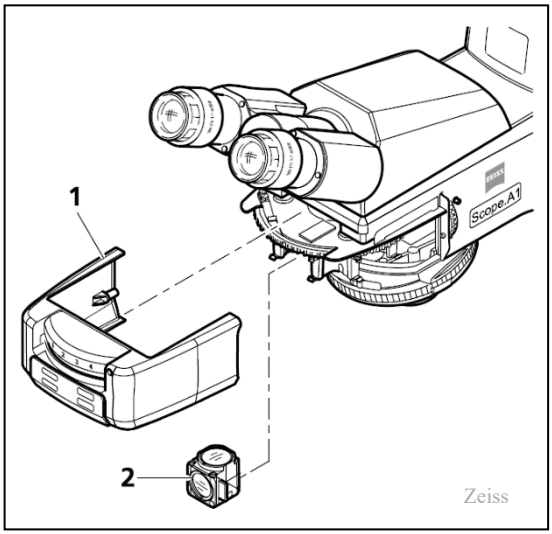


Fig. 59: Changing the reflector module in the upper stand FL-LED

In the front of the microscope a grey plastic cover, behind which the turret for the reflector modules can be found, can be pulled off in forward direction for mounting the reflector modules in the reflector turret. It is just held in place by magnets.

Remove the cover cap (indicated with 1 in Fig. 59) in front of the filter turret to get access to the reflector module ports.

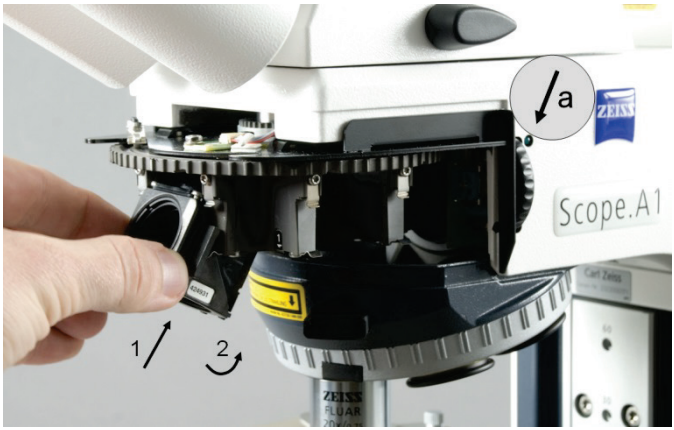


Fig. 60: Mounting the reflector module

The reflector module is inserted after turning it by 180° around its vertical axis (the reflector module is mounted with its excitation filter side Fig. 57/A7 facing to the front). It shall be inserted carefully into the upper spring elements. Then engage it firmly by gently pressing it down into the turret.

When switching to another LED light source, also the reflector module is switched. Please make sure that the numbers of the LED module positions correspond with the numbers of the reflector modules in the turret of the AxioScope.A1.

A grey circle (a) in Fig. 60 marks the switch that normally regulates the LED modules. Since the LED modules for PAM use will be connected via the top cover of the microscope with the central control unit of the imaging system (IMAG-CG), this switch is not active anymore (see chapter 6.4.4).

6.3.4 Mounting of the reflector module (Axioscope 5)

The reflector modules are delivered fully assembled by Walz. However, they are not yet installed in the microscope for safe transport. This must be done by the user before commissioning the microscope. For the IMAGING MICROSCOPY-PAM normally a Zeiss Axioscope 5 with a reflector turret with 4 coded positions is used. The turret holding the push-and-click (P&C) reflector modules can be detached from the microscope by unlocking it via a 3 mm hex nut screw marked with “lock” at the top right of the front of the reflector turret.

Fig. 61 disassembly of the filter turret of Axioscope 5

The drawer in which the reflector module (beam splitter) is mounted is located at the front of the microscope. It is equipped with a small lock on the right-hand side, which requires a 3 mm hex key. After unlocking, it pops out slightly automatically. It can then be pulled out completely.

Inside the drawer are several compartments marked with numbers. In the following, as well as in the manual, compartment no. 1 is generally referred to as the compartment in which the reflector module is located.

To adjust the focus level later using transmitted light, you must select a compartment that is not equipped with a reflector module. Otherwise, the visible image will be very dark.

In the image below the insertion of the PAM reflector module into compartment #1 is shown:

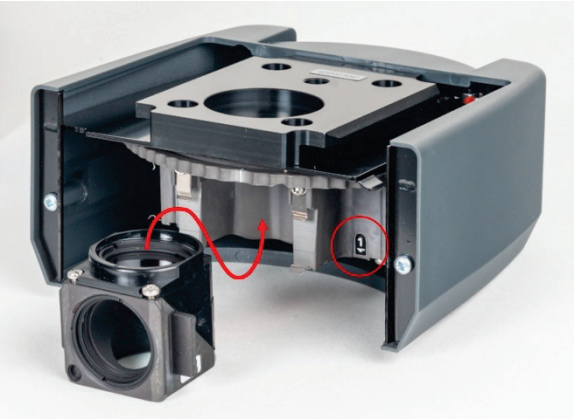


Fig. 62 Mount the push-and-click (P&C) reflector modules delivered with an IMAG-AXIOSCOPE-5 or accessory set IMAG-AX-SCOPE-5

If the reflector module is in the correct position the filter turret can be inserted again. The position in which the reflector module has been inserted is also marked on the front of the turret module (Fig. 61)

6.4 LED Modules

A wide range of wavelengths is available for the Zeiss LED modules, enabling experienced users to easily modify their epifluorescence system with additional light sources of various wavelengths. As described in this chapter, LED modules may need to be adapted. The filters used in the reflector module may also need to be adapted if excitation light sources are exchanged (see chapter 6.3.3 and 6.3.4)

6.4.1 Adjustment of brightness by grey filters (old AxioScope.A1)

Since the original LED modules from Zeiss can be far too bright for PAM purposes (especially for the measuring light, which would prevent a proper Fo determination) neutral grey filters have to be used in front of the LED lightsources for refected light – this is only relevant for users of the old AxioscopeA1. Users of the recent Axioscope 5 may jump to the next section.

A set of these filters are provided with each LED module purchased from Walz (Y = 6,6; 13,7; 23,5 and 51,2). The darkest ones are already used for dimming the LED. Two more neutral grey filters are provided for fine adjustment when other magnifications like the standard 20 x Fluar lens (Zeiss) are used. The higher the magnification used, the smaller the illuminated spot of the imaged field, which means that there is a good chance to increase measuring light intensity on the sample plane too much. In this case it might be necessary to change the filter composition in

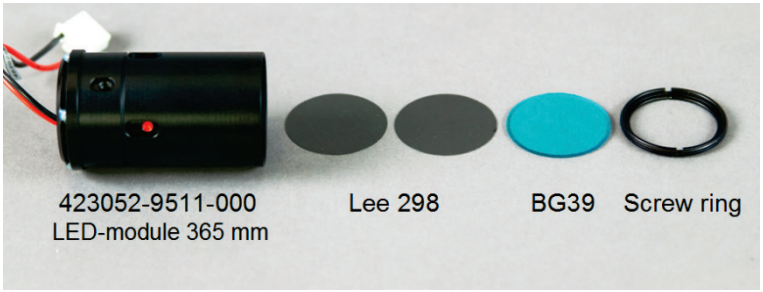
Fig. 63: LED-module filter tool for older Axioscope A1 lamps

front of the LED module by using the provided tool shown in Fig. 63. The side pointing upwards is used for mounting the filters of the LED modules.

Zeiss LED modules are shipped together with a set of filters:

The red LED module IMAG-L625M5 comes with a set of grey neutral density filters that can be used to adapt the emitted light to different magnifications. The additional KPF647,5 filter cuts off unwanted wavelengths and shall be mounted as last filter in front of the ND filters.

With the blue LED module IMAG-L470M5 also four ND filters are shipped. With this unit no additional KPF filter is necessary.



The 365 nm module comes also with light grey Lee filters. These have a transmission of 40% each in the range of 365 nm so that these filters can also be used to adapt the intense ultraviolet irradiance. The

ND filters shall be mounted between the lamp and the BG39 filter which is always recommended to be used as last optical filter

This lamp also needs a special reflector module (Beam-splitter 395 nm Zeiss 446431-0011-000). ***Make sure not to intermix this part with other colorplitter modules.***

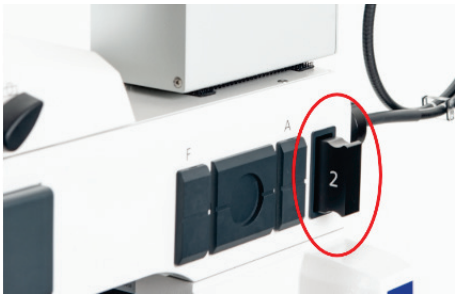
Since this is no standard part, please inquire at Walz.

6.4.2 Adjustment of brightness by grey filters (Axioscope 5)

The light intensity acting on the sample can be adjusted on one hand by settings in the software, on the other hand the microscope offers a filter slider, which has already been equipped with filters by us. This filter slider is in the rear part of the microscope and offers two positions, each equipped with ND filters of different density.

Depending on the lens used, the incident PAM light is focused on a larger or smaller point, so that the light lists to be used depend particularly on the lens used. The filter slider helps to better adjust light intensities and can be equipped with other filter combinations as needed.

In the image below, the filter slider is shown in position 2. The shipping is done with ND filters already mounted in the filter slider. In position 1 you can find an ND filter with Y=51.2 and a darker ND filter with Y=23.5 in position 2.



Please note that light calibrations done before will only apply when the instrument is set up in the same ways as it has been mounted for the calibration.

This applies to all actions that affect the light at the object level (ND filter combinations in the filter slider, LED module changes, magnification changes). For this reason, a check of the correct setup and PAR calibration is recommended.

For an overview, here is an image of the parts that come together with the filter slider (some of them are already mounted and thus not separately packed):

Fig. 64 brightness adaptation set for Axioscope 5

The milky diffuser discs shown in the image above are not required in the RGB head configuration.

Due to the fact that with different lenses, alternative ND filter (combinations) might be necessary, some spare filters with higher density are provided. All mounted filters are held in place with the help of a sealing ring shown on the right side of the image.

Please remember to recalibration the PAR lists after switching to a different filter configuration.

6.4.3 Integration of LED modules into AxioScope.A1 (old)

The integration of LED modules in the AxioScope.A1 is shown in Fig. 65.

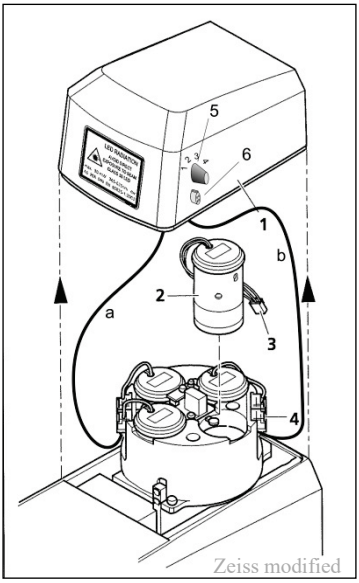


Fig. 65: Changing the LED module in the upper stand part FL-LED

- Lift the covering cap (Fig. 63 indicated with number 1) off the upper stand part.
- Remove the connection plug of the LED module to be changed number 2 and 3 (Fig. 63) from the corresponding slot and pull the LED module out of its socket.
- Insert the new LED module into the socket and plug the cable into the corresponding slot. No further adjustment is necessary.
- As the LED circuit is mechanically coupled to the reflector turret, it is necessary to make sure that LED module and

fluorescence filters on the corresponding reflector turret position are compatible.

- For better operation, the positions of the LED module and those in the reflector turret are numbered.

In the case that the AxioScope.A1 shall also be used for further epifluorescence applications beside PAM measurements, the electrical connections shown in Fig. 65 (cables **a** and **b** connecting the LED modules with the IMAG-CG unit via rotary switch indicated with number 5**)** have to be modified depending on the number of LED modules used for each application. Please ask for technical assistance at the Heinz Walz GmbH if a combined application is intended.

Please Note: If cables **a** and **b** in Fig. 65 are connected the switch for the epifluorescence lamps on the right side of the microscope is not active (Fig. 60 grey circled a).

6.4.4 Equipping the Axioscope 5 with an LED module

In contrast to the older AxioScope.A1, the Axioscope 5 can only carry a single light module for PAM applications in its reflected light slot. Walz offers two standard LED modules with different wavelengths that are ideally suited to the absorption maximum of the measured PSII. The blue module IMAG-L470M5 has a peak wavelength of 470 nm and is ideal for most samples containing green algae or diatoms, while the red module IMAG-L625M5 with an excitation wavelength of 625 nm is able to deliver slightly better signals for cyanobacteria samples. A customized reflector module is available for each of the two LED modules.

In the back of the Axioscope 5, you can see a single optical port to which the single wavelength LED lamps MC-470M5 and MC-625M5 are flanged.

For protecting the optical components, most of the retaining screws are also made of plastic. They should only be tightened carefully so that the components are fixed.

n the following image the optical port is shown in detail:

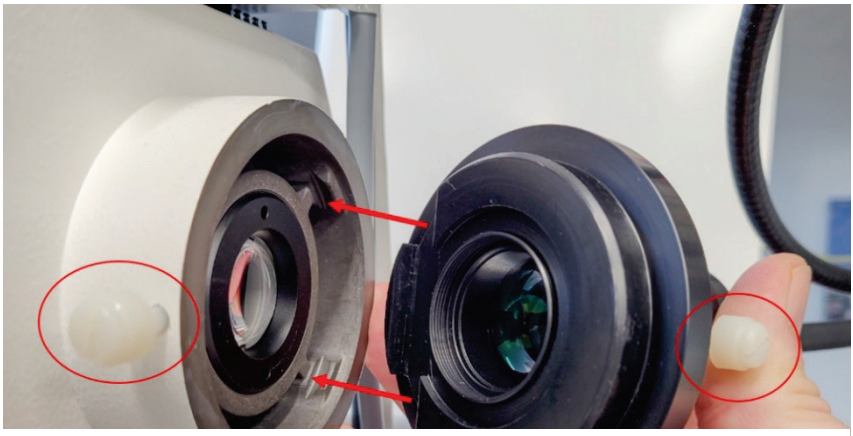


Fig. 66 Assembly of the optical port (here already with the LED module inserted). To protect the components, the screws are made of plastic. They are only used to secure the parts and should be tightened carefully.

When positioned correctly, the optical microscope port looks as in the following image:

For an orderly cable routing, but also to avoid kinks that can lead to cable breakage, cable clamps are mounted at two points of the housing (see image above).

6.4.5 Connecting LED modules with IMAG-CG

For the use together with IMAGING-PAM MICROSCOPY version the LED modules have to be connected with the central control unit IMAG-CG. The provided connection cable for the LED modules has to be connected with the 3-pin connector (Fig. 65 number 6) on the cover cap of the Zeiss AxioScope.A1 and with the 6-pin "MINI-Head" connector on the IMAG-CG control unit.

6.4.6 Switching LED modules for measurements

Switching from one LED module to another is done by turning the filter wheel on the front of the reflector turret. Additionally, the rotary switch on the right side of the covering cap (Fig. 63 indicated with number 5) has to be set to the corresponding number of the reflector module chosen, so that the LED module is engaged for the measurement.

6.4.7 IMAG-RGB

Fig. 67: IMAG-RGB Microscopy LED Lamp

The Red-Green-Blue Microscopy LED Lamp (in contrast to the IMAG-L470M5 or IMAG-L625M5) features its own LED drivers. It

is connected to the RGB-Head socket at the rear side of the IMAG-CG control unit. Optically it is connected to the epifluorescence microscope via a fluid light guide (100 cm length, 3 mm ∅). The IMAG-CG control unit provides the power and the trigger signals for driving the 3 types of LEDs. Special pigtail-LEDs exclusively developed for PAM fluorometry are used. These LEDs give exceptionally high light intensities at the exit of 1 mm ∅ plastic optical fibers. The fibers of 7 LEDs (2x Red, 3x Green, 2x Blue) are put together resulting in a 3 mm ∅ bundle, to which the 3 mm ∅ fluid light guide connects. The latter serves for thorough mixing of the three colors and for carrying the light to the epifluorescence microscope excitation entrance port.

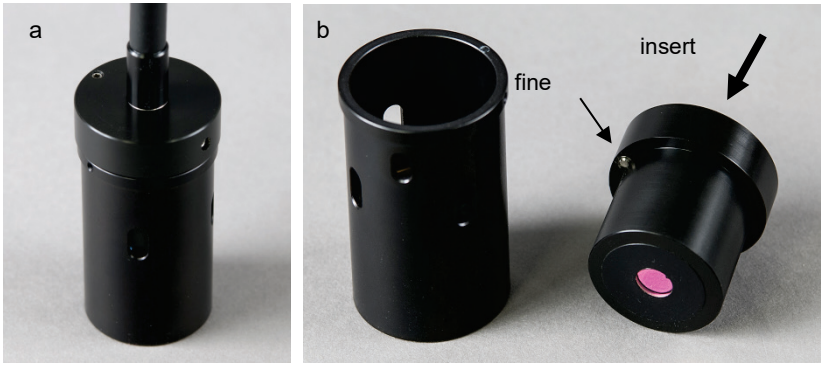


Fig. 68: Fluid Light Guide with Adapter Endpiece for connecting to excitation entrance port of Epifluorescence Microscope

The microscope side of the fluid light guide features a metal tube adapter with an aspherical lens that fits to the LED module socket of the AxioScope.A1

- Lift the covering cap (Fig. 63 indicated with number 1) off the upper stand part.

- Remove the connection plug of the LED module to be changed number 2 and 3 (Fig. 63) from the corresponding slot and pull the LED module out of its socket.
- Insert the fluid light guide metal tube into the socket.
- Close the covering cap.
- Insert the fluid light guide through the whole in the covering cap into the fluid light guide metal tube.

As the LED circuit is mechanically coupled to the reflector turret, it is necessary to make sure that LED module and fluorescence filters on the corresponding reflector turret position are compatible.

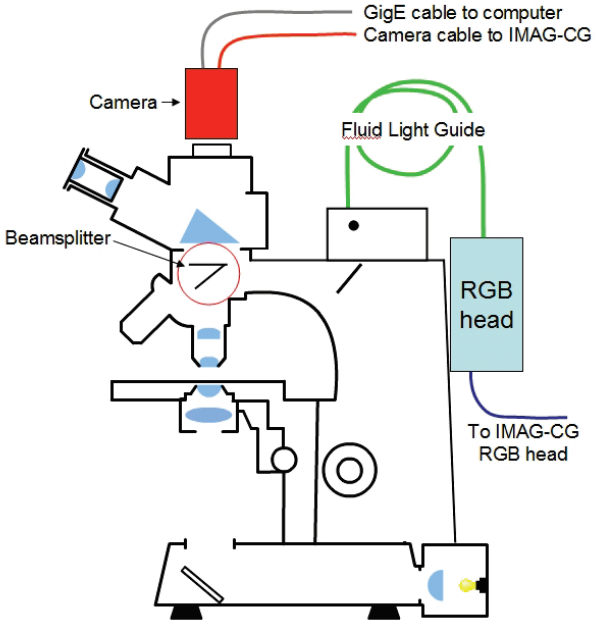


Fig. 69: Schematic presentation IMAGING-PAM MICROSCOPY-version with IMAG-RGB

7 How to get started

While some parts of this section specifically refer to the MAXI-Imaging-PAM, most information applies to all versions of the Imaging-PAM M-series. Specific information on MINI-, and MICROSCOPY-versions is presented in sections 4 - 0.

The IMAGING-PAM is readily set up and its basic operation is quite simple. The following sub-sections explain how to put the system together and how to install the software on the PC. Also some simple measurements will be described, which may help the user to become acquainted with the instrument.

7.1 Connecting the cables

There is a total of 3 (in case of MAXI-version 4) cables to be connected:

1) Camera cable between the CCD Camera and the Control Unit IMAG-CG (front side)
2) GigE Ethernet cable between Camera and PC
3) LED-Array cable connecting to the Control Unit IMAG-CG

- MAXI-version: IMAG-MAX/L or IMAG-MAX/LR cable to MAXI-HEAD socket (front side), and the second LED-Array cable connecting to the separate POWER-SUPPLY with the red plug to the red (+) socket and the black plug to the black (-) socket
- MINI-version: IMAG-MIN/B, IMAG-MIN/R or IMAG-MIN/GFP cable to MINI-HEAD socket (rear side)
- MICROSCOPY-version: IMAG-L625M5 and IMAG-L470M5 is connected via MINI-Head cable from the 3-pin connector (Fig. 65 number 6) on the cover cap of the Zeiss

AxioScope.A1 to the 6-pin “MINI-Head” connector (IMAG-CG rear side), the IMAG-RGB is connected via IMAG-RBG cable to RBG-HEAD socket (IMAG-CG rear side)

Notes:	**All cables should be connected prior to switching on the Control Unit, the separate POWER-SUPPLY and the PC is started.** **Generally, always first switch off the external POWER-SUPPLY before exchanging LED-Array cable.** **Never have more than one Measuring Head (MAXI-, MINI- or RGB-) connected at the time.**

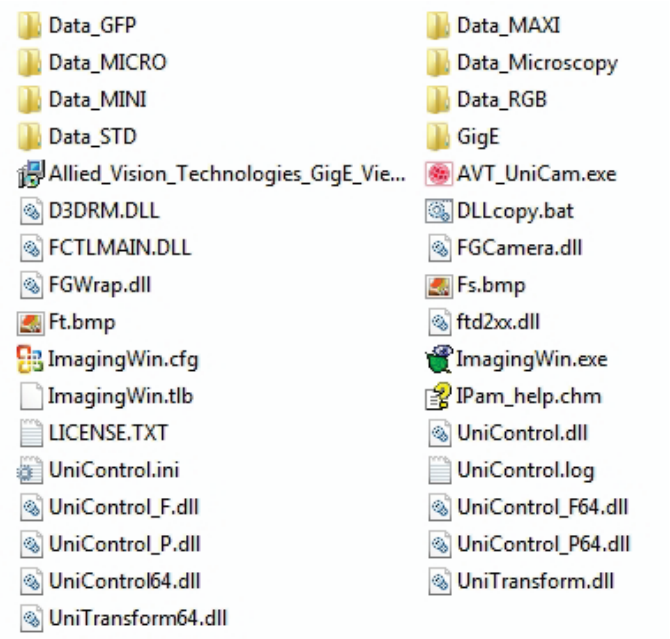
7.2 Software installation

All software required for operating the IMAGING-PAM already is installed, when the Notebook PC IMAG-PC was purchased together with the instrument. Otherwise the user can install the required software as outlined in the following sub-sections.

7.2.1 Installation and Starting of ImagingWin

The ImagingWinGigE software is delivered together with the instrument in form of a CD-ROM. For installation of ImagingWin this CD-ROM is put into the CD-drive of the PC which is going to be used in conjunction with the IMAGING-PAM. Installation occurs automatically (Autostart). If the Autostart function is not active under Windows, the Set-up file has to be started manually from the CD. A program icon (ImagingWinGigE) and a link to the ImagingWinGigE Folder are automatically installed on the Desktop.

The ImagingWinGigE folder contains all files required for operation of the IMAGING-PAM and also the Data-directories for the various types of measuring heads.



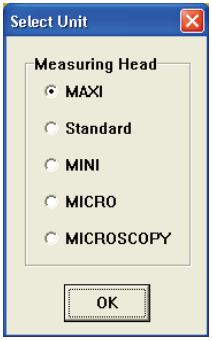
For updates of the ImagingWinGigE software update setup files can be downloaded from the Walz website (www.walz.com). Please note that the Data directories and all system settings are not affected by the Update. After clicking "Install" the Windows "Hardware Installation" may give a warning which, however, can be ignored by clicking "Continue Anyway". The installer will automatically setup

necessary camera drivers. After installation the software can be started by clicking on the desktop icon.

7.2.2 Installation of camera driver

If, for some reason, the previously installed Camera driver got lost, the re-installation can manually be done by opening the Allied_Vision_Technologies_GigE_Viewer located in the ImagingWinGigE folder.

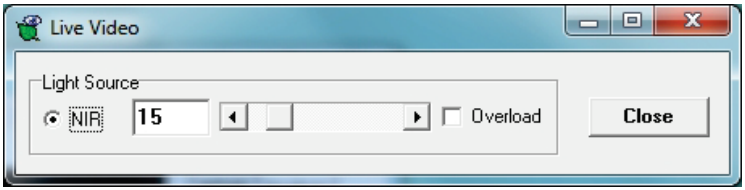
7.3 First steps and examples of routine measurements



Once the IMAGING PAM has been set up and all cables connected and software installed (see section 7.2), the first measurements can be taken to familiarise yourself with the instrument. The following description assumes the use of the MAXI measuring head with the LED array illumination unit IMAG MAX/L in conjunction with the mounting stand IMAG-MAX/GS. Before starting the programme, switch on the power at the IMAG-CG control unit (via the power switch on the front) and the external 300 W power supply (via the rear switch). When the program is started by clicking the ImagingWinGigE.exe start icon, a selection window will appear.

If you are using one of the other imaging systems (MICROSCOPY or MINI), you will be prompted to select the light colour. It is important to select the correct colour and head, as otherwise the LEDs may be damaged. After selecting the measuring head and confirming with OK, check and confirm the Maxi button with OK. The pulse-modulated fluorescence measuring light will then switch on automatically. Fluorescence is measured using relatively weak measuring light pulses at a low repetition rate

(approximately 1 Hz). This weak measuring light does not cause significant changes to the state of a leaf sample. After starting the program, the image of the fluorescence parameter Ft is displayed in the image window on the PC monitor screen. The Ft image is black if no leaf sample is present. When a leaf sample is placed on the X-Y stage plate at the slow rate of the measuring light pulses, the Ft image slowly appears on the screen. To arrange a defined position of the sample within the field of view and focus the image, it is often advantageous to switch from fluorescence imaging to near-infrared (NIR) imaging by selecting Live Video (on the right-hand side of the Image window). NIR measuring light pulses can now be used to follow image changes. The frequency of the NIR light pulses can be adjusted in the Live Video window.



The image can be **focused** by turning the **adjustment ring** of the **objective lens**. After focusing the image using NIR light, quit the Live Video window by clicking 'Close' or the exit box in the top right-hand corner. The system will then return to fluorescence measuring mode and display the focused fluorescence image. Fig. 70 shows a screenshot of a typical fluorescence image of a leaf under the above conditions.

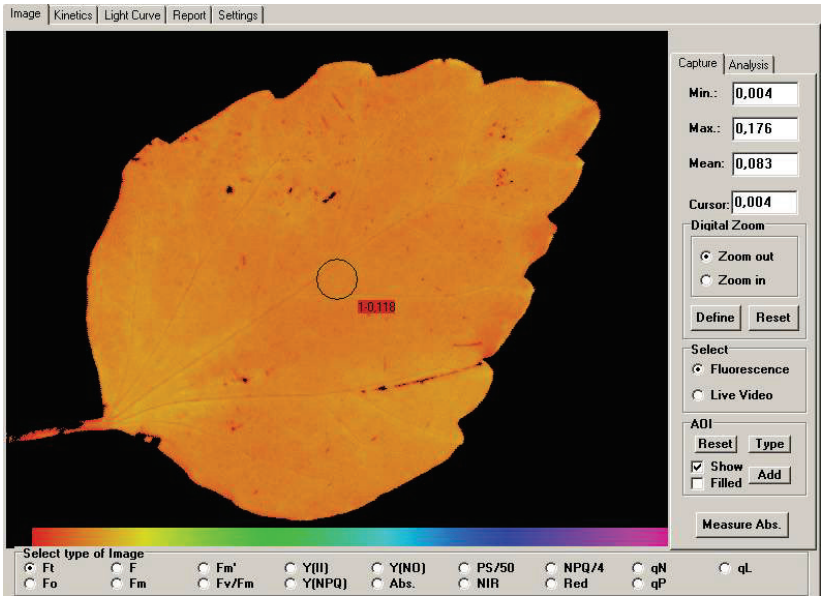


Fig. 70: PC screen shot of Ft-image of leaf

In the center of the screen by default a circular area is defined as so-called **Area of Interest (AOI).** The Ft values of all pixels within this area are averaged and the averaged value is shown in the little red box close to the AOI. Additional AOIs can be defined by the user, with various shapes and sizes (via the **AOI box** at the right-hand side of the screen). At the bottom of the image area the **false color code bar** is displayed, with the colors encoding for numerical values between 0 (corresponding to black at the left edge) and 1 (corresponding to purple at the right edge).

So far, the IMAGING-PAM has been monitoring fluorescence yield, but no actual measurement was carried out yet. With the IMAGING-PAM, just as with most other PAM fluorometers, a "measurement" means the assessment of photosynthetic parameters by fluorescence quenching analysis with the help of a saturating light flash (**Saturation Pulse**). For determination of so-called **quenching**

coefficients, measurement of the minimal and maximal fluorescence yield of a dark-adapted sample is important. dark-adaptation does not have to be strict. In most cases a few minutes adaptation to low light conditions are sufficient for serving this purpose.

Warning:

It is recommended that the eye protecting red Perspex hood is slid down before a saturation pulse is given.

The fluorescence intensity excited by the Saturation Pulse is so high that it can be readily seen by the bare eyes through the red Perspex hood, which absorbs the much stronger blue light, which would be harmful for the eyes. The **"dark fluorescence parameters"** can be assessed by an **Fo, Fm measurement**.

The corresponding push button is at the bottom of the screen, together with various other elements for system operation.

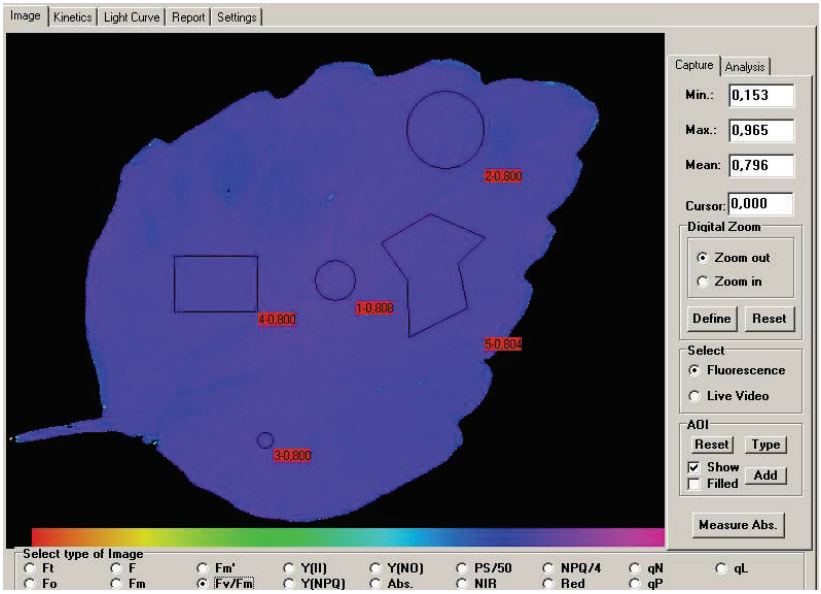


Fig. 71: PC screen shot of Image window following Fo, Fm determination with the Fv/Fm image being selected and various types of AOI being defined

In Fig. 71 the Image window following Fo, Fm determination is shown, when the **Fv/Fm-image** is selected. Fv/Fm reflects the maximal PS II quantum yield of a dark-adapted sample. With the given leaf sample, Fv/Fm is distributed quite homogenously over the whole leaf. It may be noted that after Fo, Fm determination the Fo, Fm button is not accessible anymore and that instead the **New Record button** has become accessible. The Fo, Fm determination will remain valid until a New Record is started. All F and Fm' values measured in conjunction with the help of Saturation Pulses (triggered via the **SAT-Pulse button**) are compared with Fo and Fm and consequently the quenching parameters are calculated. The user may apply some Saturation Pulses and experience how the images of the various parameters (e.g. F, Fm', Yield, qP and qN) change with pre-exposed.

When the sample is illuminated, the **effective quantum yield** is decreased, as PS II reaction centers partially close (decrease of photochemical quenching) and energy dissipation into heat increases (increase of non-photochemical quenching). Actinic illumination can be started by checking the **AL box**. Then the **PAR box** shows the PAR-value of the **incident light**. For assessment of fluorescence parameters during actinic illumination, a Saturation Pulse can be applied using the SAT-Pulse button. Fig. 72 shows an image of effective PS II quantum yield, Y(II), measured with the help of a Saturation Pulse applied after 2 min illumination at 81 µmol quanta $m^{-2}s^{-1}$.

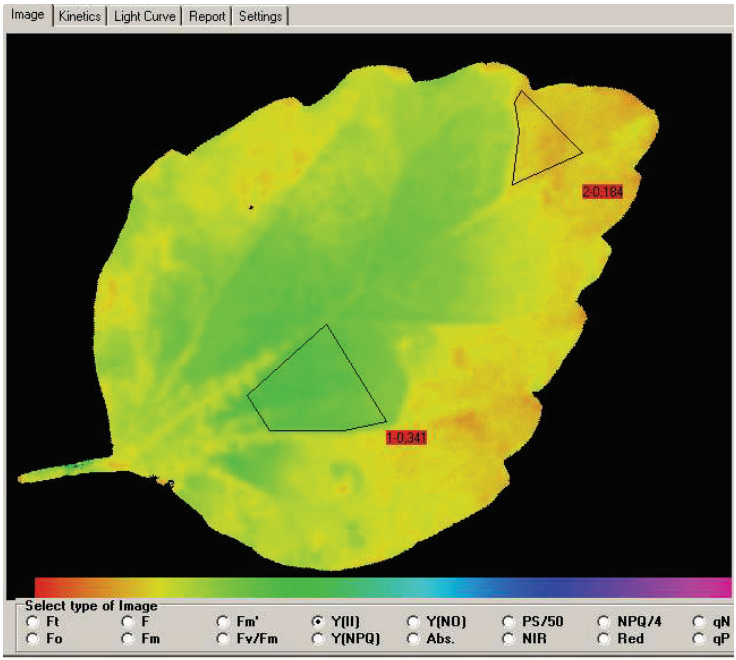


Fig. 72: Y(II) image assessed after 2 min illumination at 81 µmol quanta $m^{-2}s^{-1}$

This measurement reveals some heterogeneity in the lowering of quantum yield by illumination in different parts of the leaf. It is

generally observed that differences in photosynthetic efficiency can be distinguished best when actinic light is applied, which puts some pressure on the limiting steps of the overall process, such that electrons accumulate at the acceptor side of PS II.

Considerable heterogeneity is also displayed by nonphotochemical quenching (expressed by the fluorescence parameters qN, see also 10.1.1.15) as illustrated in Fig. 73

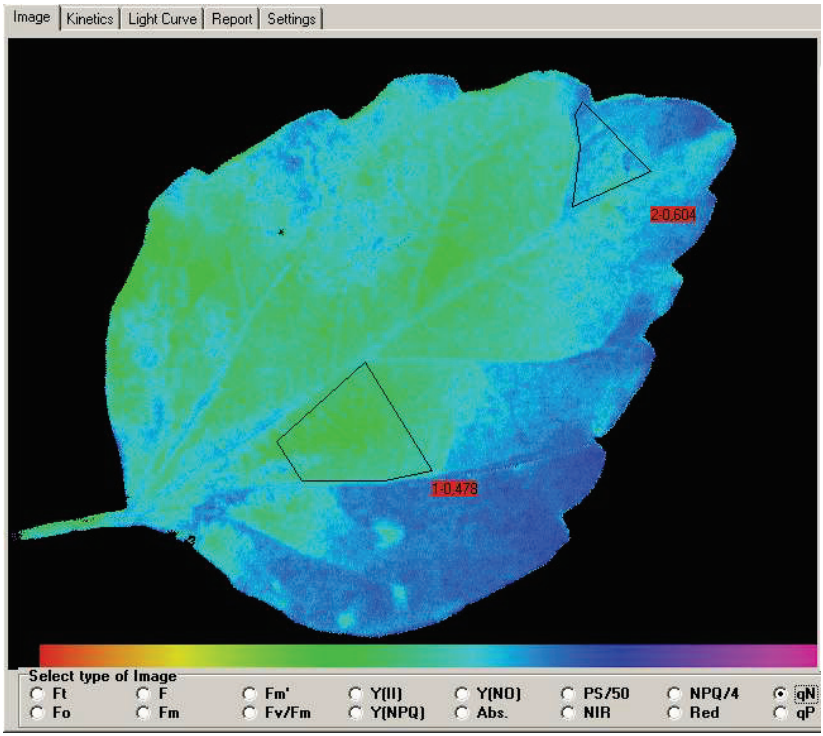


Fig. 73: Image of the coefficient of nonphotochemical quenching qN measured 2 min after onset of illumination at 81 µmol quanta m^{-2-1}

The light induced changes in fluorescence parameters are highly dynamic. When a dark-adapted sample is illuminated, fluorescence yield first rises and then drops again **(dark-light induction curves,**

Kautsky effect). **Saturation Pulse quenching analysis** reveals that characteristic changes in quantum yield (YII) and nonphotochemical quenching (qN) accompany the changes in fluorescence yield. The **Kinetics-window** serves for the study of such dark-light induction phenomena. It is opened by clicking the **Kinetics** register card at the top of the screen. For the recording of induction kinetics at least one AOI has to be defined. Then the recording of an **Induction Curve (Ind.Curv.)** can be started (click "Start").

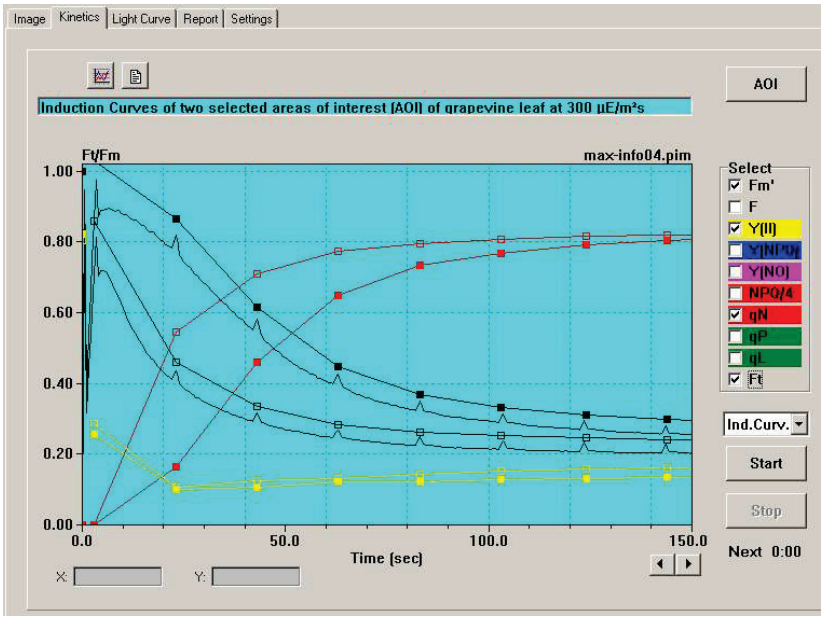


Fig. 74: Kinetics window showing Induction Curve. Two AOIs are selected, for which the averaged pixel values of Fm', Y(II), qN and Ft are displayed.

In ImagingWin, recording an induction curve constitutes a **new record**, which is initially stored in **buffer memory**. It can then be permanently saved to the hard disk. Induction curve recordings normally start with an **Fo, Fm measurement**, based on which the quenching coefficients are calculated. When the Induction Curve

recording is terminated, ImagingWin exits **Measure Mode** (the green tick box is inactive) and enters **View Mode**, which allows you to view the recorded data. A vast amount of information is stored during the course of an induction curve, which can be analysed at any time after the recording. Analysis is also possible in **Offline Mode**, i.e. without the IMAGING PAM being connected to the PC. Images of the various fluorescence parameters were captured for each Saturation Pulse. These images can be viewed by returning to the **Image Window**, where the desired parameter can be selected. When '**Go**' is activated, the consecutive images are displayed as if in a movie, starting with the data set corresponding to the Fo, Fm determination. The **Go Speed** can be modified under **Settings** (click on the relevant register card). Images can also be selected manually by deactivating 'Go' and clicking in the box to the left, where each mark corresponds to a data set associated with a saturation pulse. The current number is also shown in a separate box. In View mode, the data can be stored as a **PAM imaging (XPIM) file** on the hard disk. Individual images can also be exported as **TIFF** or **JPEG files**.

Dark-light Induction Curves provide valuable information about the various stages of the complex photosynthetic process, enabling the identification of potential limitations induced by stress parameters, for example. The IMAGING PAM enables this tool to be applied with high reproducibility using **pre-programmed Standard Induction Curves**. Induction curve parameters such as actinic light intensity, the time interval between saturation pulses, and the duration of illumination can be defined by the user in the **Settings** menu. Another standard tool for assessment of photosynthetic parameters by Saturation Pulse quenching analysis are recordings of **Rapid Light Curves** (more briefly also called **Light Curves**). For measurement of a Light Curve the user has to return to the Measure Mode and click the **Light Curve register card**. While the recording of a pronounced Induction Curve is favored by previous dark

adaptation, the opposite is true for the recording of a Light Curve, which should not be dominated by induction effects. Therefore, a Light Curves can be measured best shortly after an Induction Curve with the same sample. While the Light Curve is running, one can either follow the development of the curve on the **Light Curve window** or look at the changing images of e.g. Y(II) or qN. The Light Curve starts with an Fo, Fm determination which, however, formally is correct only, if the sample was dark-adapted. If a Light Curve is recorded after an Induction Curve using the same sample, the previously measured Fo, Fm values may be retained, provided there was no change in the position of the sample.

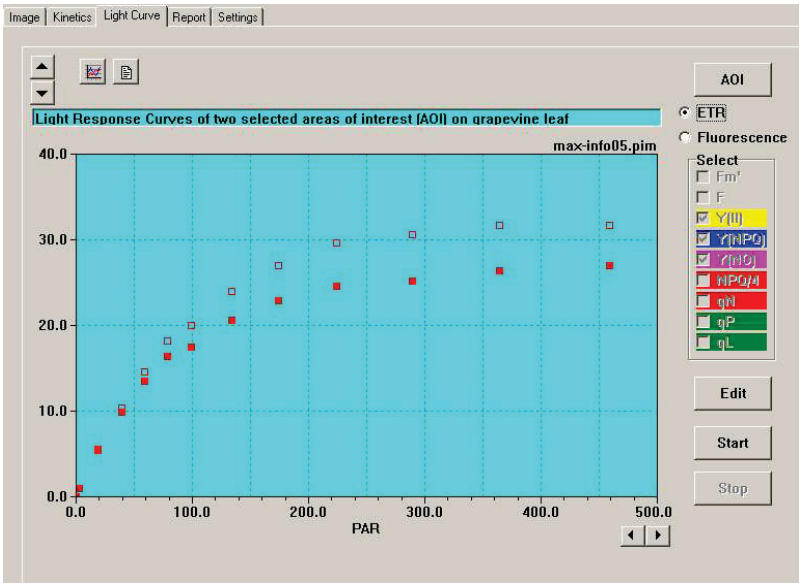


Fig. 75: Light Curve window showing the Light Curves of two AOIs, for which the averaged values of the ETR parameter (relative apparent electron transport rate) are displayed.

In Fig. 75 a Light Curve recording of the ETR-parameter is shown. ETR is a relative measure of the apparent electron transport rate. It initially shows an almost constant slope and saturates at high

light intensities, in analogy to conventional light response (PI) curves. It has to be kept in mind, however, that PI-curves are measured with much longer adaptation times at each intensity step.

The original definition of the ETR-parameter assumes a uniform absorption of incident light over the whole sample area**:**

ETR = Yield x PAR x 0.5 x Absorptivity

The Absorptivity parameter describes the fraction of incident light which is absorbed. The factor 0.5 takes into account that only half of the absorbed quanta is distributed to PS II (under steady state conditions). In most studies carried out with standard PAM fluorometers, like the PAM-2100 or MINI-PAM, it has been assumed that Absorptivity amounts to 0.84, which is the mean value for a large number of normal, healthy green leaves determined with the help of an Ulbricht Sphere.

The IMAGING-PAM offers a special routine for measuring **PAR-Absorptivity images** by comparing the remission images of diffuse red and NIR radiation, which is emitted by the same LED-Ring-Array as the blue fluorescence measuring light. Despite of its simplicity, this routine function works well, provided the intensity of the red light was appropriately adjusted with respect to the NIR light.

Additionally it is important that the sample surfaces are compatible in the sense that they must not be shiny or damp. A PAR-Absorptivity measurement is carried out after clicking the **Measure Abs. button.** First an NIR-remission image and then a R-remission image is measured, the Absorptivity-parameter is automatically calculated pixel by pixel according to the equation **Abs. = 1 - R/NIR** and the Abs.-parameter is displayed on the Image-window, as illustrated in Fig. 76

In case an Absorptivity measurement has been made prior the measurement, the actual measured Abs values will be taken for the calculation of the photosynthesis parameters like ETR.

Any pigment that absorbs Red more than NIR light, i.e. generally all photosynthetically active pigments, will decrease R with respect to NIR, thus decreasing R/NIR and increasing the derived Abs.-value. On the other hand, pigments that absorb Red and NIR light similarly, as e.g. necrotic spots, will not cause R/NIR to deviate substantially from unity and thus Abs. will remain close to zero. Notably, a white and a black piece of paper give similar Abs.-images with pixel-values close to zero. This illustrates that the Abs. parameter does not assess general Absorptivity of a sample for visible light, as sensed by the human eye, but rather specifically the Absorptivity of photosynthetically active light. Therefore, the Abs. parameter as determined by the IMAGING-PAM may be considered a close estimate of PAR-Absorptivity. This approach is based on the empirical fact that pigments that contribute to the absorption of PAR do not show significant absorption bands in the near-infrared (NIR) spectral region. On the other hands, pigments that absorb NIR are likely to also absorb Red light.

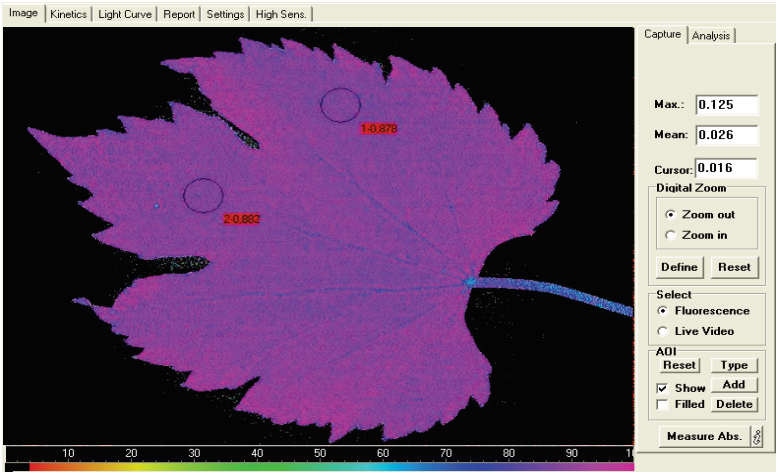


Fig. 76: Image of PAR-Absorptivity determined by the Measure Abs. routine

In the example of Fig. 76 Absorptivity is distributed quite homogenously (pixel values around 0.9) over the whole leaf area. In other cases, substantial heterogeneities can be observed, e.g. induced by viral or fungal infections and stress induced damage which leads to formation of necrotic spots.

With information on PAR-Absorptivity, it is possible to calculate a relative apparent rate of photosynthetic electron transport:

PS = Yield x PAR x 0.5 x Abs.

This parameter is calculated by ImagingWin and can be selected on the Image-window. As all imaged parameters have to be normalized to values between 0 and 1 (for the sake of a uniform false color scale), the calculated PS-values are divided by the expected maximal rate, the preset value of which is 50.

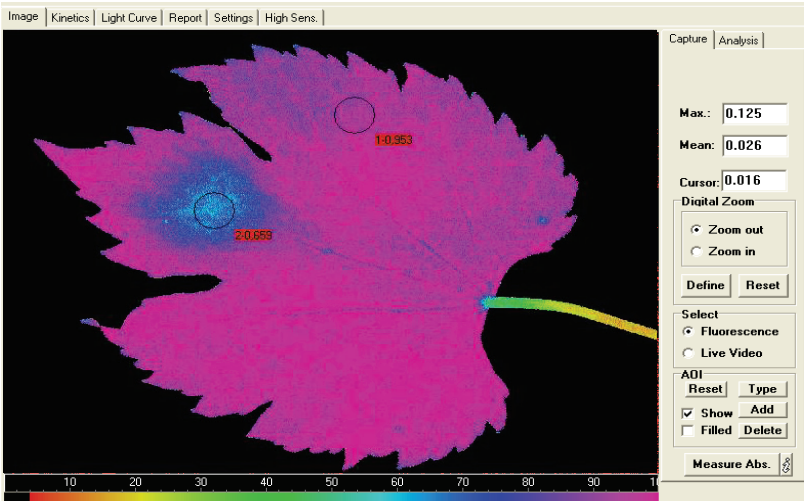


Fig. 77: Image of the relative apparent rate of photosynthetic electron transport as measured by the PS/50 parameter

The PS/50 image displayed in Fig. 77 reveals that a homogeneously green looking leaf may show distinct heterogeneities in photosynthesis.

These first measurements on one hand demonstrate the simplicity of measurements with the IMAGING-PAM and on the other hand give a first impression of the vast potential of this tool for assessment of photosynthetic parameters. This introduction should enable the user to get acquainted with the instrument and to start carrying out own experiments. For quantitative work some more information may be required. In the following Chapter 8 the numerous functions and features of the ImagingWin software are described systematically in more detail. Unavoidably there will be some overlapping with the information given in this Chapter 7.

8 ImagingWin

Except for the POWER on/off switch on the Control Unit and the External 300 W Power Supply, the MAXI-IMAGING-PAM is fully operated via PC using the ImagingWinGigE software. Fig. 78 shows the user interface of ImagingWinGigE after start of the program, as seen on the PC monitor screen. The current version offers the option of detaching the image window to the left of the screen and displaying it next to the data or settings window. For reasons of space, we will limit ourselves to the integrated, classic display in this manual. You can switch between the different views using the tabs at the top of the window.

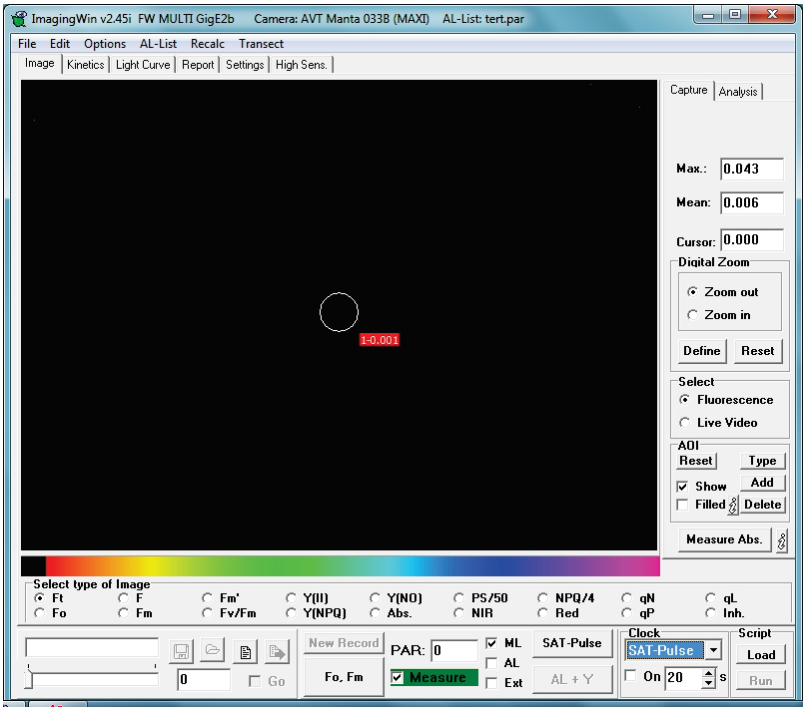


Fig. 78: User surface of ImagingWinGigE after start of the program

The screen is divided into three parts. A topmost part, containing the menu bar (see chapter 11). A major upper part, the content of which changes depending on the particular window selected by the various **register cards 10** (Image, Kinetics, Light Curve, Report, Settings and High Sens, chapter 10:) and a bottom part, which relates to **system operation** (like saving data, starting measurements etc.) remains unchanged when different windows are selected (chapter 9).

1) After start of the program, the upper part of the screen by default shows the **Image-window**. The other windows can be installed by clicking the corresponding register cards. The various windows will be explained in detail in separate sections below.

2) At the bottom of the screen different types of functional elements essential for operation of the IMAGING-PAM are located:

   - the elements at the left side relate to the **recorded data** (viewing, saving, opening and export of data)
   - in the middle the functional elements are located which serve for defining a new recording (**New Record; Fo, Fm; Measure**)
   - the elements to the right relate to the **various types of light sources** (PAR, ML, AL, Ext, SAT-Pulse, AL+Y, Clock)
   - the remaining elements on the right side are for operating the ImagingPam using **script files** (Load, Run)

3) The top of the screen locates the menu File, Edit, Options, Al-List, Recalc and Transect.

9 ImagingWin - System Operation

9.1 Definition of New Record

9.1.1 Fo, Fm

The determination of Fo and Fm is of central importance for recordings with the IMAGING PAM. Only after the appropriate determination of Fo and Fm using a dark-acclimated sample will the subsequently measured values of the fluorescence parameters qP, qN and NPQ be meaningful. All data recorded after an Fo, Fm determination are stored as one 'record' in the current buffer memory (see below) and can eventually be saved as a PAM image file (PIM). Please note that upon Fo, Fm determination, all previously stored data in the buffer memory will be erased. Therefore, the user is prompted: 'Save previous record?' If this question is answered with 'No', the previously recorded data will be irrevocably deleted. When a kinetics recording (see 10.2) or Light Curve recording (see 10.3) is started, the user is asked: 'Do you want to keep the previously recorded Fo, Fm?' No Fo, Fm determination is possible during a running record. The Fo and Fm images, which can be selected in the Image window, are a prerequisite for calculating the Fv/Fm images and the quenching coefficients qP, qN and NPQ. The Fv/Fm image defines not only the maximal PS II quantum yield, but also the sample limits. This definition is used to suppress noise outside the sample limits in Y(II) images.

9.1.2 New Record

When a new record is started, the previously recorded data stored in buffer memory is erased to make room for the new data. Therefore, the user is asked: 'Save previous record?' If this question is answered with 'No', the previously recorded data is irrevocably deleted. While a new record is normally started by an Fo, Fm determination, it is also possible to keep the previously determined Fo, Fm values (see above). It is also possible to carry out measurements using Saturation Pulses without a previous Fo, Fm determination. In this case, however, no quenching coefficients can be calculated, and the noise suppression based on the Fv/Fm image (see above) will not work either. It is not possible to carry out a later Fo, Fm determination within a running record. As soon as a new Fo, Fm determination is carried out, a new record is started. The start of an **Induction Curve** (under Kinetics, see 10.2) or a **Light Curve** (see 10.3) is equivalent to the start of a New Record.

Previously defined areas of interest (AOI, see 10.1.2.2) are not erased upon start of a New Record, such that several Records (e.g. Light Curves and Induction Curves) can be measured for the same AOIs. AOIs can be reset and newly defined at any time, in the Measure- as well as in the View-mode.

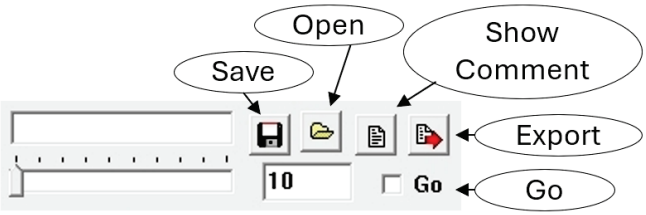
9.1.3 Measure

The Measure checkbox can be used to switch between Measure and View modes. In Measure mode, only the images recorded during the last measurement (last Saturation Pulse) are displayed. In View mode, all previously recorded data for a record can be viewed. The functions located in the box on the left apply in View mode. While actual measurements are possible in the Measure-mode only, the various types of

illumination are not affected by switching to the View-mode. In this way, it is possible to keep a sample in a defined light state, while viewing previously recorded data. If the user wants to stop illumination (e.g. in order to save battery power), the various types of illumination (ML, AL and Clock) have to be switched off manually. The **Save**-icon (see 9.2) is also accessible in the Measure-mode. When it is clicked, the Measure-mode is temporarily quit and the View-mode installed (see 9.2) for data storage. Once the data have been saved, Measure Mode is automatically reinstalled so that the running record can continue. Therefore, data can be saved successively during a recording.

9.2 Functions applying to the View-mode

The **View-mode** is automatically installed when the Measure-mode is quit (**Measure checkbox**). Data previously stored in the **Buffer-Memory** can be viewed on the Image-, Kinetics-, Light Curve- and Report-windows. For Kinetics and Light Curve at least one Area Of Interest (AOI) must be defined (see 10.1.2.2). Previously defined AOIs can be erased and new AOIs can be defined at any time. The data in the Buffer-Memory are numbered according to the time of measurement. A measurement is defined by application of a Saturation Pulse. In the upper **display line,** the number of measurements with date and time is shown. Using the **arrow bar** below, a particular measurement can be manually selected. To the right of the display line the current number of measurements is displayed in a separate box (also active in the Measuring-mode).



Save The data transiently stored in Buffer-Memory can be permanently saved on hard disk in form of a **PAM Image (PIM) file**. Data saving is also possible in the Measure-mode during the course of a Record (see 9). Data are saved in the Data-directory of the corresponding Imaging-PAM version. Additionally, to the Data file a commend (.txt) file for experimental descriptions can be edited and saved.

Open Data stored in form of a PAM Image (PIM) file on hard disk can be opened by loading into the Buffer-Memory. Then, if desired, also new AOIs may be defined.

Show comment Opens up the text file of the current PAM Image file.

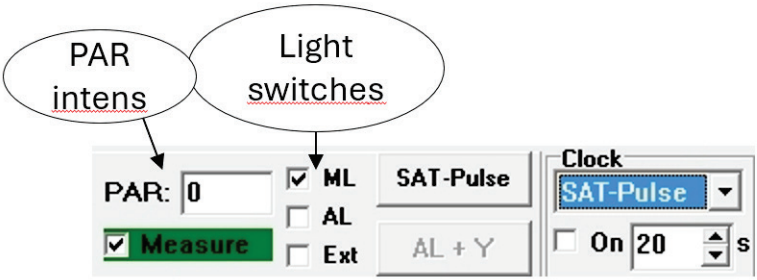
Export Data stored in the Buffer-Memory can be exported in the form of **JEPG-** or **TIFF-files**. A JEPG-file serves for exporting one particular image, which was selected for display on the Image-window and is relatively small (c. 100 KB). On the other hand, TIFF-files are rather large (c. 10 MB), as they essentially contain the same information as the original PIM-files. Each TIFF file consists of a series of images of the following parameters: Fo,

Fm, NIR, Red, F1, Fm'1, F2, Fm'2, F3, Fm'3 etc. In principle, on the basis of these images, images of all other fluorescence parameters as well as of PAR Absorptivity can be derived (for formulas see section 10.1.1). TIFF-images are monochrome without false color coding. They are suited for being used in conjunction with other image analysis programs, like “ImageJ”.

Go When Go is started, the images stored in Buffer-Memory are automatically displayed one after the other at a rate determined by the **Go Speed** (under Settings). After showing the last measurement, Go automatically starts again with the first measurement. Please note that the **Yield-filter** (see 10.5.12) slows down the image build-up of all calculated parameters and, therefore, should be switched off when high Go Speeds are chosen.

9.3 Light controls

The Imaging-PAM employs the same LEDs for pulse-modulated Measuring Light (ML), Actinic Light (AL), (Ext) and Saturation Pulses.



ML Checkbox for switching **Measuring Light on/off** at a pulse frequency defined under Settings.

AL The checkbox is used to switch the **Actinic Light on or off**, or to start a period of actinic illumination. The duration of this period is defined under Settings. When the Actinic Light is switched on, the ML frequency is automatically switched to the maximum setting (8).

Ext Checkbox for switching an **External Light Source on/off** intensity and width is defined under Settings.

PAR Display of light intensity (**Photosynthetically Active Radiation**) in µmol quanta m^{-2} s^{-1}, which is corresponds to the intensity of Actinic Light as well as (although to a much lesser extent) also by the intensity and frequency of the Measuring Light, as defined by the intrinsic **PAR-List** (under Options). In the case of MAXI- and MINI-versions the displayed values are calculated on the basis of PAR-values measured at the given fixed distance between LED-lamp and sample plane with the help of a

micro quantum sensor. With the MICROSCOPY-version it also depends on the choice of objective lens. With each measurement (defined by a Saturation Pulse) the momentary PAR-value is stored. It is also displayed in the View-mode. The PAR-values for the 20 AL-intensity settings, as well as for ML-frequency 8 (equivalent to AL0), are stored as default.par file in the Data folder of each ImagingPam-version. It can be viewed and/or modified under AL-List (LED currents/PAR values see chapter 11.3). A modification of the original values can e.g. become necessary, when the optional Filter Plate IMAG-MAX/F is used (see 3.2).

SAT-Pulse Key for starting a single **Saturation Pulse**, which defines a Measurement (i.e. determination of F and Fm' as well as on-line calculation of the derived fluorescence parameters), with the obtained data being stored in the Buffer-Memory.

AL + Y Key for starting a **period of actinic illumination**, the length of which is defined under Settings (Act. Light Width) and at the end of which a **Saturation Pulse** is applied. The AL + Y key is not active when Act. Light Width is set to zero (indefinite).

Clock When the Clock is switched on, the **selected Clock item** is repeated with the set interval until manually switched off again. The **Clock interval** can be set between 5 s and 3600 s (1 hour). There is the choice between four different **Clock items**: **SAT. Pulse, AL, AL + Y** and **Ft only.** While the "SAT. Pulse" and "AL + Y" Clocks involve the repetitive application of Saturation Pulses and, hence, correspond to the measurement of fluorescence parameters, this is not

the case for the "AL" Clock. A particular case is the "Ft only" Clock, which allows repetitive measurement of Ft without application of a Saturation Pulse. In the absence of actinic illumination, this allows to follow changes in Fo or Fo'-images. In the case of the "AL" and "AL + Y" Clocks, it should be made sure that the Clock interval is longer than the Act. Light Width.

Using the SAT-Pulse clock not only single Saturation Pulses but also sequences of defined numbers of Saturation Pulses can be applied (see 10.5.1).

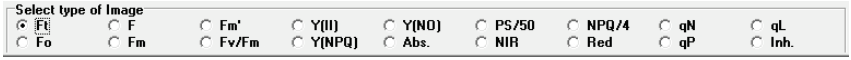
10 ImagingWin - Register Cards

10.1 Image-window

The major part of the Image-window is occupied by the actual Image, at the bottom of which the **false color code bar** is located. The standard false color code ranges from black via red, orange, yellow, green, blue and violet to purple. These colors code for **numbers between 0 and 1**. Hence, all measured or calculated parameters are normalized to values between 0 and 1. The correspondence between color and numerical value can be evaluated with the help of a **"Ruler"** which can be installed above the false color bar via **Options** in the menu**.** Instead of a false color bar also the corresponding black-and-white bar (**grey scale**) can be installed (via the B/W check box under Settings/Display). In the middle of the Image, by default an **area of interest (AOI**) is defined in form of a standard circle which is accompanied by a little red box displaying the averaged value of the selected fluorescence parameter within this AOI.

Below the Image-area the various parameters are listed, images of which can be selected by clicking the corresponding radio buttons **(Select type of Image)** (see 10.1.1). At the right hand side of the Image-area a number of functional elements are located which serve for **image capture and analysis** (see 10.1.2).

10.1.1 Different types of images



Under **Select type of Image** one out of 18 different parameters can be selected, the image of which is displayed on the Image-window. The meaning of the various parameters will be briefly described in the following subsections.

10.1.1.1 Current fluorescence yield, Ft

The current fluorescence yield, Ft, is continuously monitored in the Measure-mode (see 9), when the Measuring Light (ML) is switched on. While images of Ft are not continuously stored in the Buffer-Memory, at any time the current Ft image can be stored by applying a Saturation Pulse. Then the current Ft-image is stored in form of an F- or Fo-image. The latter applies, if the Saturation Pulse is given in conjunction with an Fo, Fm-determination (see 9). It is also possible to measure Ft-images without application of a Saturation Pulse with the help of the "Ft only Clock" (see 9.3).

Kinetic changes of Ft can be recorded on the Kinetics-window in conjunction with measurements of dark-light induction curves or light response curves for selected areas of interest (AOI). In this case, Ft-values are stored continuously, i.e. also between Saturation Pulses.

10.1.1.2 Dark fluorescence yield, Fo

The dark fluorescence yield, Fo, can be assessed after dark adaptation using the Fo, Fm-key. After dark adaptation normally all PS II reaction centers are open and maximal photochemical quenching is observed. This does not necessarily mean that Fo is the minimal fluorescence yield. Fluorescence yield can drop below the Fo-level by strong non-photochemical quenching induced during illumination. When an Fo measurement is triggered, the current Ft is averaged for 3 s and the averaged value is denoted Fo. In Microscopy-applications, when dealing with low signal levels, Fo-averaging can be applied, which allows assessment of Fo at substantially enhanced sensitivity (see 10.6.1). Fo determination is essential for correct calculation of the quenching coefficient qP (see 10.1.1.16).

10.1.1.3 Fluorescence yield, F

The fluorescence yield, F, is assessed like all fluorescence parameters (except for Ft) in conjunction with the application of a Saturation Pulse. When a Saturation Pulse is triggered, the current Ft is averaged for 3 s and the averaged value is denoted F. Like all fluorescence parameters measured in conjunction with a Saturation Pulse, F images are stored in the Buffer Memory.

10.1.1.4 Maximal fluorescence yield, Fm

Maximal fluorescence yield, Fm, can be assessed after dark adaptation using the Fo, Fm-key. The Fm-value is assessed at the plateau level reached during application of a Saturation Pulse. During the Saturation Pulse the Measuring Light frequency automatically is switched to the maximal setting. Assessment of Fm involves averaging of 3 image recordings. In special applications, when dealing with low signal levels (e.g. MAXI-version with algae suspensions in multiwell plates or MICROSCOPY-version), a "Special SP-Routine" can be applied, which allows assessment of Fm at substantially enhanced sensitivity (see 10.6.1).

After dark adaptation normally the extent of energy-dependent nonphotochemical quenching is minimal. Fo, Fm-determination at the start of a New Record (see 9) is essential for correct calculation of the quenching parameters qP, qN and NPQ. The Fm-image measured at the start of a Record remains unchanged until a New Record is started by a new Fo, Fm- determination. In this respect Fm differs from Fm', the images of which change with every Saturation Pulse (see below).

10.1.1.5 Maximum fluorescence yield, Fm'

In illuminated samples, the maximum fluorescence yield, Fm', is observed, which normally is lowered with respect to Fm by non-

photochemical quenching. Its value is assessed at the plateau level reached during application of a Saturation Pulse. During the Saturation Pulse the Measuring Light frequency automatically is switched to the maximal setting. Assessment of Fm' involves averaging of 3 image recordings. In special applications, when dealing with low signal levels (e.g. MAXI-version with algae suspensions in multiwell plates or MICROSCOPY-version), a "Special SP-Routine" can be applied, which allows assessment of Fm' at substantially enhanced sensitivity (see 10.6.1).

Depending on the state of illumination at the moment the Saturation Pulse is applied, a given sample can show an infinite number of different Fm'-images. Conversely, the same sample is characterized by unique Fo and Fm images, which are determined using a dark-adapted sample (see 10.1.1.4 and 10.1.1.2).

10.1.1.6 Maximal PS II quantum yield, Fv/Fm

Maximal PS II quantum yield, **Fv/Fm**, is determined after dark-adaptation. It is calculated according to the equation:

Fv/Fm = (Fm - Fo)/Fm

After dark adaptation normally all PS II reaction centers are open (F = Fo) and non-photochemical energy dissipation is minimal (qN = NPQ = 0) and maximal fluorescence yield, Fm, is reached during a Saturation Pulse. In this state the fluorescence increase induced by a Saturation Pulse (variable fluorescence, Fv) as well as the PS II quantum yield (ΔF/Fm = Fv/Fm) are maximal. The Fv/Fm image is measured in conjunction with an **Fo, Fm-determination**. It remains unchanged until the next Fo, Fm-determination. In this respect, the Fv/Fm image differs from the Y(II) image which changes with every Saturation Pulse (see below).

The contrast between the photosynthetically active object and the background matrix is enhanced, by the definition: Fv/Fm = 0 if Fm <0.048. All pixel for which this limit is not reached, are displayed in black. In this way, unavoidable noise associated with the Fm-determination can be suppressed. The resulting **"noise mask"** is saved for a given Record and also applied to Y(II), Y(NPQ) and Y(NO) images. Please note that this approach requires that the sample does not move during a given Record. If sample movement cannot be avoided, quenching analysis is not possible and measurements should be carried out without Fo, Fm-determination.

10.1.1.7 Effective PS II quantum yield, Y(II)

The effective PS II quantum yield is calculated according to Genty et al. (1989) by the formula:

Y(II) = (Fm'-F)/Fm'

As this fluorescence parameter is derived from a ratio of fluorescence intensities, any inhomogeneities of fluorescence excitation intensity or chlorophyll concentration will disappear and any remaining inhomogeneities can be interpreted in terms of differences in activity.

A given sample can show an infinity of different Y(II)-images, depending on the state of illumination at the very moment when the Saturation Pulse is applied. A unique state is given after dark adaptation when the effective PS II quantum yield is maximal (see 10.1.1.6).

Y(II) measurements normally are preceded by an Fo, Fm-measurement. In this case the contrast between the photosynthetically active object and the background matrix is enhanced, by the definition: Fv/Fm = 0 if Fm<0.048. All pixel for

which this limit is not reached, are displayed in black in Fv/Fm as well as in Y(II) images ("noise mask", see 10.1.1.6).

In principle, a quantum yield may vary between 0 and 1. If, for example Y(II) = 0.5, this means that one half of the absorbed quanta are converted into chemically fixed energy by the photochemical charge separation at PS II reaction centers. The other half of the quanta is dissipated into heat and fluorescence. The sum of all quantum yields always amounts to 1. Based on the work of Kramer et al. (2004) Photosynthesis Research 79: 209-218 two other types of quantum yield can be defined, **Y(NPQ)** and **Y(NO)**, which represent the nonregulated and regulated energy dissipation at PS II centers, respectively (see 10.1.1.8 and 10.1.1.9), adding up to unity with the photochemical quantum yield:

Y(II) + Y(NPQ) + Y(NO) = 1

10.1.1.8 Quantum yield of regulated energy dissipation, Y(NPQ)

The quantum yield of regulated energy dissipation in PS II, Y(NPQ) can be calculated according to Kramer et al. (2004) by the equation:

Y(NPQ) = 1 - Y(II) - 1/(NPQ+1+qL(Fm/Fo-1))

For the validity of this equation it is essential that the PS II pigments of the investigated sample are organized according to the "Lake model" (Stern-Volmer approach), which may be assumed for most higher plant leaves. The NPQ parameter is a measure of nonphotochemical fluorescence quenching (see 10.1.1.13), reflecting down-regulation of PS II as a protective mechanism against excess light intensity. The qL parameter is a measure of the fraction of open PSII centers in the "Lake model" (see 10.1.1.17).

A high Y(NPQ) value on one hand indicates that the photon flux density is excessive and on the other hand shows that the sample has

retained the physiological means to protect itself by regulation, i.e. the dissipation of excessive excitation energy into harmless heat. Without such dissipation there would be formation of singlet oxygen and reactive radicals, which cause irreversible damage.

In Y(NPQ) images all pixel are set to 0 (black) for which Fv/Fm = 0 ("noise mask", see 10.1.1.6).

10.1.1.9 Quantum yield of nonregulated energy dissipation, Y(NO)

The quantum yield of nonregulated energy dissipation in PS II, Y(NO) can be calculated according to Kramer et al. (2004) by the equation:

Y(NO) = 1/(NPQ+1+qL(Fm/Fo-1))

For the validity of this equation it is essential that the PS II pigments of the investigated sample are organized according to the "lake model" (Stern-Volmer approach), which may be assumed for most higher plant leaves. The NPQ parameter is a measure of nonphotochemical fluorescence quenching (see 10.1.1.13), reflecting down-regulation of PS II as a protective mechanism against excess light intensity. The qL parameter is a measure of the fraction of open PSII centers in the "lake model" (see 10.1.1.17).

A high Y(NO) value indicates that both photochemical energy conversion and protective regulatory mechanisms are inefficient. Therefore it is indicative of the plant having serious problems to cope with the incident radiation. Either it is already damaged or it will be photodamaged upon further irradiation. Extremely high values of Y(NO) e.g. can be induced by PS II herbicides, which not only block PS II reaction centers, but also prevent the build up of a transthylakoidal proton gradient. The latter is an important prerequisite for energy-dependent non-photochemical quenching.

In Y(NO) images all pixel are set to 1 (purple) for which Fv/Fm=0 ("noise mask", see 5.4.1.6).

10.1.1.10 Absorptivity, Abs.

Measurement of the absorptivity parameter requires a special set of red and near-infrared (NIR) LEDs, which are integrated into the LED arrays of the **blue measuring light version** of the MAXI and MINI heads. The red measuring light versions, as well as the microscopy version of the Imaging PAM M series, do not support this function.

The absorptivity (Abs.) image is a measure of the fraction of incident red light absorbed by the leaf sample. This is recorded using the **'Measure Abs.'** function (see10.1.2.1). When this key is pressed, the sample is first illuminated with red light, followed by NIR light, and the **red** and **NIR images** are recorded. Apparent absorptivity is then calculated pixel by pixel from the R and NIR images using the formula:

Abs. = 1 - R/NIR.

This measurement is based on previous calibration of the instrument, which involves appropriate adjustment of the R-intensity with respect to the NIR-intensity. The instrument is properly calibrated, when the R- and NIR-images of a white piece of paper (or white flower petal) show similar brightness values. Then R/NIR is close to unity and Abs. = 1 - R/NIR is close to zero. Any pigment that absorbs Red more than NIR light, i.e. generally all photosynthetically active pigments, will decrease R with respect to NIR, thus decreasing R/NIR and increasing the derived Abs.-value. On the other hand, pigments that absorb Red and NIR light similarly, as e.g. **necrotic spots**, will not cause R/NIR to deviate substantially from unity and thus Abs. will remain close to zero. Notably, a white and a black piece of paper give similar Abs.-images with pixel-

values close to zero. This illustrates that the Abs. parameter does not assess general Absorptivity of a sample for visible light, as sensed by the human eye, but rather specifically the Absorptivity of photosynthetically active light. Therefore, the Abs. parameter as determined by the IMAGING PAM may be considered a close estimate of **PAR-Absorptivity**. This approach is based on the empirical fact that pigments that contribute to the absorption of PAR do not show significant absorption bands in the near-infrared (NIR) spectral region. On the other hands, pigments that absorb NIR are likely to also absorb Red light.

For technical reasons, the measurement of PAR-Absorptivity is carried out using red light (660 nm), whereas blue light is employed for actinic illumination (450 nm). This approach may be justified by the fact that the same pigments (mainly chlorophyll a and b) are responsible for absorption at 660 as well as at 450 nm. PAR-Absorptivity complements the information provided by the Yield-image on the lateral distribution of effective PS II quantum yield. The incident PAR is known as it is defined by the MAXI IMAGING PAM, provided that illumination by ambient light can be neglected. With knowledge of Yield, PAR and Abs. the apparent rate of photosynthetic electron transport rate (PS-parameter) can be estimated (see 10.1.1.11).

The absorbed PAR may be overestimated when leaves contain PAR-absorbing pigments which do not transfer the absorbed energy to the photosynthetic reaction centers. A special case is given when leaves are red colored, e.g. due to anthocyanin accumulation in the vacuoles of the epidermis. On one hand, this will cause a low Abs.-value, as a large fraction of the Red will be remitted. On the other hand, also the amount of blue light penetrating to the mesophyll will be lowered, leading to correspondingly low values of absorbed PAR and fluorescence yield.

It should be noted that the absorbance value does not necessarily correlate with chlorophyll content per leaf area, as commonly determined by extraction. For example, if the leaf surface is covered in reflective hairs (pubescence), this will strongly affect photosynthetically active radiation (PAR) absorptivity, thus lowering the measured absolute values without affecting the leaf's chlorophyll content. The same leaf will normally show considerably lower absorption values on the underside than on the upper side.

10.1.1.11 Apparent rate of photosynthesis, PS/50

An apparent rate of photosynthesis (PS) can be calculated on the basis of the measured effective PS II quantum yield, the incident photon flux density (PAR) and the PAR-Absorptivity (Abs.):

PS = 0.5 x Y(II) x PAR x Abs. µequivalents m^{-2} s^{-1}

The incident PAR is known for the defined distance between LED-Ring-Array and sample plane. The PAR-Absorptivity previously has to be measured using the **Measure Abs.** routine (see 10.1.1.10). It is assumed that 50 % of the absorbed PAR is distributed to PS II. As the PS calculated in this way can reach values around 50, the parameter PS/50 is displayed in order to depict the apparent rate of photosynthesis with the help of the false color code for values ranging between 0 and 1.

The PS-parameter measured with the IMAGING-PAM in most respects closely corresponds to the ETR-parameter previously defined for measurements of the apparent rate of photosynthetic electron transport:

ETR = 0.5 x Yield x PAR x 0.84 µequivalents m^{-2} s^{-1}

This commonly used definition assumes a PAR-Absorptivity of 0.84, i.e. that 84 % of the incident photons of photosynthetically active radiation is absorbed by the leaf. This value may be

considered typical for a "standard green leaf". In reality, however, PAR-Absorptivity may vary considerably, e.g. between upper and lower leaf side, as well as between different species and leaves at different developmental stages. The latter is particularly evident during senescence. Even the same leaf may show considerable lateral heterogeneity of PAR-Absorptivity, in particular caused by virus infection and other plant diseases that disturb pigment synthesis.

10.1.1.12 NIR light remission, NIR

NIR light remission (NIR) is measured in conjunction with the "Measure Abs." function (see 10.1.2.1). This provides an indication of the amount of light that is not absorbed by photosynthetically active pigments. For this purpose, 780 nm light is used. Remission includes the reflection and backscattering of the 780 nm light received by the CCD detector. Reflection mainly occurs at the leaf surface, i.e. before the incident light has reached the cell layers containing photosynthetic pigments. Therefore, it should be similar for 660 nm and 780 nm light. Conversely, backscattering differs greatly between 660 nm and 780 nm light because most of the 660 nm light, unlike the 780 nm light, is absorbed by photosynthetic pigments. In this respect, backscattering is similar to transmission, which, in a leaf, is strongly influenced by light scattering, causing a considerable increase in the path length of the measuring light within the leaf.

The intensity of the NIR-measuring light can be adjusted under **Live Video** (see 10.1.2.3). It should be made sure that there is no overload, particularly under conditions when the non-modulated background signal is relatively large (due to ambient light). While the non-modulated background signal does not contribute to the intensity values of the NIR-image, it does add to an overload of the overall signal. Once the instrument is calibrated for measurements of **PAR-Absorptivity** (see 10.1.1.10), changes in NIR-measuring light

intensity should be avoided, as otherwise the Red-measuring light intensity would have to be readjusted.

Please note that the NIR-image is not corrected for heterogeneity of intensity distribution, which in this case mostly consists in a decrease of brightness on the fringes of the image caused by the vignetting effect of the objective lens. As essentially the same effect is observed in the R-image, it does not affect the ratio R/NIR and the calculated Absorptivity parameter Abs. = 1 - R/NIR.

NIR-images cannot be measured with the MICROSCOPY-versions of the Imaging-PAM.

10.1.1.13 Nonphotochemical quenching, NPQ/4

The NPQ parameter provides a measure of nonphotochemical quenching which in contrast to qN does not require knowledge of Fo' (see 10.1.1.2). It is defined according to the equation:

NPQ = (Fm-Fm')/Fm'

In contrast to the quenching coefficient qN, the parameter NPQ can reach values higher than 1. In practice, NPQ rarely exceeds 4. Hence, images of **NPQ/4** are displayed, with values ranging between 0 and 1, that can be displayed using the standard false color code.

The definition of NPQ implies a matrix model of the antenna pigments (Stern-Volmer quenching). With NPQ that part of nonphotochemical quenching is emphasized which reflects heat-dissipation of excitation energy in the antenna system. NPQ has been shown to be a good indicator for "excess light energy" which in leaves is primarily dissipated via zeaxanthin (xanthophyll cycle) in the presence of a transthylakoidal ΔpH. On the other hand, NPQ is relatively insensitive to that part of nonphotochemical quenching which is associated with qN-values up to 0.4, reflecting thylakoid membrane energization.

Assessment of NPQ requires previous determination of Fm with the same sample after dark adaptation, i.e. under conditions when per definition NPQ = 0.

10.1.1.14 Red light remission, R

Red light remission (R) is measured alongside the "Measure Abs." function. (see 10.1.2.1). It is an inverse measure of the absorption of photosynthetically active radiation (PAR) by the sample under investigation. The 660 nm light emitted by the red LEDs in the LED panel is strongly absorbed by chlorophyll. Consequently, only a small proportion of the incident red light is reflected and backscattered from the sample. The same is true of the 450 nm light used for fluorescence excitation and actinic illumination. However, unlike 660 nm light, 450 nm light cannot penetrate the red filter protecting the CCD or CMOS detector. The intensity of the red light is adjusted so that a highly scattering white sample (such as a white piece of paper or a white flower petal) produces the same signal as the near-infrared (780 nm) reference light. The 780 nm light is emitted by LEDs adjacent to the 660 nm LEDs in the LED array (seqtion 3.2). Both types of LED emit rather diffuse light, thus providing homogeneous illumination of the sample. Unlike the 660 nm light, the 780 nm light is not absorbed by leaves as chlorophyll absorption does not extend to this wavelength range. Please note that the R image is not corrected for heterogeneous intensity distribution, which in this case mostly consists of a brightness decrease at the image's edges caused by the objective lens's vignetting effect. As the same effect is essentially observed in the NIR image, it does not affect the R/NIR ratio or the derived absorptivity parameter, Abs = R/NIR.

Red-images cannot be measured with the MICROSCOPY-versions of the Imaging-PAM.

10.1.1.15 Coefficient of nonphotochemical quenching, qN

The coefficient of non-photochemical quenching, qN, is defined by the equation:

qN = (Fm-Fm')/(Fm-Fo')

qN can vary between 0 (defined for dark adapted state) to 1 (all variable fluorescence quenched). The above definition takes into account that not only variable fluorescence (induced upon reaction center closure), but also the dark-level fluorescence (all centers open) can be quenched non-photochemically, primarily by increased heat dissipation induced during illumination. For correct determination of Fo', it would be necessary to switch off the actinic light and to quickly reoxidize the PS II acceptor side with the help of far-red light, before non-photochemical quenching can relax. This approach, however, is not feasible with the IMAGING-PAM, as the far-red light would penetrate to the CCD-detector and cause serious disturbance of fluorescence imaging. Therefore, instead of measuring Fo', this parameter is estimated using the approximation of Oxborough and Baker (1997):

Fo' = Fo/ (Fv/Fm + Fo/Fm')

This approximation relies on the assumption that the same mechanism that causes quenching of Fm' with respect to Fm is also responsible for Fo-quenching. The quenching coefficient qN is quite sensitive to changes in the energy status of the chloroplasts (energy-dependent quenching). Such changes are readily induced by various environmental stress factors causing stomatal closure, switching from CO_2-dependent to O_2-dependent electron flow and down-regulation of the rate of energy conversion in PS II. Hence, qN is an indicator of stress induced limitations and, actually, has proven to be the most sensitive parameter for early detection of such limitations by fluorescence imaging.

Assessment of qN requires previous Fo, Fm-determination with the same sample after dark adaptation, i.e. when qN = 0 per definition.

10.1.1.16 Coefficient of photochemical quenching, qP

The coefficient of photochemical quenching, qP, is a measure of the overall "openness". Red-images cannot be measured with the MICROSCOPY-versions of the Imaging-PAM. They vary between 0 and 1. Calculation of qP requires knowledge of the fluorescence parameter Fo' (minimal fluorescence yield of illuminated sample, which is lowered with respect to Fo by non-photochemical quenching):

qP = (Fm'-F)/(Fm'-Fo')

Correct Fo'-determination requires application of far-red light, which would disturb the fluorescence imaging. However, as the same mechanism causing Fo-quenching is also responsible for quenching of Fm' with respect to Fm, it is possible to estimate Fo' from Fm' measurements (Oxborough and Baker 1997):

Fo' = Fo/ (Fv/Fm + Fo/Fm')

Assessment of qP requires previous Fo-Fm determination with the same sample after dark adaptation, i.e. when qP = 1 per definition.

While the definition of qP is based on the "puddle model" of PS II, the antenna pigment organization in leaves is more realistically described by the "lake model". This means that the antenna of individual PS II reaction centers are connected, so that the excitation energy can be transferred with high probability from closed reaction centers to neighboring open centers. Therefore, the fraction of open PS II centers is overestimated by qP. The fraction of open PS II

centers estimated on the basis of the "lake model" is described by the quenching coefficient qL (see below).

10.1.1.17 Coefficient of photochemical quenching, qL

The coefficient of photochemical quenching, qL, is a measure of the fraction of open PS II reaction centers, which can vary between 0 and 1. Its definition is based on the "lake model" of PS II antenna pigment organization. Calculation of qL requires previous determination of the fluorescence parameter Fo' (minimal fluorescence yield of illuminated sample, which is lowered with respect to Fo by nonphotochemical quenching):

qL = (Fm'-F)/(Fm'-Fo') x Fo'/F = qP x Fo'/F

Correct Fo'-determination requires application of far-red light, which would disturb the fluorescence imaging. However, as the same mechanism causing Fo-quenching is also responsible for quenching of Fm' with respect to Fm, it is possible to estimate Fo' from Fm' measurements (Oxborough and Baker 1997):

Fo' = Fo/ (Fv/Fm + Fo/Fm')

Assessment of qL requires previous Fo-Fm determination with the same sample after dark adaptation, i.e. when qL = 1 per definition.

When during illumination nonphotochemical quenching is generated, generally Fo'<F and, therefore, also qL<qP. The difference between these two coefficients of photochemical quenching increases with the connectivity between PS II reaction centers.

10.1.1.18 Inhibition, Inh.

The Inhibition (Inh.) parameter describes the **inhibition of PS II quantum yield, Fv/Fm or Y(II)**, relative to a control reference AOI, the number of which can be selected under Settings / Inh. Ref. AOI

(see 10.5.11). After start of the program AOI #1 is the control reference for calculation of the Inh. image according to the equation:

$$\mathbf{Inh.} = (Y_{control} - Y_{sample}) / Y_{control}$$

This parameter is particularly important for assessment of phytotoxicity with **multiwell plates** using the MAXI-version. In this application wells are filled with algae suspensions and the inhibitory effect of phytotoxicant addition relative to a control sample is assessed. In this case AOI #1 is defined for the well of the control sample, which always displays the highest Y(II) value.

Images of Inh. can also be informative in applications with other objects, like leaves, using all versions of the Imaging-PAM. In phytopathological studies, for example, the inhibition of an infected area relative to a control area (defined as AOI #1) can be depicted.

Like all other imaged parameters, the Inh. ranges from 0 (black) to 1 (purple). In order to assure a good contrast between sample and background, in the case of Inh. images the latter is white instead of the usual black.

10.1.2 Image capture and analysis

The functional elements for image capture and analysis are located at the right hand side of the Image-window. The standard Image-window is shown when **Capture** is selected. The measured image of a particular parameter can be modified for emphasizing certain features when **Analysis** is selected. The various functional elements are described in the following sub-sections.

10.1.2.1 Measure Abs.

This function requires a special set of Red and NIR LEDs that are integrated in the LED arrays of the MAXI- and MINI-Heads in

the **blue measuring** light version. The MICROSCOPY-versions of the Imaging-PAM M-series does not support this function.

Using the **Measure Abs. key** an automatic routine for the measurement of a **PAR-Absorptivity (Abs.) image** can be started (see also 10.1.1.10). This measurement involves consequent illumination of the sample with Red and NIR light, resulting in the capture of **Red** and **NIR remission images** (see 10.1.1.14 and 10.1.1.12), from which the **Abs.-image** is calculated pixel by pixel using the formula:

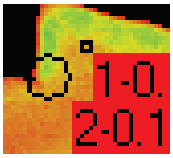
Abs. = 1 - R/NIR.

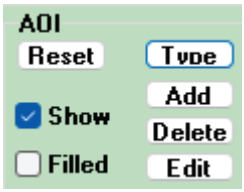
As the Abs., Red and NIR images are erased upon start of a New Record, it is recommended to carry out an Abs. measurement routinely after start of a New Record (see 9).

The Measure Abs. routine can give meaningful results only, if the relative intensities of the NIR and Red measuring light are properly adjusted (see also 10.1.1.10). Appropriate settings of NIR and Red intensity, as well as of Red Gain, were determined at the factory for each individual IMAGING-PAM and are documented on a sticker fixed to the corresponding LED-Array. These settings are preinstalled on the Notebook PC IMAG-PC, if this is purchased together with the instrument. They are listed in the **INI-file** within the ImagingPAM directory. Correct **calibration** can be ascertained by the user by measuring Red and NIR images of a white piece of paper. While it appears unlikely that the settings of NIR and Red intensities change with time, small corrections of **Red Gain** may be required after some time, due to ageing of the LEDs. For this purpose, the Red Gain is accessible under Settings/Absorptivity (see also 10.5.3). No corrections may be necessary, if the Red and NIR values do not differ by more than 5 %. If necessary, also the values of Red and NIR intensities can be corrected. These parameters are accessible under Settings/Absorptivity.

10.1.2.2 Area of Interest, AOI

The displayed image is composed of 640 x 480 (i.e. 307200) pixels. Each pixel captures specific fluorescence information, such that in principle e.g. 307200 Light Response Curves could be recorded. In practice, however, it is necessary to reduce this vast amount of information. For this purpose, special **Areas of Interest, AOI**, can be defined. All pixel values contained in an AOI are averaged and the averaged value is shown in a little box adjacent to a particular AOI. In this way, the pixel noise is considerably reduced.

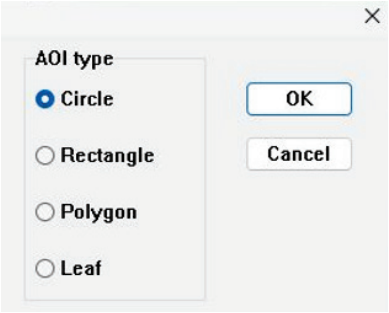




The definition of at least one AOI is required for recordings of Induction Curves (see 10.2) and Light Curves (see 10.3). After start of the program a **standard AOI circle** is installed by default in the center of the image area. This AOI can be removed with the help of the **Reset**-button. New AOIs can be installed with the help of the **Add**-button. After clicking "Add", a standard circle can be moved with the help of the mouse cursor to the desired position. With the help of the **+ key** the circle diameter can be increased. With the **- key** it can be decreased. The AOI position and size are confirmed by mouse click (left or right). The AOI-size will remain unchanged when further AOIs are added, unless it is modified using +/-. The last added AOI can be removed via **Edit/Undo** (Menu). All AOIs can be removed by **Reset**. Any particular single AOI can be deleted via **Delete**. After clicking the Delete button a **Delete-hand** appears that can be moved to a particular AOI using the mouse. Please note that the pointing finger has to cross the border of the AOI. When the **Show**-checkbox is deactivated, the AOIs disappear, but can be recalled at any time by checking the box. The status of the Filled checkbox determines whether or not the area is filled with the color corresponding to the

averaged parameter value. When "Filled" is activated, any heterogeneity or structure within the AOI disappears. Only when "Filled" is inactivated it is possible to see a small AOI covered by a larger AOI.

With the help of the Type-button different AOI types can be selected. Besides a Circle also a Rectangle or Polygon can be chosen. The minimal AOI size is one pixel with a rectangular and a diameter of 10 pixels with a circular AOI.



The **AOI Type** has to be selected before clicking "Add"; after clicking "Add" the Type-selection is not accessible.

For definition of the size and position of a **Rectangle AOI**, after clicking "Add" the mouse cursor is moved to one of the envisaged corners, which is fixed by a mouse click (left or right). Then the desired shape and size of the Rectangle can be arranged by mouse movement. The final state is fixed by another mouse click.

For definition of the size and position of a **Polygon AOI**, after clicking "Add" the mouse is moved to one of the envisaged corners, which is fixed by a mouse click (left or right). Then the mouse cursor is moved to the next corner, which is fixed by mouse click and so on. The last corner is marked by a **double-click**.

Up to 100 AOI can be defined. A special routine is provided for definition of an **AOI-panel**, as e.g. required for assessment of samples in multiwell plates. This routine is accessible under Options/Define AOI array geometry/Create AOI array (see chapter 11.2).

A rather new feature is the "**Leaf**" type. In this AOI type the software uses the polygon functionality to automatically draw a polygon around connected fluorescing pixels in the image

10.1.2.3 Select: Fluorescence or Live Video

After the programm has started, the measuring system is in **Fluorescence Mode**. This means that the fluorescence yield is assessed using pulse-modulated **blue measuring light**. This enables the current Ft image to be captured continuously. A low pulse frequency is used to avoid the measuring light having an actinic effect. Consequently, changes to the fluorescence image are significantly dampened, making it difficult to track the image while moving the sample or attempting to focus.

When **Live Video Mode** is selected, the blue fluorescence measuring light is switched off and the **NIR light** is switched on instead. This light is neither visible to the human eye nor sensed by the plant. However, the NIR is detected by the camera, enabling **imaging of the leaf (in monochrome)** at a relatively high measuring pulse frequency without preilluminating the photosynthetic apparatus. Therefore, Live Video Mode is useful for positioning the leaf sample in the field of view and **focusing the image**.

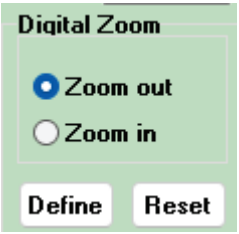
When Live Video is activated, the Live Video window appears, in which the **NIR intensity** can be adjusted.

Activating the **'Overload' box** on the Live Video window indicates that the amount of light detected by the CCD camera is too high, which will likely disturb the measured NIR image. The overall signal consists of ambient background light (e.g. daylight) and remitted NIR light. Therefore, in the event of an overload, either the ambient light should be reduced (recommended) or the NIR intensity should be decreased.

Live video images can also be obtained using continuous light from any external light source if this light can pass through the filters in front of the CCD camera. This feature is particularly important for **Microscopy** applications using the standard through-light **condenser illuminator** of the microscope. This allows samples to be easily centered and focused using the PC monitor screen.

To exit Live Video Mode and return to Fluorescence Mode, click the **Close** button on the right or the **Exit** button in the top-right corner of the **NIR Intensity** Adjustment window.

10.1.2.4 Zoom



The standard display setting for images (zoom out) uses all 640 x 480 pixels — 307,200 pixels in total.

While individual pixels cannot be seen without zooming in, they become more visible when the zoom factor is increased. Clicking 'Zoom in' applies the standard zoom factor of 2. This displays a quarter of the image in the center. At this magnification, the individual pixels are just visible since the image resolution itself does not change. Users can define the zoom factor by clicking the 'Define Zoom' button, which is only possible in the 'Zoom Out' position. After clicking 'Define Zoom', move the mouse cursor to one of the corners of the intended zoom image. Clicking the left or right mouse button

fixes this corner. You can then adjust the size of the zoom image by moving the diagonal arrow to the diagonally opposite corner of the image rectangle. The final state is fixed by clicking the mouse again. To change the zoom factor, first select 'Zoom Out' and then click 'Define Zoom' again. The 'Reset' button restores the standard zoom with a zoom factor of 2 (displaying the central quarter). Software version 2.57q43 introduced an additional zoom function for cameras such as the IMAG-K9. This camera usually operates in **2x2** pixel binning mode. For zoom purposes, however, this can be switched off to achieve a 2x zoom level with the same resolution. You can find more details in the next section (10.1.2.5)

10.1.2.5 Zoom 2x2

Unlike the “Zoom” from the previous chapter, the “Zoom 2x2” does not result in a loss of resolution and can be used for measurement but not for recorded data in the view mode.

When the “Zoom 2x2” function is activated, the camera switches the internal 2x2 pixel binning to an unbinned mode. With the same resolution of the displayed image, only a quarter of the previous image section is then captured, resulting in a magnification factor of 4.

Definition of binning: 2x2 binning in cameras like the IMAG-K9 refers to a technique in which four adjacent pixels (two in width and two in height) are combined into a single, combined pixel. This reduces the resolution of the image, but at the same time increases light sensitivity and improves noise performance. The resulting image quality has less detail, but better signal quality in low light conditions.

Binning is what happens inside the camera during exposure. For this reason, it must be activated before a measurement. The internal signal amplification is automatically adjustet so that the set FT values remain the same.

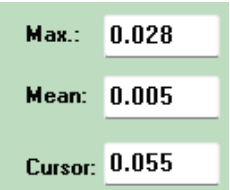
After clicking the Zoom 2x2 button a frame indicating the zoomed image section appears in the center of the image window, which can be freely* positioned within the inner image area after it has been positions with the cursor by just left-clicking. If it shall just be placed reproducibly in the center, press the Zoom 2x2 button and type a “c” to directly drop it in the middle of the image.

* *in the HEXAGON-IMAGING-PAM No significant vignetting of the image can be detected in the ... , so the selection frame for 2x2 binning can be placed anywhere in the entire image field. With devices such as the Mini or MAXI imaging head, lens-related vignetting occurs, so the 2x2 zoom selection field can only be placed in the central image section where no visible vignetting is noticeable.*

Once the new image section is defined the image switches to the new section (AOIs that have been placed before are kept in size and position relative to the software window, they are not zoomed.

To leave the Zoom 2x2 mode again, just click “Zoom All” (same button as before).

10.1.2.6 Cursor



The **Cursor box** shows the numerical value of the selected parameter at the cursor position which can be changed by mouse movement. When the cursor enters an AOI the cursor box will show the same averaged value as shown in the box close to the AOI, provided "Filled" is active (see 10.1.2.2).

The boxes above the Cursor box show the parameter values applying to the whole image:

Max. : the maximal value of all pixels

Mean : the mean value of all pixels

Cursor : value of the current cursor position

10.1.2.7 Analysis

The **Analysis function** normally is used in conjunction with **Expanded Color** display (see 10.5.8). When **Analysis** instead of **Capture** is selected, the color scale of a displayed image can be modified. Using the **Low** and **High** scroll boxes, the **Low-High limits** of the color scale can be defined. The numbers correspond to the scale of pixel values ranging from 0.000 to 1.000. In the image displayed under Analysis all pixels with values within the Low-High limits are displayed in red color, while the rest of the pixels is displayed in black-and-white (standard grey scale). The Low-High limits defined under Analysis are effective for the Expanded Color display under Capture. The closer the low and high limits are with respect to each other, the more expanded is the color display. In this way, small differences in pixel values can be emphasized by enhanced color differences, thus increasing the contrast.

Using the false color scale maximal contrast is obtained in the range of very low values (from yellow to red and black). Therefore, in

order to obtain maximal contrast in the display of a selective lowering of a fluorescence parameter in a particular region of a leaf, the "normal" range of pixel values should be shifted to yellow while the lowered range of pixel values should be shifted to red-black. This can be achieved by suitable shifting of the Low and High limits under Analysis. Under Settings the **Expanded Color** display has to be selected and the image viewed under **Capture**. An example is given in Fig. 79 shows a Y(II)-image in normal Color display of a leaf in which the veins display lowered Yield-values. In Fig. 80 the Analysis-image and the settings of the Low-High limits are displayed. Fig. 81 shows the same image with Expanded Color display under Capture.

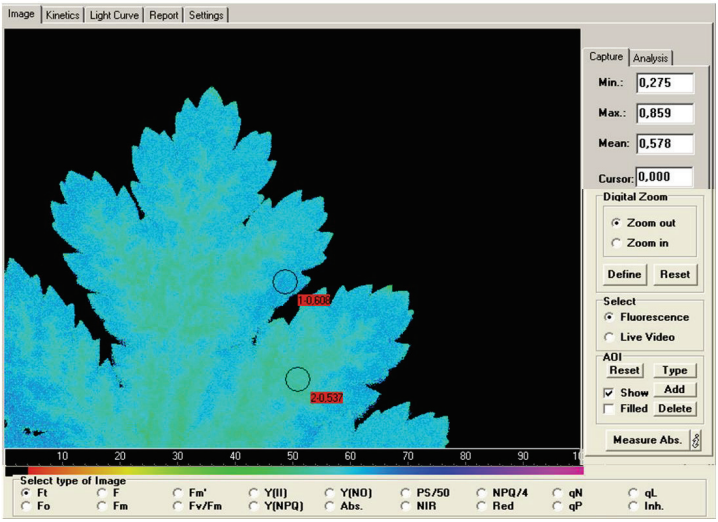


Fig. 79: Normal color display (under capture)

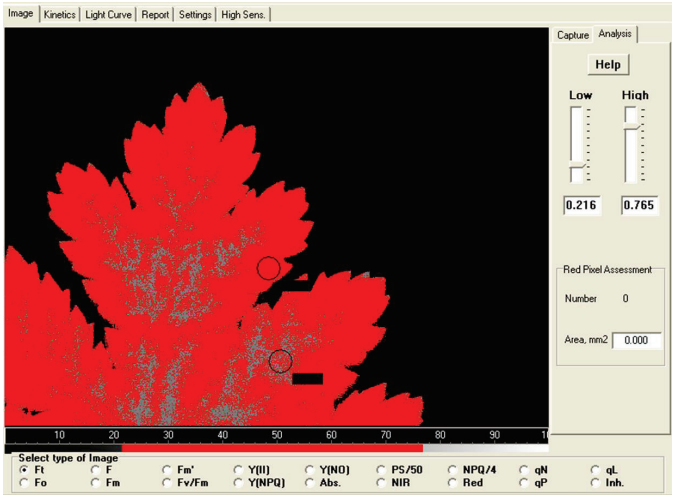


Fig. 80: Display under Analysis with particular settings of Low-High limits

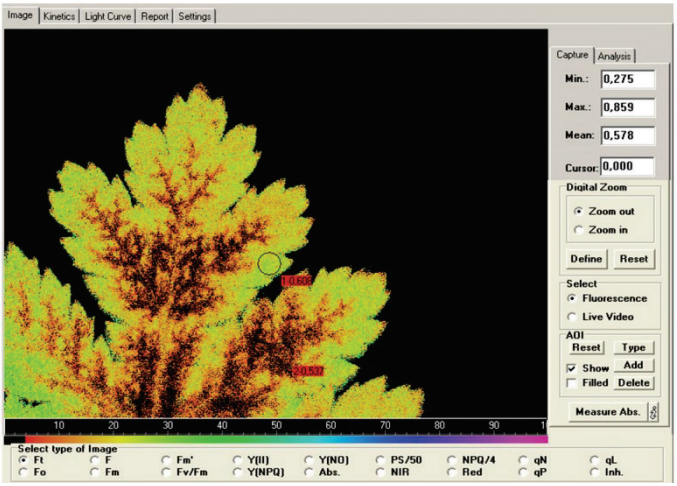


Fig. 81: Expanded Color display (under Capture)

10.2 Kinetics window

On the **Kinetics window** the changes of fluorescence parameters are plotted versus time. Following every Fo, Fm-determination, i.e. after start of a new Record, all measurements are saved in the **Buffer Memory**, as protocolled in the lower left corner of the ImagingWin user surface (see 9.2) and in more detail in the **Report file** (see 10.4). The same information can be displayed in form of kinetic curves on the **Kinetics window**. Registration of the kinetic data also occurs in the background, i.e. when the Kinetics window is not active. Display of data in the Kinetics window requires that **at least one AOI** is selected. If this is not the case, there is a corresponding warning.

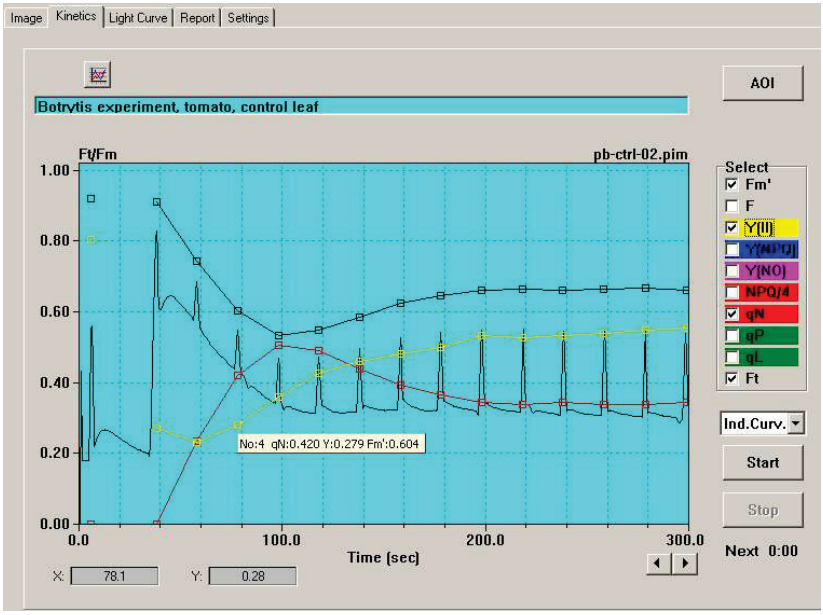


Fig. 82: Kinetics window with display of standard Induction Curve

A typical Record of a standard dark-light Induction Curve is displayed in Fig. 82. For the sake of clarity only part of the available fluorescence parameters is displayed (Fm', Y(II), qN and Ft) and

only one AOI is selected. The **Ft-parameter** (momentary fluorescence yield at any time, t), differs from all other parameters in that it is measured continuously, i.e. not only in conjunction with Saturation Pulses. However, it should be noted that Ft is recorded exclusively for the **selected AOIs**. Hence, in contrast to all other parameters, it is not possible to display Ft images for AOIs defined after the recording. Also in contrast to all other parameters Ft is not recorded in the background after Start of a New Record (Fo, Fm-determination).

For the sake of a uniform ordinate scale reaching from 0 to 1, Ft is referenced to the Fm-value, i.e. the ratio Ft/Fm is plotted versus time. Hence, before a Kinetics curve can be recorded, Fm must be determined, which is done automatically upon start of a recording via an Fo, Fm determination, unless the user wants to keep previously determined Fo, Fm values. With the definition of Fo, Fm a new Record is started and the buffer memory with the previously recorded data is erased. Therefore, the user is reminded to save the data before the Fo, Fm-measurement is initiated or the current Fo, Fm can be confirmed.

For example, it can be advantageous to retain the previously recorded Fo, Fm when an induction curve is measured following a defined pre-illumination. In this case, Fo and Fm can be measured before the preillumination, meaning that the Fo and Fm determination preceding the induction curve recording can be omitted. Then Ft-normalization as well as calculation of quenching parameters and of Fv/Fm will be based on the previously determined Fo and Fm values. If the FoFm image is to be used further in the following measurement, it is essential to ensure that the sample has not moved in the meantime.

When the AOI button is pressed, a new window opens showing the user the defined AOIs and offering further options (such as deactivation or grouping).

In the upper part of the AOI window the data point symbols of the selected AOI number are shown.

Clicking onto AOI numbers will activate or deactivate them - at the same time, the corresponding flags of the AOIs change color in the image window (flags of active AOIs are displayed in red, flags of inactive AOIs in black).

When the **AOI button** is pressed, the AOI window is opened, which shows in its lower part a list of all AOIs previously defined on the Image-window. By clicking a particular number, the corresponding AOI can be selected for data display in the Kinetics window. The data of several or all AOIs may be superimposed. In the upper part of the AOI window the data point **symbols of the selected AOI number** are shown. Examples are given for one out of 4 selected AOIs and 8 out of 8 selected AOIs being active for display, respectively. Please find a more details on the recent window version in section 11.2.6

The time-dependent changes of **eleven different fluorescence parameters** may be displayed in the Kinetics window. For display the corresponding check box has to be marked. As pointed out above, the Ft parameter can be displayed for AOIs only which were defined before start of the Record.

One out of **three different types of Kinetic recordings** can be selected (Induction Curve, Manual recording and Induction + Recovery). After Start of a recording, it can be terminated via the **Stop**-button. **Next** refers to the remaining time until the next measurement takes place (i.e. application of next Saturation Pulse).

Ind. Curve: An Induction Curve is a preprogrammed dark-light induction curve (Kautsky effect), the parameters of which can be defined by the user (under **Settings/Act. Light/Slow Induction**). After Start of an Induction Curve, normally first an **Fo, Fm determination** is carried out (unless the user prefers to keep the previously determined Fo,Fm values, see above). Actinic illumination is started after the **Delay**-time at an intensity defined by **Act. Light Int**. Saturation Pulses for quenching analysis are given repetitively at defined **Clock**-intervals. The length of the recording is defined by the **Duration**-parameter. An Induction Curve is terminated automatically at the end of the preprogrammed Duration-period. It can be terminated earlier with the help of the **Stop**-button, however not before the Delay-time is passed.

Manual: The Manual recording corresponds to a chart recording. When the Ft checkbox is activated, for all AOIs the time courses of the averaged Ft pixel values are displayed. After Start of a Manual registration, normally first an Fo, Fm determination is carried out. The user may also decide to keep the previously determined Fo, Fm values. It is up to the user when to start actinic illumination, to apply a single Saturation Pulse or repetitive Saturation Pulses using the Clock (at lower right corner of the screen). A Manual registration is terminated by the **Stop**-button.

Ind.+Rec.: An Induction Curve + Recovery is a preprogrammed dark-light induction curve (Kautsky effect) followed by a light-dark induction curve which provides information on the **dark-recovery** of fluorescence parameters after a period of illumination, the parameters of which can be defined by the user. The **Duration**-parameter (defined under Settings/Slow Induction) refers to the period of illumination (just like in the case of a normal Induction Curve). In the time period following termination of actinic illumination 16 Saturation Pulses are applied with the time between Saturation Pulses being exponentially increasing. In this way the rapid recovery kinetics can be recorded briefly after light-off and during registration of the slow recovery kinetics the actinic effect of the Saturation Pulses is minimized. Fig. 83 shows a typical recording of an Induction Curve+Recovery. In this example the Duration-parameter was set to 600 sec.

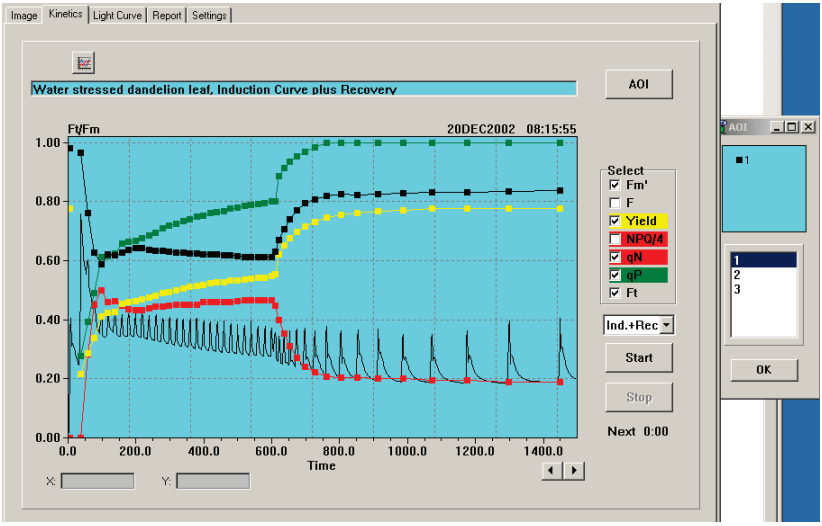
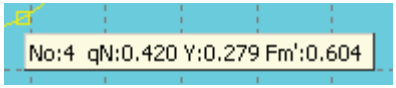


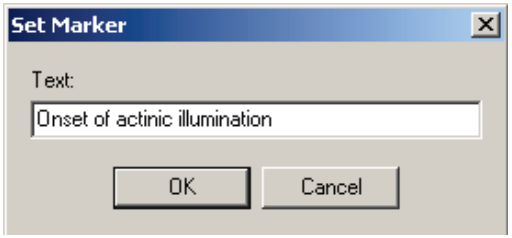
Fig. 83: Typical Induction Curve+Recovery

When the **Autoscale** icon is clicked the time scale automatically is changed such that the data fill the Kinetics screen.

The **X-Y boxes** show the coordinates of the cursor position, with X corresponding to the time (sec) and Y corresponding to the pixel value of the selected fluorescence parameter.



When the **cursor** is moved on top of a data point, for a period of 10 sec the number of the measurement and the values of the selected parameters are shown.



An **event marker** can be set in form of a **vertical red line** and a corresponding **event text** may be entered. For this purpose, the cursor has to be moved to the time of the event and the right mouse has to be clicked. Then the Set Marker box is opened, into which the event text may be entered. After confirmation by OK, the red vertical line is installed and whenever the cursor comes close to this line the event text is displayed for 10 sec. The event marker is saved in the pim-file.

ETR = 0.5 x Yield x PAR x 0.84 µequivalents m^{-2} s^{-1}

(see also section 10.1.1.11). While ETR Light Curves resemble conventional light response curves, it should be realized that the illumination periods normally are too short to assure true steady state conditions. Normally ETR Light Curves are distorted by dark-light

induction effects. The latter can be minimized by using pre-illuminated samples.

10.3 Light Curve window

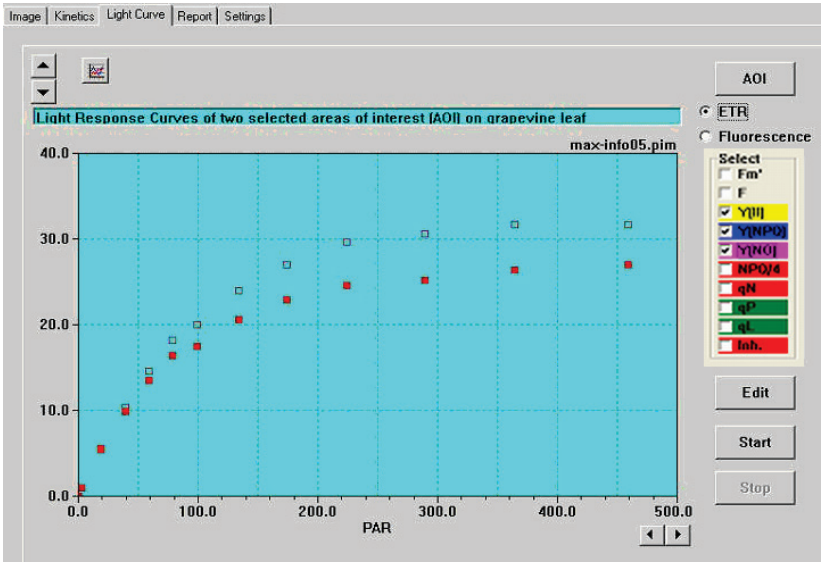


Fig. 84: Light Curve window with display of typical Light Curve of the ETR-parameter

Recording of a **Light Curve** consists of a number of illumination steps, at the end of which the effective PS II quantum yield as well as various other parameters are determined with the help of a Saturation Pulse. Either the **ETR parameter** or the various **Fluorescence parameters** can be displayed. The x-axis corresponds to incident PAR, as defined by the previously defined PAR list. The current PAR list can be viewed under Options/PAR-List (see chapter 11). The definition of ETR is:

The maximal ETR reached upon light saturation of photosynthetic electron flow at high PAR values strongly depends on correct determination of rather small ΔF values with the help of saturation pulses. It is important to realize that close to light saturation an underestimation of Fm' by a few percent will induce a large underestimation of Y(II) and, hence, also of ETR. In this

context, the Fm factor is important see 10.5.13), which allows to correct for underestimation of Fm and Fm' due to the unavoidable heating of the LEDs during a Saturation Pulse. In High Sensitivity or Microscopy applications using the Special SP-Routine (see 10.6.1) also the Fm Normalization Factor has a strong influence on correct assessment of Fm and Fm'.

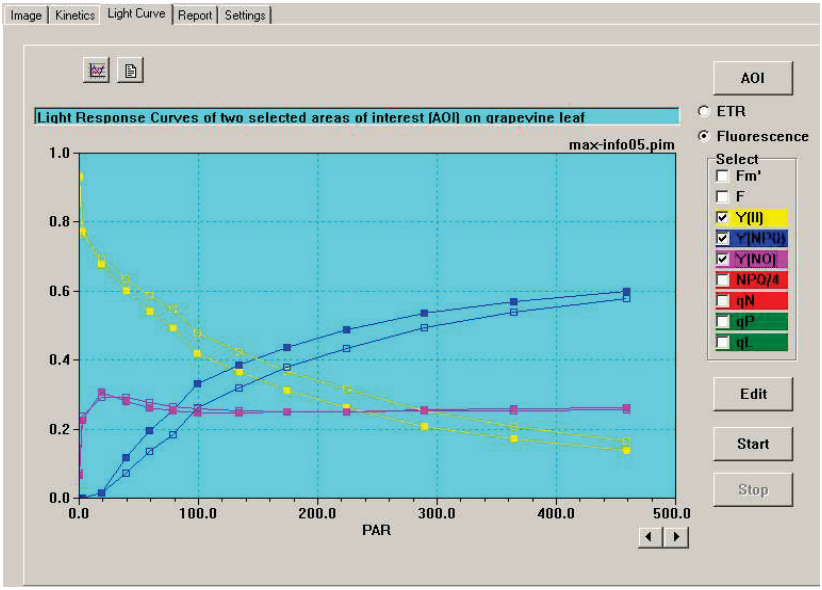


Fig. 85: Light Curve window showing the Light Curves of two AOIs, for which the averaged values of the PS II quantum yield parameters Y(II), Y(NPQ) and Y(NO) are displayed.

More detailed information on the physiological reactions taking place during the course of a Light Response Curve is provided by Light Curves of the various **Fluorescence parameters**. As illustrated in Fig. 85, with increasing PAR the **Y(II)** parameter continuously decreases, whereas the **Y(NPQ)** parameter shows an almost antiparallel increase and the **Y(NO)** parameter is almost constant. The curves for the 2 selected AOIs are similar, but not identical. The three quantum yields always add up to a total of 1. Their relative

values give important information on the partitioning of excitation energy between photochemical utilization, Y(II), regulated heat dissipation, Y(NPQ) and unregulated heat dissipation.

For the sake of clarity, in the example of Fig. 87: Report window showing the Record of the Light Curve recording displayed in Fig. 61 only part of the available fluorescence parameters is displayed.

As outlined above, the user may choose between display of **ETR** or of various **Fluorescence** parameters. Display of ten different fluorescence parameters is possible. For display the corresponding check box has to be marked. The parameters Fm', F, Y(II), Y(NPQ), Y(NO), NPQ/4, qN, qP and qL were already described in detail (see section 10.1.1). In principle, all fluorescence parameters may be displayed on top of each other.

Display of data in the Light Curve window requires that **at least one AOI** is selected. If this is not the case, there is a corresponding warning. A Light Curve is started via the **Start** button and can be terminated at any time with the help of the **Stop** button. Recording of a Light Curve constitutes a **New Record**. Therefore, unless any preceding Record already was stored, the user is asked **"Save previous Record?"**. Furthermore, as Light Curves often are measured with pre-illuminated samples, the user is asked **"Do you want to keep previously determined Fo, Fm?"**. During recording of a Light Curve the number of the current illumination step is indicated in the **Step**-box. **Next** refers to the remaining time until the next measurement takes place (i.e. application of next Saturation Pulse).

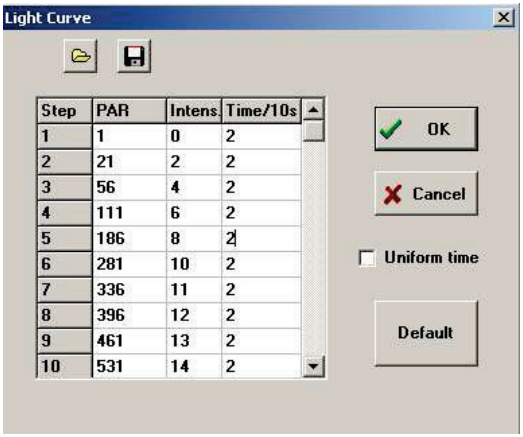
Upon Start automatically the ETR scale limit is set to 50, which can be either changed manually or using the **Autoscale** icon.

Above the Light Curve the time and date of the recording are documented. The user may also write a short comment into a **"text field"**, which is saved together with the Light Curve Record. The text also may be entered or modified in the View-mode. The same text is automatically also written into a corresponding text field above the Report file (see 10.4).

After termination of a Light Curve recording the **View-mode** is installed. Then the Light Curve Record can be saved using the **Save** icon (below Light Curve window).

In the View-mode an extensive analysis of the vast information stored during a Light Curve can be carried out. For software versions released before mid-2025 the window that opens looks like the one shown in the following image. Users who are using newer software will see an expanded AOI window that offers even more options. Please refer to section 11.2.6 for a more detailed description.

When the **AOI button** is pressed, the AOI window is opened, which shows in its lower part a list of all AOIs that previously were defined on the Image-window. By clicking a particular number, the corresponding AOI is selected for data display in the Light Curve window. The data of several or all AOIs may be superimposed. In the upper part of the AOI window the data point symbols of the selected AOI number are shown. Examples are given for one out of 4 selected AOIs and 8 out of 8 selected AOIs being active for display, respectively. Please find a more details on the recent window version in section 11.2.6



When the **Edit**-button is clicked, a separate window is opened, in which the user may **define the Light Curve parameters**, i.e. the number of illumination steps, the Intensity-setting at each step and the time between consecutive steps. **Up to 20 different illumination steps** can be defined. In order to modify a current setting (Intensity or Time/10 s), it first has to be selected by cursor/left mouse click and then the modified setting has to be entered. Please note that the PAR values for the selected Intensity settings are derived from the PAR list (see Options, section 11). It becomes effective with the next mouse click. When the **Uniform time** box is checked, the last entered time setting will be applied for all steps as soon as the Time/10 s cell of another step is clicked. When the **Default** button is pressed a Standard Light Curve featuring 12 Steps is defined, which has proven to give good results with "normal leaves". This Standard Light Curve is terminated at intensity setting 16 (approximately 700 µmol quanta m^{-2} s^{-1} PAR). In practice, it rarely makes sense to go beyond this intensity setting, as the effective quantum yield becomes rather low and the noise in Yield-determination correspondingly high. In order to **terminate a Light Curve**, under Time/10 s a **zero (0)** has to be entered.

In some applications it may be of interest to record a Light Curve with PAR values first increasing until saturation is reached and then decreasing again, in order to evaluate the capacity of the sample to recover from light saturation. Such an **"Up-Down Light Curve"** can

be readily programmed by the user, e.g. by substituting in the Default list the 0 time value in step 13 by the value 2.

The Light Curve Parameters defined under Edit can be saved in an **lcp-file** from which they can be reloaded at any later time. In this way, different sets of Light Curve parameters can be optimized for different types of plants (e.g. sun and shade plants) and called up readily without loosing much time. The lcp-files are saved in the Data-directories of the various Measuring-Heads.

10.3.1 Fitting Routine for RLC

For imaging devices with a recent firmware a fitting routine is available. Light curve experiments are an established tool for analyzing the photosynthetic performance of organisms. The “Light Curve” tab provides a predesigned procedure to execute a light curve protocol. Relevant parameters (number and duration of light steps, initial PAR, and recovery curve) can be adjusted in the menu that opens of the button “Edit” (on the right side of this tab) is clicked. For further details on settings please also refer to the light curve section of the recent Imaging-PAM manual.

The measuring routine “Light Curve” exposes the sample to increasing intensities of actinic illumination. Usually, the time interval of each intensity level is too short for full equilibration of photosynthetic reactions. Therefore, these so-called “Rapid Light Curves” (RLC) provide information on the present state of photosynthesis and are not to be confused with classical photosynthetic light response curves in which photosynthetic rates under steady state conditions are plotted against light intensities. By

plotting ETR versus PAR (see previous section), Rapid Light Curves provide subsequent key parameters:

- α, electrons/photons: Initial slope of RLC which is related to quantum efficiency of photosynthesis.
- ETR_m, µmol electrons/($m^2 \cdot s$): Maximum electron transport rate.
- I_K, µmol photons/($m^2 \cdot s$): Minimum saturating irradiance.

For the above mentioned parameters a fitting routine according to the Eilers und Peeters model, published in 1988. The function is still under construction, but the fitting process works well and the calculated data are then listed in the first line of the light curve listing of the report.

For Fitting a rapid light curve experiment, just press the button “Fit” shown in the image below:

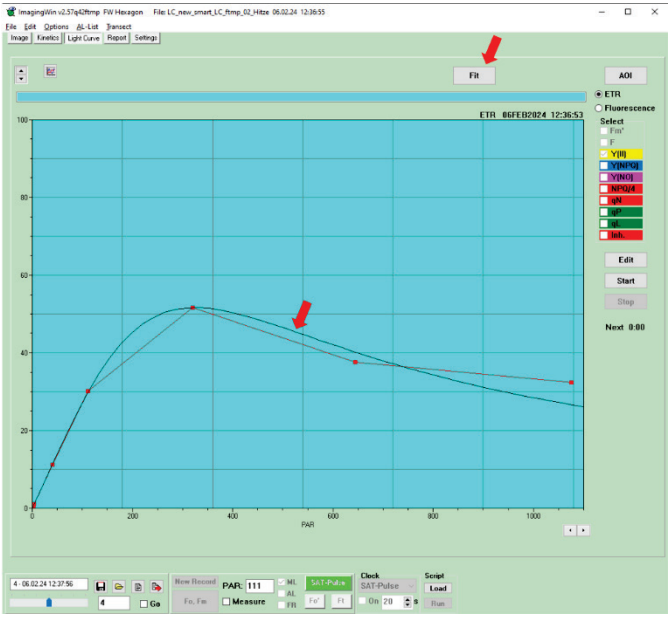
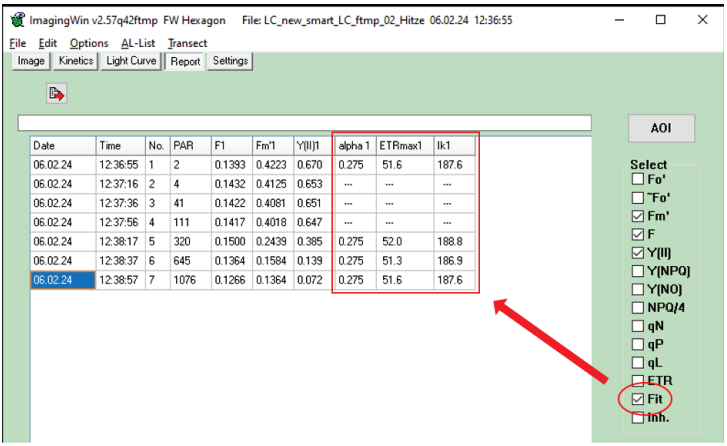


Fig. 86: Fitting a Rapid Light Curve according to the Eillers & Peeters model (1988) using the “Fit” function of ImagingWin

To export the fit data it is necessary to activate the parameter “Fit” in the “Report” tab shown in the following image:



Date	Time	No.	PAR	F1	Fm'1	Y(II)1	alpha 1	ETRmax1	Ik1
06.02.24	12:36:55	1	2	0.1393	0.4223	0.670	0.275	51.6	187.6
06.02.24	12:37:16	2	4	0.1432	0.4125	0.653	---	---	---
06.02.24	12:37:36	3	41	0.1422	0.4081	0.651	---	---	---
06.02.24	12:37:56	4	111	0.1417	0.4018	0.647	---	---	---
06.02.24	12:38:17	5	320	0.1500	0.2439	0.385	0.275	52.0	188.8
06.02.24	12:38:37	6	645	0.1364	0.1584	0.139	0.275	51.3	186.9
06.02.24	12:38:57	7	1076	0.1266	0.1364	0.072	0.275	51.6	187.6

In the first row of the Light Curve report (shown above) the final fitted values for the key parameters alpha, ETRmax and Ik are listed. In these early stages of the light curve a reliable fit cannot be calculated and thus rows do not contain any data.

From row 5 on a curve fit can be calculated and first approaches are listed in the columns for alpha, ETRmax and Ik. Now the “**Smart Light Curve**” feature comes into play. From the time/PAR on where Ik does not change more than 2% the light curve is automatically stopped to avoid a too high light impact for the sample (e.g. photoinhibition) and also to save time. The final fitting values for the parameters alpha, ETRmax and Ik are now written into the first line of the report for each active AOI. In this example only a single AOI has been active to keep the table small.

Some Papers on Rapid Light Curves

P.H.C. EILERS and J.C.H. PEETERS (1988) Model for the relationship between light. Intensity and the rate of photosynthesis in phytoplankton. Ecological Modelling, 42 (1988) 199-215

Fouqueray M, Mouget J-L, Morant-Manceau A, Tremblin AG (2007) Dynamics of short-term acclimation to UV radiation in marine diatoms. J Photochem Photobiol B: Biology 89: 1–8

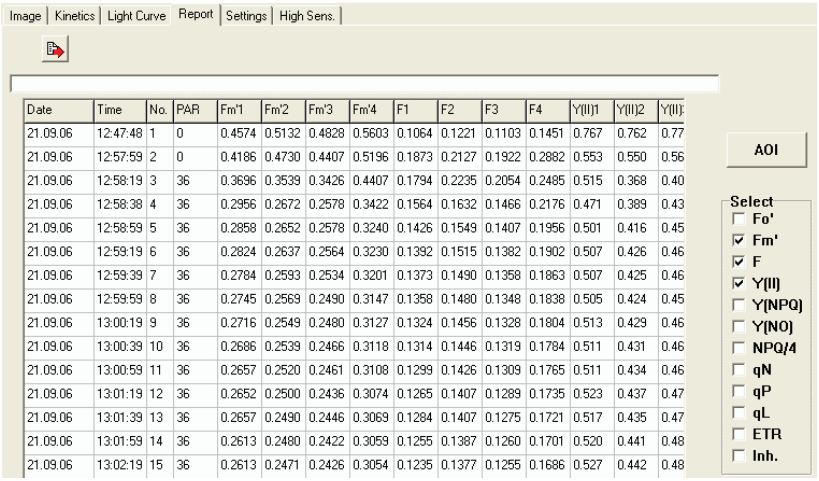
Perkins RG, Mouget J-L, Lefebvre S, Lavaud J (2006) Light response curve methodology and possible implications in the application of chlorophyll fluorescence to benthic diatoms. Marine Biol 149: 703-712

Ralph PJ, Gademann R (2005) Rapid light curves: A powerful tool to assess photosynthetic activity. Aquat Bot 82: 222-237

Rascher U, Liebig M, Lüttge U (2000) Evaluation of instant light-response curves of chlorophyll fluorescence parameters obtained with a portable chlorophyll fluorometer on site in the field. Plant Cell Environ 23: 1397-1405

10.4 Report window

In the Report window the data of the current **Record** are displayed in form of listed parameter values. These lists can be transferred to spread sheet programs, like Excel. At the top of the Report window there is a text field, into which a comment can be written. The same text automatically is written into the corresponding text field on top of the Kinetics or Light Curve window, if the particular Record was started under Kinetics or Light Curve. Alternatively, the text can be also written into the corresponding text field in the Kinetics or Light Curve windows and then will automatically also appear in the text field above the Report.



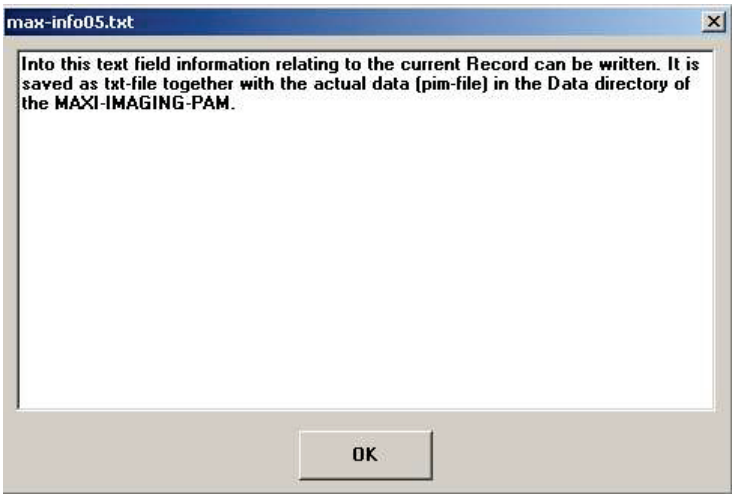
Date	Time	No.	PAR	Fm'1	Fm'2	Fm'3	Fm'4	F1	F2	F3	F4	Y(II)1	Y(II)2	Y(II):
21.09.06	12:47:48	1	0	0.4574	0.5132	0.4828	0.5603	0.1064	0.1221	0.1103	0.1451	0.767	0.762	0.77
21.09.06	12:57:59	2	0	0.4186	0.4730	0.4407	0.5196	0.1873	0.2127	0.1922	0.2882	0.553	0.550	0.56
21.09.06	12:58:19	3	36	0.3696	0.3539	0.3426	0.4407	0.1794	0.2235	0.2054	0.2485	0.515	0.368	0.40
21.09.06	12:58:38	4	36	0.2956	0.2672	0.2578	0.3422	0.1564	0.1632	0.1466	0.2176	0.471	0.389	0.43
21.09.06	12:58:59	5	36	0.2858	0.2652	0.2578	0.3240	0.1426	0.1549	0.1407	0.1956	0.501	0.416	0.45
21.09.06	12:59:19	6	36	0.2824	0.2637	0.2564	0.3230	0.1392	0.1515	0.1382	0.1902	0.507	0.426	0.46
21.09.06	12:59:39	7	36	0.2784	0.2593	0.2534	0.3201	0.1373	0.1490	0.1358	0.1863	0.507	0.425	0.46
21.09.06	12:59:59	8	36	0.2745	0.2569	0.2490	0.3147	0.1358	0.1480	0.1348	0.1838	0.505	0.424	0.45
21.09.06	13:00:19	9	36	0.2716	0.2549	0.2480	0.3127	0.1324	0.1456	0.1328	0.1804	0.513	0.429	0.46
21.09.06	13:00:39	10	36	0.2686	0.2539	0.2466	0.3118	0.1314	0.1446	0.1319	0.1784	0.511	0.431	0.46
21.09.06	13:00:59	11	36	0.2657	0.2520	0.2461	0.3108	0.1299	0.1426	0.1309	0.1765	0.511	0.434	0.46
21.09.06	13:01:19	12	36	0.2652	0.2500	0.2436	0.3074	0.1265	0.1407	0.1289	0.1735	0.523	0.437	0.47
21.09.06	13:01:39	13	36	0.2657	0.2490	0.2446	0.3069	0.1284	0.1407	0.1275	0.1721	0.517	0.435	0.47
21.09.06	13:01:59	14	36	0.2613	0.2480	0.2422	0.3059	0.1255	0.1387	0.1260	0.1701	0.520	0.441	0.48
21.09.06	13:02:19	15	36	0.2613	0.2471	0.2426	0.3054	0.1235	0.1377	0.1255	0.1686	0.527	0.442	0.48

Fig. 87: Report window showing the Record of the Light Curve recording displayed in Fig. 61

Due to the fact that an infinite number of AOIs can be defined by the user, in principle also an infinite number of Report files can be derived from a Record stored in a PAM-Image (pim) file. When the AOI button is clicked the numbers of the presently defined AOIs are shown. Those AOIs, the data of which shall be displayed on the Report window, can be selected by left mouse click. They are shown

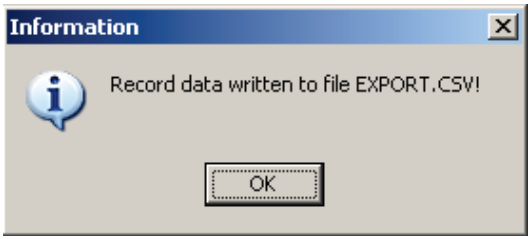
in the upper box with their respective symbols in the displays of the same Record on the Light Curve (or Kinetics) window. Under **Select** it is possible to select the fluorescence parameters that shall be listed in the Report.

Together with a Record also a **Comment File** can be saved, which is stored as a txt-file under the same name as the corresponding pim-file (PAM image).



This comment file can be added or edited at any time in the View-mode and will be automatically saved as txt-file in conjunction with the corresponding pim-file. Considering the vast amount of data which can be collected with the IMAGING-PAM under largely different measuring conditions, this comment file is of considerable importance for later assessment of the results.

When the **Export** icon is clicked, a routine for transfer of the Report file into an external spread sheet program, like Excel, is started. Please note that the exported Report corresponds to the selection of AOIs and fluorescence parameters displayed in the Report-window.



After confirmation by OK the Record (with the information specified in the Report window) is first transferred to the file **export.csv** (comma separated values) in the ImagingPam directory. From there it can be transferred to other programs, like **Excel**. If Excel is installed on the PC, the Report-data are automatically opened under Excel when export.csv in the ImagingPam directory is double clicked.

Settings: mi2, mf1, ai3, aw0, icmax, g3, d2, si10, bo-, rg440, al:default.par, ff1.000, fmf1.000

At the bottom of the Report window the **Settings** in abbreviated form are listed, which apply to the conditions of measurement Nr. 1 (Fo, Fm determination) of the given Record. The meaning of the abbreviations is as follows:

mi	Measuring light intensity
mf	Measuring light frequency
ai	Actinic light intensity
aw	Actinic width
Icmax	status of Image Correction
g	gain
d	damping
si	saturation pulse intensity
sw	saturation pulse width
bo	booster

rg	red gain
fmf	Fm-factor
ff	F-factor(not applied under standard settings, see 10.5.14 for explanations)
fmnf	Fm Normalization Factor
mifo	measuring light intensity for Fo measurement
gfo	gain for Fo measurement
mifm	measuring light intensity for Fm measurement
gfm	gain for Fm measurement
foav	number of Fo averages (the last 6 items apply only for the MAXI- and MICROSCOPY-versions, if the Special SP-Routine is activated)

10.5 Settings window

The Settings window shows all instrument settings, which can be modified by the user, and in the Measure-mode also provides information on the battery status.

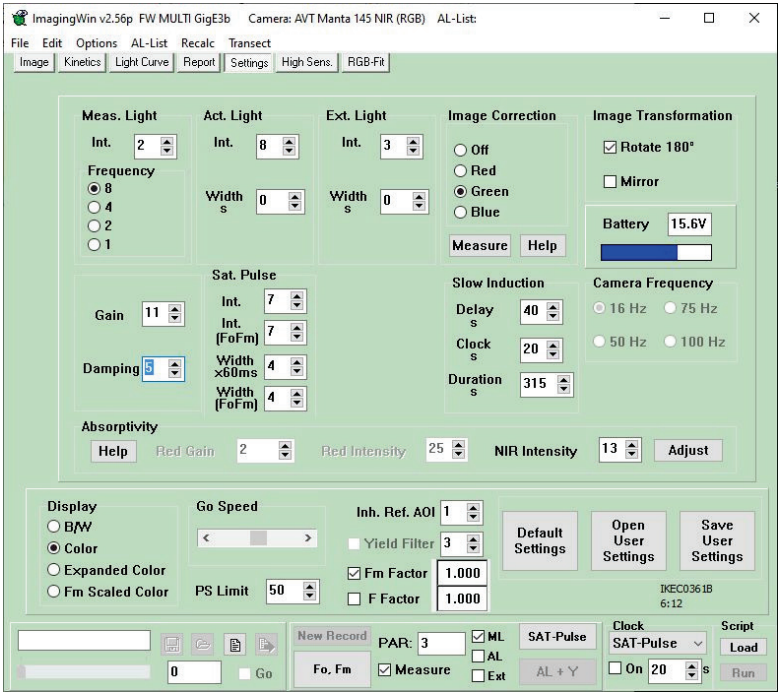


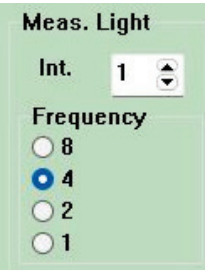
Fig. 88: ImagingWin user surface with Settings window being selected

In Fig. 88 the Settings window with standard settings is depicted. Standard settings can be reinstalled at any time by clicking the Default Settings button. Most settings relate to light parameters (but not the light list).

10.5.1 Light parameters

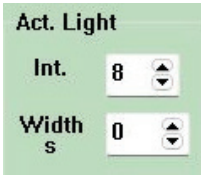
The IMAGING-PAM features four different types of light: **Measuring Light**, **Actinic Light, External Light** and **Saturation**

Pulses. Except of the external light all three types of light are derived from the same source, the **LED Illumination Unit** (array or single LED depending on the particular version of the Measuring Head).



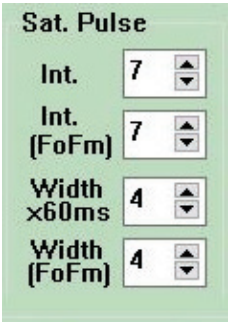
The **Measuring Light** is pulse modulated. It consists of relatively short (in the order of 100 µsec) but very short LED pulses (in the order of 100 µsec). While these pulses are quite intense, they are applied at a relatively low repetition rate (frequency) of 1 to 8 Hz and, hence, do not have much actinic effect. The Measuring Light is automatically switched on after start of the program. It can be manually switched off/on via the **ML** check box (see 9.3). Its **Intensity** and **Frequency** can be set by the user. Standard settings are Intensity 2 and Frequency 1 (in conjunction with Damping 2) for MAXI-, and MINI-versions. At these settings the actinic effect of the Measuring Light is negligibly small. In the case of the MICROSCOPY-versions, standard settings are Intensity 3 and Frequency 8 (in conjunction with Damping 5) for the sake of an improved Signal/Noise at low signal levels. The Meas. Light Int. determines the amplitude of the fluorescence signal. Normally a signal amplitude of 150 - 200 units is optimal, assuming maximal stimulation of fluorescence yield during a Saturation Pulse by a factor of 4 - 5. While signal saturation occurs at 1000 units, a certain noise band has to be taken into account which depends on the Damping (see 10.5.2). When dealing with weakly fluorescent objects (like diluted algae suspensions in black multiwell plates using the MAXI-version) or in Microscopy-applications a Special SP-Routine is provided, which involves automated switching to a high setting of Meas. Light Int. at lowered Gain-setting. In this way, the Signal/Noise of Fm, Fm', Fv/Fm and Y(II) measurements can be considerably enhanced. At a given Meas. Light Int. setting the amplitude of the fluorescence signal can be adjusted by the Gain (see

10.5.2). Meas. Light Int. up to setting 20 can be selected. In this way, also weakly fluorescent objects can be imaged. It should be kept in mind, however, that with objects showing a light induced fluorescence increase part of this increase will already occur during an individual Measuring Light pulse, if Meas. Light Int. is too high. In this case, the F-value will be overestimated and the saturation pulse induced fluorescence increase as well as the PS II quantum yield, Fv/Fm or Y(II), will be underestimated. In principle, it is possible to correct for this effect by the **F Factor** (see below).



The **Actinic Light** drives photosynthesis. It is switched on manually by the **AL** check box or the **AL+Y** button (see 9.3). The **Intensity** and the **Width** of actinic illumination can be defined by the user. When the Width is set to 0, actinic illumination will not be terminated until manually stopped by the user by clicking the AL check box. One out of 20 settings of Act. Light Int. can be selected. The PAR-value corresponding to a particular setting is shown in the PAR-field when Actinic Light is switched on (see 9.3). The list of PAR-values corresponding to all intensity settings can be viewed and edited under Options/PAR-List (Menu at upper edge of the user surface, see chapter 11).

Saturation Pulses (SP) are applied for determination of maximal fluorescence yield (Fm or Fm'). Also the fluorescence yield, Ft, observed briefly before triggering of the SP is assessed. By application of a SP a **Measurement** is defined, with the resulting data being saved in the buffer memory. Ten **Intensity** settings of SP are available, with the maximal setting 10 being standard. In most practical applications the best results are obtained with maximal SP intensity. This, however, is true only, if appropriate use of the Fm-factor correction (see 10.5.13) is made.



During a Saturation Pulse the LEDs are driven with very high current, which leads to a temperature increase of the light emitting chip. Therefore, the intensity of the emitted light is transiently lowered by about 5 % in the older LED array generations, but not in the younger LED arrays built from the year 2020 on. This results in a corresponding decrease of the intensity of the Measuring Light, which is driven by the same LEDs, thus causing underestimation of the Fm value measured during a Saturation Pulse. This effect can be compensated by the Fm Factor (see below). In ImagingPAM software higher than version 2.56 it is also possible to apply different intensities of the SP depending on situation in which AL is on or off (e.g. during Fo Fm determinations). By applying lower SP intensities for Fv/Fm measurements high-energy-fluorescence quenching can be avoided.

The **Width** of Saturation Pulses can be changed from 240 to 840 ms (4 x 60 ms to 14 x 60 ms), standard **Width** of Saturation Pulses is 720 ms (12 x 60 ms).

Please also note that users with software versions higher than 2.56 have a firmware version built-in which enables them to get rid of the Fm factor (chapter 10.5.13). From this Firmware version on, Fm factors are determined before shipping the instrument and stored in the EEPROM of the IMAG-CG central control unit. This means that central units can no longer be freely exchanged between measuring heads. The relevant factors are already stored in the memory of the central units when they are delivered. If a measuring head is purchased without a central unit, it is delivered with a corresponding data record that must be stored in the memory of the central unit before use.

10.5.2 Gain and Damping

The **Gain** determines the amplitude of the **fluorescence signal, Ft**, at a given setting of Measuring Light Intensity (see 10.5.1). Twenty settings are available, with standard setting 2 for MAXI- and MINI-versions. In the case of the MICROSCOPY-versions, when dealing with relatively low signal amplitudes, the Gain 9 is set by default. The Gain should be set such that in the absence of actinic illumination the fluorescence amplitude (Ft = Fo) is in the range of 150 - 200 units.

When dealing with weakly fluorescent objects (like diluted algae suspensions in black multiwell plates using the MAXI-version) or in MICROSCOPY-applications a Special SP-Routine is provided, which involves automated switching to a high setting of Meas. Light Int. at lowered Gain-setting (see 10.6.1). In this way, the Signal/Noise of Fm, Fm', Fv/Fm and Y(II) measurements can be considerably enhanced.

The time response of fluorescence measurement can be slowed down by **Damping**. The selected Damping-setting determines the time resolution with which changes of the Ft-image can be viewed and also the amount of noise visible in the recorded images. Five settings (0 - 4) are available, with standard settings 2. It has to be considered that time resolution not only depends on Damping, but on Measuring Light frequency as well (see 10.5.1). Hence, in order to see rapid changes in the Ft-image, e.g. while moving a sample, both low Damping and high Measuring Light frequency settings have to be selected. In the Live Video mode (see 10.1.2.3), which applies near-infrared instead of blue light, Damping is low and Measuring Light frequency high. Therefore, the Live Video mode is best suited for positioning samples in the field of view and for focusing images.

10.5.3 Absorptivity (not available for HEXAGON-IMAGING-PAM)

For correct assessment of PAR-Absorptivity the pixel values with a white piece of paper of the Red image and the NIR image are supposed to be close to identical (5%) at the given **Red** and **NIR**

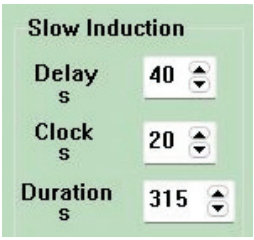
Intensities (e.g. 0.700). Therefore the NIR and RED image needs to be adjusted with the NIR Intensity, Red Intensity and Red Gain settings.

Adjust opens up a prompt for comfortable setting-changes without switching between the image and the Settings window.

The changes in NIR and Red Settings are recorded in connection with the Absorptivity-measurement (**Measure-Abs.** function, see 10.1.2.1 and 10.1.1.10). The **Red Gain** setting allows **fine adjustment** of the intensity of the Red image.

Whereas the steps with which the LED intensities can be set are relatively coarse, a fine adjustment via Red Gain is possible. Such adjustment may become necessary with ageing of the LEDs.

Please note that the NIR Intensity accessible under Settings/Absorptivity can be set independently from the intensity of the NIR light used for measurements of Live Video images (see also chapter 10.1.2.3).

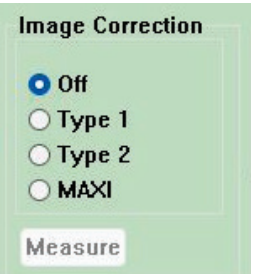


10.5.4 Slow Induction parameters

The **Slow Induction Parameters** apply to recordings of preprogrammed dark-light **Induction Curves** in the **Kinetics** window. After Start and automatic Fo, Fm determination (see 10.2) some time is given

for fluorescence yield to decline back close to the original Fo-level, before Actinic Light is switched on. This **Delay**-time can be defined by the user, with the default value being 40 s. The **Clock**-time determines the time interval between repetitive Saturation Pulses, with which F and Fm' are measured. At a given length of actinic illumination, the Clock-time also defines the number of measurements made in the course of an Induction Curve. The **Duration**-time corresponds to the overall length of a recording, including the Delay-time and the actinic illumination time. The recording of a standard Induction Curve (under default settings) involves a total of 15 measurements, with the first corresponding to Fo, Fm determination and the remaining 14 carried out after start of actinic illumination.

10.5.5 Image Correction



With the help of **Image Correction** unavoidable inhomogeneities of measuring sensitivity over the imaged area can be compensated. One part of such inhomogeneities originates from spatial differences in Measuring Light intensity and another part is due to the unavoidable vignetting-effect of the objective lens. For compensation, correction images can be measured with the help of a sample, which shows uniform fluorescence emission over the whole imaged area. For this purpose, normal white printing paper may serve which at high Meas. Light Intensity emits sufficient fluorescence for a good quality fluorescence image measured at high Gain setting.

Correction images have to be measured under identical optical conditions at which the actual experiments are done. In the case of the MAXI-version this applies particularly to the **working distance**, as the inhomogeneities due to Measuring Light intensity are minimal

at 17 - 19 cm distance between the exit plane of the LED-Array and the sample plane. Using the standard Mounting Stand with Eye Protection (IMAG-MAX/GS) the working distance is fixed at **optimal 18.5 cm** (standard distance). Also the default PAR-list determined at the factory applies for this standard distance (see 11). Hence, unless there are compelling reasons to do otherwise, the standard working distance of 18.5 cm should be used, even when the Measuring Head is mounted independently from the standard Mounting Stand (see 3.5).

In the case of the MICROSCOPY-versions the working distance is determined by the **focusing position**. As inhomogeneities (due to dust etc.) will be emphasized in the fully focused position, a position should be chosen where the image is just out of focus.

With every type of Measuring Head three different correction images can be stored: **Type 1**, **Type 2** and **Maxi** (or **Mini, IMAG-L450, RGB).**

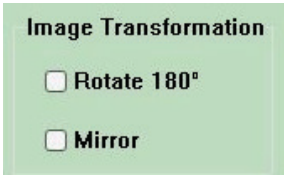
For **measuring Image Correction** please proceed as follows:

- set the optical conditions under which the actual measurements are going to be done (working distance, focusing position, see above)
- select Type 1, Type 2 or Maxi/Mini/IMAG-L470/RGB (under Settings/Image Correction)
- in the case of MAXI- and MINI-versions place at least two layers of white paper (e.g. folded DIN-A4) into sample plane; in the case of the MICROSCOPY-version the plastic fluorescence standard
- put the image somewhat out of focus to avoid imaging fine structures of the white paper tissue or dust etc. on the surface of the fluorescence standard
- press Measure (under Settings/Image Correction)

The measured correction image will be saved until it is overwritten by a new measurement. The correction images will remain valid as long as the same optical parameters apply (LED Illumination Unit, working distance, focusing position, camera objective lens, microscope objective lens).

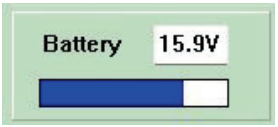
While the Image Correction can compensate for heterogeneities in Measuring Light intensity, the corresponding heterogeneities of Actinic Light intensity unfortunately cannot be corrected. In principle, higher PAR values tend to induce lower values of Y(II) and higher values of Y(NPQ) (see section on Light Curves 10.3). With all Measuring Heads of the Imaging-PAM M-series maximal deviations of PAR values from the mean value are small (not exceeding 10 %) and, hence, can be ignored in most applications.

10.5.6 Image Transformation



The display of images can be changed by the **Image Transformation** function in order to account for different positions of the camera with respect to the user. Images can be rotated by 180° or mirrored along the vertical midline. Default settings (**Rotate 180°** and **Mirror** boxes not checked) apply for use of the standard configuration, with the camera pointing downwards and its wide side pointing towards the user. If, for example, in a special application the Measuring Head would be pointing upwards, the shifting of a sample to the left and the right (or up and down) would cause opposite changes of the displayed image. In this case**,** checking **Rotate 180°** as well as **Mirror** would be appropriate.

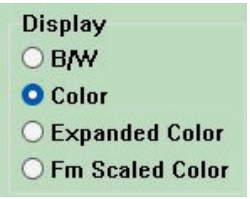
10.5.7 Battery



The **Battery**-status is indicated by a blue bar, the height of which represents the remaining capacity. In addition, the battery voltage is shown. Please note that the status display is updated in the **Measure-mode** only. The display is updated every minute, except during Light Curve or Induction Curve recordings. When the **Battery Charger 2120-N** is connected, high capacity is indicated even when the battery is only partially charged. Hence, the true Battery-status should be evaluated with the Battery Charger being disconnected. When 14.0 V is reached there is a Low Battery warning. At 13.5 V the instrument is automatically switched off. The instrument should not be stored with a discharged battery, which should be recharged every three months.

Note:	Charging of the internal Li-ion battery should be avoided, when the IMAGING-PAM is switched on, as this may lead to malfunctioning.

10.5.8 Display parameters



The user may choose between three different types of image **Display**.

B/W: black-and-white; gray scale, ranging from black through shades of gray to white. This scale normally provides less contrast than a false color scale. At the bottom of the scale a cut-off filter is installed, which transforms all pixel values ≤ 0.040 into zero (black) for the sake of background noise suppression (see below).

Color: standard scale of false colors, ranging from black (pixel values ≤ 0.040) via red, yellow, green and blue to pink (0.999). At

the bottom of the scale a **cut-off filter** is installed, which transforms all pixel values ≤ 0.040 into zero (black). This filter serves the purpose to suppress the background noise and to give optimal contrast between leaf area and background. Even a non-fluorescent background area gives a weak signal due to unavoidable noise and some reflected leaf fluorescence. While the position of the cut-off filter is fixed under Color-display, it can be shifted under Expanded Color/Analysis (see below).

Expanded Color: Expanded Color can be activated in the **view mode**. When selected, the Low and High cut-off limits defined by the user under Analysis become effective. All pixel values below the Low-limit are displayed in black and all pixel values above the High-limit are displayed in white. For pixel values within the cut-off limits, the same false color as for the standard Color display is used (ranging from black via red, yellow, green and blue to purple).

With Low and High limits approaching each other, smaller differences in pixel values are required to give different colors. This may help to increase contrast. On the other hand, it is also possible to lower or completely remove the Low cut-off limit which under normal Color-display by default is set to 0.040.

False color scale ranging from black via red, yellow, green and blue to pink with **Low and High cut-off** limits defined by the user under **Analysis** (see 10.1.2.7). All pixel values below the Low-limit are displayed in black and all pixel values above the High-limit are displayed in white. With Low and High limits approaching each other, smaller differences in pixel values are required to give different colors. This may help to increase contrast. On the other hand, it is also possible to lower or completely remove the Low cut-off limit which under normal Color-display by default is set to 0.040 (see above).

Fm Scaled Color: The Fm Scaled Color can be activated in the **view mode** and serves for emphasizing structures with high fluorescence yield and suppressing structures with low fluorescence yield. This can be particularly useful in Microscopy applications with focused objects displaying high fluorescence yield. As fluorescence yield depends on the angle of incidence of the measuring light, Fm Scaled Color images give a 3-D impression.

When Fm Scaled Color is active, the pixel intensities (see info on Brightness, Settings window upper right corner) are scaled according to Fm (or Fm'), if the displayed parameter involves assessment of Fm (or Fm') with the help of a saturation pulse.

The fluorescence parameters, images of which can be measured with the Imaging-PAM, can be divided into two groups: 1) directly measured parameters like Fo, F, Fm and Fm'. 2) derived parameters like Fv/Fm, Y(II), NPQ etc. The latter are based on ratios of the directly measured parameters and, hence, lack information on the fluorescence yield. For example, in Microscopy applications a focused object displaying high fluorescence yield shows the same Fv/Fm as another object which is out of focus. Display of the latter, which greatly disturbs the image, is suppressed by the Fm Scaled Color function, as the Brightness with which it is displayed is low.

This function is automatically installed in the **View mode** and cannot be used in the Measure mode.

10.5.9 Go Speed

The **Go Speed** refers to the rate with which consecutive images are displayed when the **Go-function** is activated in the **View-mode** (see 9.2). At maximal speed spatiotemporal variations of fluorescence parameters can be presented in a similar way as a video movie. On the other hand, lower speeds are required to evaluate the

observed variations. Please note that the maximal speed with which calculated parameters like Y(II) can be displayed, depends on PC processor frequency. For high speed display of Y(II)-images the **Yield Filter** (see 10.5.12) should be **inactivated**.

10.5.10 PS Limit

The estimated rate of photosynthetic electron transport, PS, is calculated according to the equation:

PS = 0.5 x Y(II) x PAR x Abs. µequivalents m^{-2} s^{-1}

(see also 10.1.1.11)

In order to display images of this parameter on a false color scale ranging from 0 to 1, the PS value is divided by a number, which corresponds to the expected limit of maximal PS, the **PS Limit**. The **standard setting is 50**, which means that the pixel value 1 is reached when PS/50 = 1. Limits of 50, 100, 150, 200 and 250 can be defined.

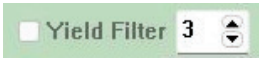
10.5.11 Inh. Ref. AOI

The image of the Inh. parameter is calculated pixel by pixel relative to the Inh. Ref. AOI, which normally corresponds to a control AOI. An AOI number between 1 and 100 can be selected. AOI #1 is set by default upon start of the program.

This parameter is particularly important for assessment of phytotoxicity with the MAXI-version using multiwell plates. The Inh. parameter describes the relative inhibition of PS II quantum yield with respect to a control (see also 10.1.1.18):

Inh. = ($Y_{control}$ - Y_{sample}) / $Y_{control}$

10.5.12 Yield Filter



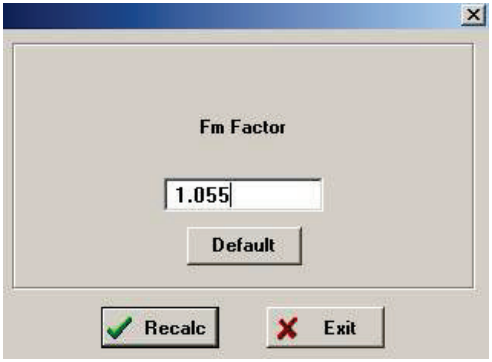
The **Yield Filter** may serve for suppression of noise in Y(II)-images, which is mainly due to the noise in the Fm and Fm' images measured during the relatively short Saturation Pulses. The Yield-filter is effective in the **View-mode** only, i.e. when images are called up from buffer memory (see 9.2). Filter settings 0-5 are available, with standard setting 3. Noise reduction is achieved by averaging the value of every individual pixel with those of a defined number of neighboring pixels. With increasing filter setting the number of pixels within an averaged domain increases (setting 1, 8 neighbors; setting 2, 24 neighbors; setting 3, 48 neighbors; etc.). This unavoidably leads to some loss in spatial resolution. Furthermore, depending on the noise structure, the averaged domains may form patterns which sometimes can be more disturbing than the original noise. Please note that the Yield-filter slows down the build-up of the Y(II) image. This may limit the rate with which consecutive Y(II) images can be displayed using the Go-function (see 10.5.9).

10.5.13 Fm Factor

Please also note that users with software versions higher than 2.56 have a firmware version built-in which enables them to get rid of the Fm factor (chapter 10.5.13). From this Firmware version on, Fm factors are determined before shipping the instrument and stored in the EEPROM of the IMAG-CG central control unit.

This means that the Fm factor remains switched off or 1.000 for typical experiments.

The Fm Factor compensates for underestimation of Fm and Fm' caused by the decrease of Measuring Light intensity during a Saturation Pulse, which is related to the unavoidable heating of the LEDs. In the case of the blue LED-Array Illumination Unit of the MAXI-IMAGING-PAM, at the standard setting 10 of SP Intensity this lowering amounts to 5 - 6 %. Hence, in the MAXI-version the standard value of the Fm Factor set by default is 1.055. For the other versions different values apply, that depend on the color of the applied LEDs. Red LEDs generally show more heat induced lowering of intensity than blue LEDs. The extent of SP induced lowering of ML intensity can be estimated with the help of the plastic fluorescence standard that is provided with the instrument (see below). After Reset Settings (see 10.5.15) the Fm Factor is active.



When Fm Factor is active, the Fm (or Fm') values documented in the Report (and used for calculation of other fluorescence parameters) are derived from the product of the actually measured Fm (or Fm') and the Fm Factor.

A new Fm Factor can be defined by the user for the current Record in the Measure as well as in the View mode. In the Measure mode, however, this definition has to be done before the first measurement (normally Fo,Fm determination). A corresponding dialog window is opened by a left mouse click on the current value in the **Fm Factor box**. Alternatively, this window can be also opened

via **Recalc** in the Menu (see 11.4). The current value can be erased and the new value written into the box. The new value is confirmed and the previously measured data recalculated upon pressing the **Recalc** button. The new Fm Factor remains installed until manually changed or reset to the standard value of 1.055 via the Default button.

Note:	Recalculation of data on the basis of a new Fm Factor always applies to the whole Record. If the user tries to change the Fm factor after Fo, Fm determination, there is a corresponding warning: **"For changing Fm factor start new record"**.

The importance of the Fm Factor increases with PAR, as $\Delta F = Fm'-F$ as well as $Y(II) = \Delta F/Fm'$ decrease and eventually approach zero. For example, when at high PAR values the true ΔF approaches 5.5 % of Fm', without this correction the apparent value of ΔF would approach 0, whereas the true ΔF would approach 0.055. Correct assessment of such low Y(II) values in any case is problematic due to the unavoidable noise limiting Fm' determination. A systematic underestimation of Fm' and Y(II), however, should be avoided in view of its pronounced effect on Light Response Curves.

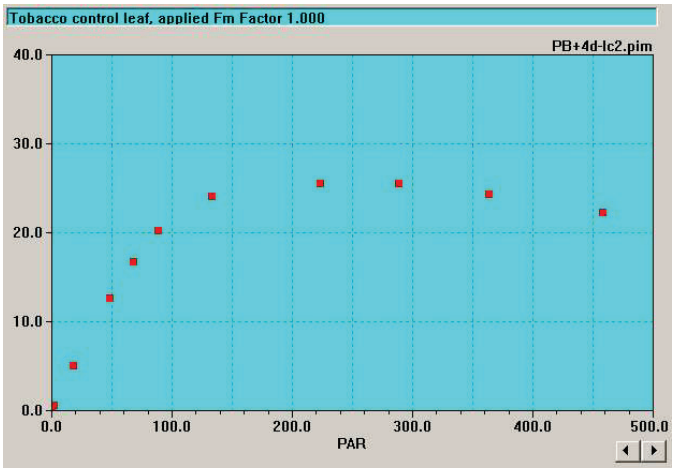


Fig. 89: ETR Light Curve recalculated with Fm Factor 1.000

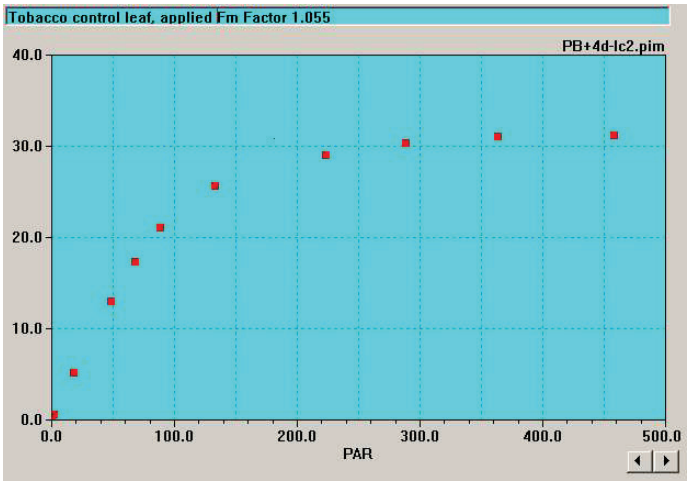


Fig. 90: ETR Light Curve based on the same original data as in Fig. 89, recalculated with Fm Factor 1.055

In Fig. 89 - Fig. 90 the same original Light Curve recording is displayed after recalculation with Fm Factor = 1.000 (Fig. 89) and Fm Factor = 1.055 (Fig. 90). With Fm Factor 1.000 maximal ETR amounts to a relative value of 26 and there is an apparent decline of

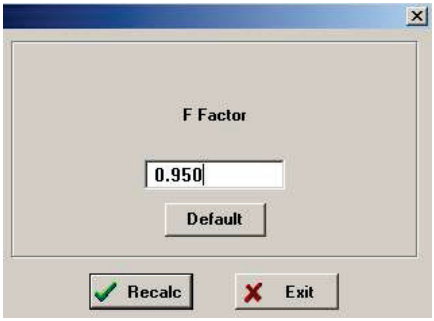
ETR at PAR values exceeding 250 µmol quanta m^{-2} s^{-1} , which could be misinterpreted to reflect "photoinhibition". On the other hand, using the standard value of 1.055, maximal ETR amounts to a relative value of 31, where the light response curve saturates at about 350 µmol quanta m^{-2} s^{-1}, without any decline apparent at higher PAR values. In practice, if there is uncertainty about the correct Fm Factor to be applied for previously stored data, the data may be recalculated using several different values. If the chosen Fm Factor is too high, in a Light Curve this is reflected by a biphasic response without saturation even at maximal PAR-values.

The actual extent of Measuring Light lowering during a Saturation Pulse can be estimated with the help of the plastic **fluorescence standard** delivered with the instrument. As the fluorescence yield of this standard in contrast to that of a living leaf does not change upon illumination with a Saturation Pulse, in first approximation it may be assumed that the observed decrease of fluorescence intensity is a measure of the decrease in Measuring Light intensity. Formally it corresponds to the **Fo/Fm** or **F/Fm'** ratio obtained with the fluorescence standard, which can be calculated from the values listed in the **Report** file.

10.5.14 F Factor

The F Factor can be applied for compensation of the actinic effect of the Measuring Light pulses, which tends to cause an overestimation of Fo or F and a corresponding underestimation of Fv and Y(II). The F Factor is always < 1. It corresponds to the factor with which the measured Fo or F value has to be multiplied in order to obtain correct values. In contrast to the Fm Factor the F Factor is not an instrument parameter and it cannot be assumed to be constant. It depends on the physiological condition of the sample and in particular on the state of PS II reaction centers. Therefore, the user must decide himself,

whether in a particular application the F Factor correction is advantageous or not. It cannot be recommended to be used in conjunction with Light Curves. The F Factor check box is not activated upon Reset Settings (see 10.5.15). It is also possible to **recalculate previously recorded data** on the basis of a new F Factor defined by the user.



A new F Factor can be defined for the current Record in the Measure as well as in the View mode. In the Measure mode, how-ever, this definition has to be done before the first measurement (normally Fo,Fm determination). A corresponding dialog window is opened by a left mouse click on the current value in the **F Factor box**. Alternatively, this window can be also opened via **Recalc** in the Menu (see section 11.4). The current value can be erased, and the new value written into the box. The new value is confirmed and the previously measured data recalculated upon pressing the **Recalc** button. The new F Factor remains installed until manually changed or reset to the standard value of 0.950 via the Default button.

Note:	Recalculation of data on the basis of a new F Factor always applies to the whole Record. If the user tries to change the F-factor after Fo, Fm-determination, there is a corresponding warning: **"For changing F factor start new record"**.

Two types of actinic effects of the Measuring Light can be distinguished:

1) Accumulation of closed PS II reaction centers due to repetitive illumination with Measuring Light pulses. This effect increases with Measuring Light intensity, pulse frequency and the extent of dark adaptation of the sample. It does not play any role, when the overall PAR is high during actinic illumination. Furthermore, in the case of the Imaging-PAM even at the maximal ML Frequency there are relatively long dark times between ML pulses, so that under normal physiological conditions the accumulation of reduced primary acceptors is insignificantly small.

2) Closure of a significant fraction of PS II centers during each individual Measuring Light pulse. This effect increases with Measuring Light intensity and is favored by a large functional absorption cross section of PS II. Notably, it also occurs at minimal pulse frequency of the Measuring Light and does not become irrelevant during actinic illumination, as long as there are open PS II reaction centers. In experiments with the Imaging-PAM this effect can be quite significant, as relatively strong ML pulse intensity has to be applied to obtain high quality images at the given low repetition rates (limited by transmission of large data volume). The F Factor correction is essential when in experiments with low fluorescence samples a high Measuring Light intensity is chosen. In principle, the system sensitivity can be increased either via higher settings of Gain or Meas.Light Int.. However, at high Gain, also the noise is increased. On the other hand, at high Meas.Light Int. the overestimation of F (or Fo) becomes rather large. In first approximation the effect is linear with intensity. Hence, when an increase from setting 1 to 2 results in an apparent 5 % increase of Fo, this will amount to about 20 % at setting 5.

The F Factor can be determined by the user for a particular type of sample and illumination conditions by measuring the F (or Fo) (a)

at Meas. Light Int. setting 1 and high Gain setting (averaging over several measurements) and (b) at a higher Meas. Light Int. setting and lower Gain. For comparison, the data first have to be normalized at Fm' (or Fm). Once the F Factor has been determined, its use provides an elegant way for high quality imaging of fluorescence parameters of weakly fluorescing samples, without sacrificing the correctness of F (or Fo) measurement. However, the proper use of this correction factor requires some experience and background knowledge on the physiological background.

10.5.15 Reset Default Settings, Open or Save User Settings

The buttons Default Settings, Open User Settings and Save User Settings facilitate the handling of the settings adjustments. Upon Default settings, the standard settings defined at the factory are reset. Different standard settings may apply for different types of Measuring Heads. These settings have proven optimal for imaging of fluorescence parameters of typical samples using various versions of the Imaging-PAM. Please note: The Default Settings button does not reset the PAR-List, Image Correction and Absorptivity adjustments

The Save and Open User Settings buttons store and open individually preset settings. Please note these buttons also store or reopen the active PAR-List!

10.6 High Sens. window

The High Sens. window is implemented for the MAXI- and MICROSCOPY-versions only. It features a number of functions for signal enhancement and noise suppression that are quite useful in applications dealing with weakly fluorescent samples, imaging of

which requires high Gain settings. In the case of measurements with the MAXI-version using multiwell plates or other objects with reflecting surfaces the use of the Filter Plate IMAG-MAX/F is recommended in order to avoid mirror reflections of the LED Array.

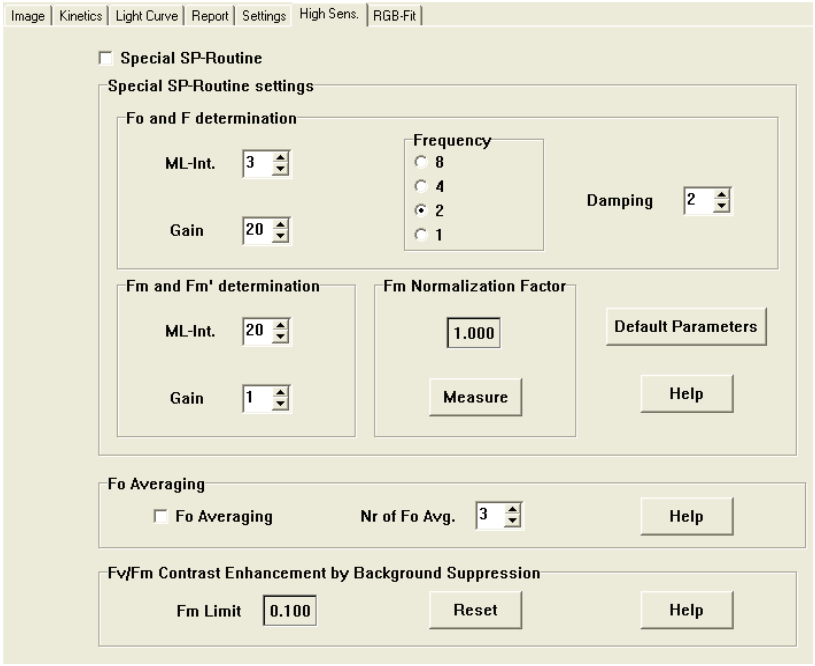


Fig. 91: ImagingWin user surface with High Sens. window being selected

Fig. 91 shows the High Sens. window for the MICROSCOPY-version after enabling the Special SP-Routine (checkbox in upper left corner). Essentially the same window also applies for the MAXI-version except that the bottom box (Fv/Fm Contrast Enhancement by Background Suppression) is missing.

10.6.1 Special SP-Routine

Upon start of the program the Special SP-Routine is disabled. After being enabled via the corresponding checkbox, the **Special SP-**

Routine settings apply. The Special SP-Routine serves for **improvement of signal/noise** in measurements of all parameters involving the application of Saturation Pulses (SP).

Rationale: While Fo or F measurements can be disturbed by high measuring light (ML) intensities (unintended closure of PS II centers), Fm or Fm' measurements always profit from high ML intensity (closure of PS II centers by SP is intended; higher signal/noise ratio). With the help of the Special SP-routine ML intensity can be automatically increased during an SP. To avoid signal saturation, simultaneously the Gain setting is correspondingly decreased. The measured Fm or Fm' values are automatically corrected by the **Fm Normalization Factor,** which previously has to be measured for the selected settings (see under Measure chapter 10.5.13). The Fm Normalization Factor assures that Fm and Fm' values measured without and with Special SP-Routine are identical.

Fo and F determination: The ML-Int. and Gain settings for "normal" measurement of fluorescence yield (continuously monitored Ft) can be selected. Default values are ML3 G9 for MICROSCOPY- and ML2 Gain10 for MAXI-versions. Please note that the selected settings for ML-Int., Gain, ML-Frequency and Damping are equivalent to those displayed on the Settings-window. Any change of these settings on the High Sens. window will lead to a corresponding change on the Settings-window and *vice versa*.

Fm and Fm' determination: These ML-Int. and Gain settings apply during the SP only. Default values are ML20G1 for MICROSCOPY- and ML15G1 for MAXI-versions. At such high settings of ML-Intensity even weakly fluorescent samples give satisfactory images and at minimal Gain setting the noise is quite low.

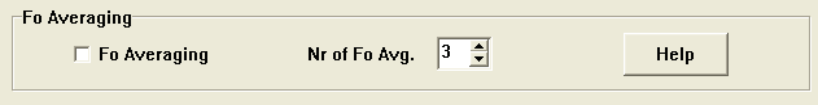
Measure: For measuring the **Fm Normalization Factor**, please replace sample by plastic **fluorescence standard** delivered with the instrument, define an AOI and press the Measure button. Then a

measurement of Fo and Fm with the selected Special SP-routine parameters is carried out. The normalization factor that gives Fm = Fo with the fluorescence standard is automatically calculated.

Please note that the correct Fm Normalization Factor depends on the current **Fm Factor** (see 10.5.13). The same Fm Factor that was effective during measurement of the Fm Normalization Factor, should also be effective when this is applied in conjunction with the Special SP-routine. In contrast the Fm Normalization Factor is not influenced by the **F Factor**, as an inert fluorescence standard different from a living sample does not show any ML pulse induced fluorescence increase. Therefore, during measurement of the Fm Normalization Factor the F Factor is automatically disabled.

NOTE: The user may check on the correctness of the current Fm Normalization Factor by carrying out a Fo, Fm measurement with the plastic fluorescence standard using the Special SP-Routine. As the fluorescence standard does not show any variable fluorescence, Fo and Fm should be close to identical.

10.6.2 Fo Averaging



Fo Averaging can be disabled/enabled via the **Fo Averaging checkbox**. When enabled, Fo averaging is applied in conjunction with every **Fo, Fm determination**. The progress of averaging can be followed on the Ft image (Image window).

The quality of the Fo images is enhanced by averaging over a number of images and by selecting a high **Damping** setting. Maximal **ML Frequency setting 8** is recommended to minimize the averaging time. Even at maximal frequency the time between ML

pulses is sufficiently long for reoxidation of reduced PS II acceptors. The time required for a single Fo measurement increases with Damping setting and amounts to 12 seconds at Damping 5 and ML Frequency 8.

No of Fo Avg.: Numbers between 1 and 5 can be selected. The time required for Fo determination is proportional to the number of averages (e.g. 3 x 12 = 36 seconds with setting 3 at Damping 5 and ML Frequency 8).

The current setting of No of Fo Avg. also applies for measurements of **RGB images** using the MICROSCOPY/RGB-version (see 10.7). This is true irrespectively of whether Fo Averaging is enabled or not.

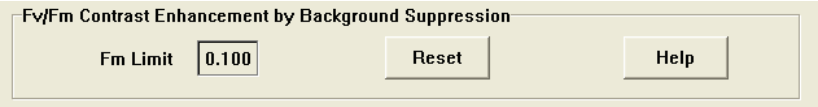
10.6.3 Fv/Fm Contrast Enhancement by Background Suppression

This function specifically applies to **Microscopy applications** and, hence, is active in the Microscopy-versions of the program only. It is used in the **View-Mode** (Measure-checkbox disabled). Please note that under Settings the **Yield Filter** checkbox must be enabled.

Rationale: In epifluorescence microscopy, images inevitably show background fluorescence from **scattered/reflected** light, as well as from non-focused areas. While this 'unwanted' fluorescence is usually weak and only disturbs Fo and Fm images to a small extent, it can have a significant impact on **Fv/Fm** and **Y(II)** images. These images are calculated using ratios of fluorescence values, meaning they lack information on the original signal amplitudes. This results in a loss of contrast, meaning that structures that can be clearly seen in Fo or Fm images may not be visible in Fv/Fm and Y(II) images. Fv/Fm pixels associated with low Fm pixel values can be eliminated by setting an appropriate Fm limit. This suppresses the disturbing

effect of a weakly fluorescing background and greatly enhances the contrast of Fv/Fm images. The same applies to Y(II) images.

Definition Fm Limit: For all pixels displaying FM values below this limit, the Fv/FM value will be displayed as black. The default Fm limit value is 0.100 and can be reinstated via the 'Reset' function.



A new Fm limit value can be entered by clicking on the “Fm limit value” field with the left mouse button. This opens a dialog box in which you can enter the new value and confirm it with “OK.”

10.7 RGB-Fit window

The RGB-Fit window is implemented for the MICROSCOPY-version with the Red-Green-Blue LED lamp IMAG-RGB only. It supports the automated measurement of RGB images and the consecutive deconvolution into three different pigment types, like diatoms, green algae and cyanobacteria. For this purpose, the RGB head uses a **red LED with an emission peak at 620 nm, a green LED (520 nm) and a blue LED with a peak at 460 nm**.

Rationale: Deconvolution of fluorescence images into **three different pigment types** is based on differences in **fluorescence excitation spectra**, similarly as with the PHYTO-PAM Chlorophyll Fluorometer. For example, cyanobacteria display maximal fluorescence yield with red-orange excitation (around 620 nm, phycocyanin absorption) and almost no fluorescence with blue excitation, as they are lacking Chl b and most of Chl a is associated with the weakly fluorescing PS I. On the other hand, chlorophytes and diatoms are characterized by strong fluorescence excitation by

blue light. While diatoms are effectively excited by green (525 nm) light, green light is distinctly less effective with green algae.

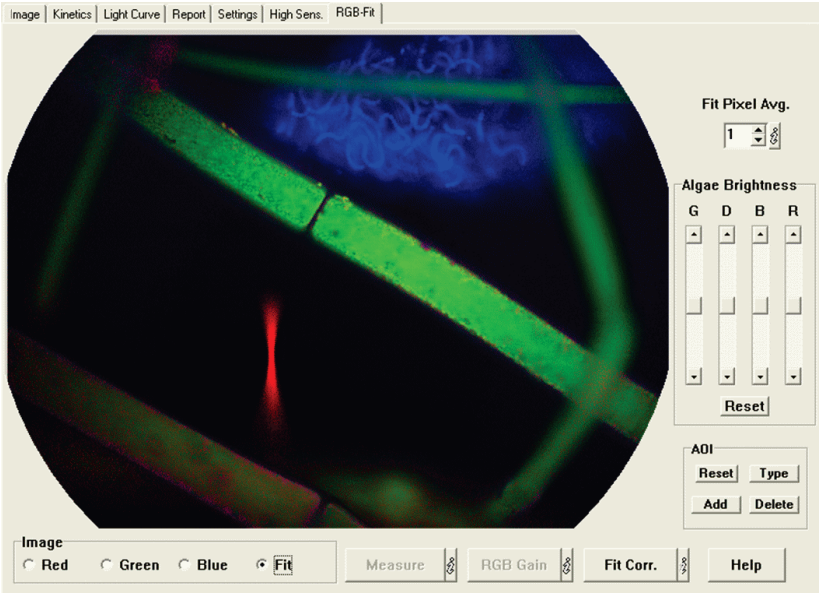


Fig. 92: RGB-Fit window showing RGB-Fit image with deconvoluted diatoms, chlorophytes and cyanobacteria

The Measure RGB routine serves for obtaining RGB images that can be deconvoluted for display of the four different algae groups (green algae, diatoms, blue-green algae and red algae) in false colors (green, yellow, blue and red, respectively) with the help of the preprogrammed fitting routine.

When the Measure button is pressed, automatically Red ML and Red Image Correction is installed and a Red image is measured. This measurement involves the averaging of Ft images over 15 s. Consequently, in the same way Green and Blue images are measured. The pixel intensity values of the obtained images differ from those of the Ft images, unless the current Red and Blue Gains are 1.000 (see info text on RGB Gain).

After RGB images are measured, the Fit image is calculated and displayed. The Fit image is influenced by the current Fit Corr. factors (see corresponding info text) and the current value of Fit Corr. Avg. (see corresponding info text). The intensity of a particular pixel is scaled according to the averaged intensity of the same pixel in the RGB images.

The time required for measurement of RGB images depends on the **Nr of Fo Avg**. set under **High Sens./Fo Averaging** (see 10.6.2). Please note that ML Frequency should be at maximal setting 8, as otherwise RGB measurements take very long. While measurements of RGB images formally are equivalent to Fo measurements, this does not mean, that the same low setting of ML Intensity (at relatively high Gain) is appropriate. Actually, it is recommended to apply **Gain 1**, where the **maximal signal/noise** ratio is obtained and to increase ML Intensity until sufficiently high fluorescence signals are observed. For this purpose, the user should view the Ft-image on the Image-window and manually switch between R, G and B excitation, in order to make sure that fluorescence signals are appropriate with all three excitation wavelengths.

10.7.1 RGB Gain

The pixel intensity values of the Red, Green and Blue images are derived from the pixel intensities of the corresponding Ft images multiplied by the Red, Green and Blue Gains.

Upon pressing the RGB Gain button a window appears that shows the current values of Red, Green and Blue Gains. While the Green Gain is fixed to 1.000, the Red and Blue Gains can be modified either manually or via a special Measure routine (see below). Upon instrument delivery Red and Blue Gains are set to 1.000, as the Red and Blue ML intensities are adjusted to give appropriate R/G and

B/G ratios for optimal fitting using the standard Axiostar Plus Epifluorescence microscope with the Fluar 20x/0.75 objective. With other lenses for optimal fitting the Gains have to be adjusted (see below).

Defined R/G and B/G ratios are a prerequisite for proper fitting (deconvolution of differently pigmented algae). Adjustment of RGB Gain may serve for compensating variations in RGB intensities. Such variations may occur with ageing of ML LEDs and/or Liquid Light Guide, choice of different objective lenses etc.

Note

The RGB Gain values have an influence on the RGB images measured for the purpose of RGB Fit.

10.7.2 Fit Correction

The fit conditions for deconvolution of the 4 major groups of algae are preprogrammed and are not directly accessible to the user. The Fit Correction offers an indirect way to modify the fit conditions.

The deconvolution into the 4 main groups of algae is based on fit conditions that relate to intensity ratios of individual pixels in the RGB images. These ratios can be modified by RGB Fit Correction factors. Fitting then is based on the correspondingly modified RGB images, with the pixel intensity values of the stored RGB images being multiplied by the RGB Fit Correction factors.

Upon pressing the Fit Corr. button, a window appears that shows the current values of Red, Green and Blue Fit Corr. factors. While the Green factor is fixed to 1.000, the Red and Blue factors can be modified by the user. Upon start of the program all factors are set to

1.000. In this respect the Fit Corr. factors differ from the RGB Gain factors, which are saved in the Ini-file (see info icon for RGB Gain).

Defined R/G and B/G ratios are a prerequisite for proper fitting (deconvolution of differently pigmented algae). In practice, even within the same group of algae there may be some variation of R/G and B/G signal ratios due to different pigment compositions and concentrations. Adjustment of Fit Corr. factors may serve for optimizing deconvolution by modifying the fitting conditions. This function can be also applied for analysis of saved data in the View mode. In this respect the Fit Corr. factors differ from the RGB Gain factors that can be applied in the Measure mode only.

Note

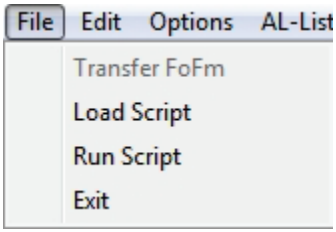
The Fit Corr. factors do not have any influence on the RGB images measured for the purpose of RGB Fit. This contrasts with the RGB Gain factors.

Definition of AOIs is fully equivalent on RGB-Fit and Image-windows (see also 10.1.2.2). With the help of the Fit image it is possible to define various AOIs that specifically represent particular organisms (e.g. a diatom attached to a filamentous green alga). Consequently the photosynthetic performance of these particular AOIs can be selectively analyzed by measuring various fluorescence parameters, recording Induction Curves (see 10.2) and measuring Light Curves (see 10.3).

11 ImagingWin - Menu Bar

The Menu Bar presents the menu File, Edit, Options, Al-List, Recalc and Transect including its functions as described in the following chapter.

11.1 File

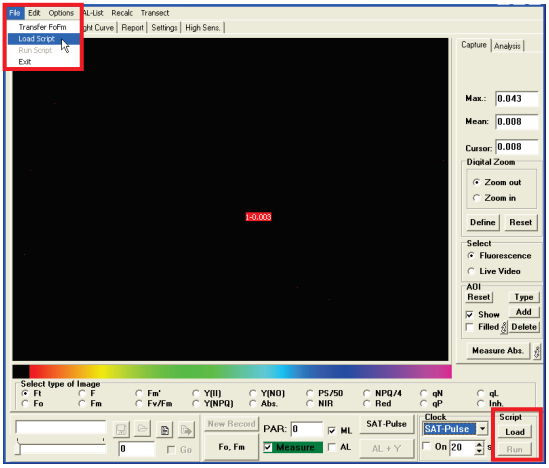


11.1.1 Transfer FoFm

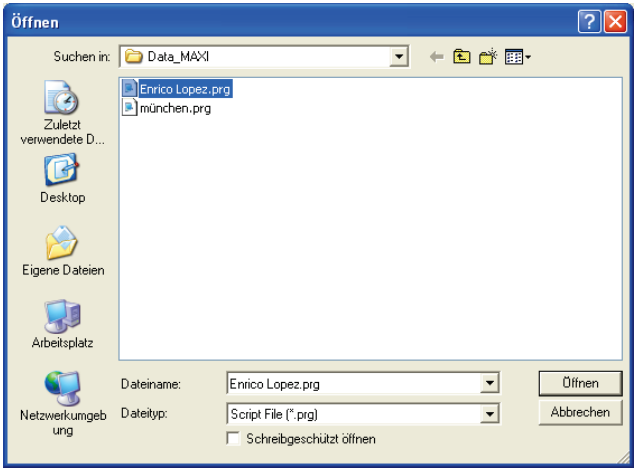
In the view mode a FoFm measurement can be transferred to another .pim file by enabling Transfer FoFm in the file menu. Data will be recalculated with the given parameter.

11.1.2 Using Skript files - Load Script/Run Script

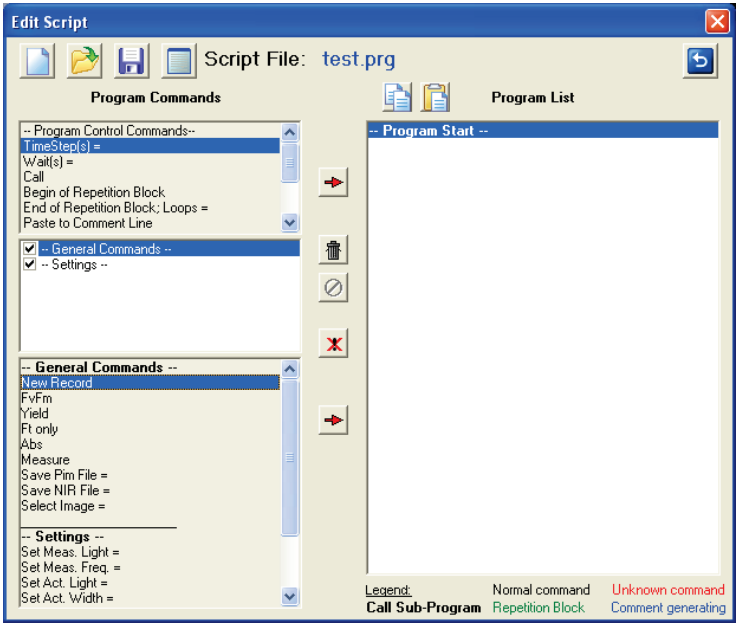
There are two ways to load an existing or to create a new script file. The load script command in the file menu or push the load script file button in the bottom right corner:



A new window opens up that shows the existing script files:



After choosing a file or entering a new file name and clicking “open” the editor is started:



Script File: Open, New, Save, Comment & Back

Four commands are provided for the management of script files

New Script File. The command clears the <Script File Window> and prompts for a new script file name.

Open Script File. Click to open a stored script file with name format <filename.PRG>. The default directory for script files is C:\ImagingPamGigE\Data_MAXI (or respective others depending on the instruments configuration). Other directories can be defined using the Windows graphical user interface.

Save Script File. Saves <Script File> to default or user defined directory.

Script File Comment. Clicking this icon displays the content of an editable text file called <filename.TXT> which is associated with the script file <filename.PRG>.

Editing Tools & Back

Copy Command. The command stores one or several lines of the current script file into a RAM clipboard. To execute the copy command, select one or several lines using the mouse cursor (Left click once to pick one line). Hold down <Shift> key and select first and last line of a series of script file commands to select a group of connected lines. Hold down <Ctrl> key to select several scattered lines. Click <Copy Command> icon. The selected commands are now available for pasting within the current or into another script file using the <Paste Command>.

Paste Command. To paste previously copied commands, select a line in the target script file and click the <Paste Command>. The pasted lines will be written below the selected line.

Insert Command. To insert a new command in the program list, mark by a left mouse click a command line in the <Script File Window>, and then select by left click in the <Command Box> the command to be inserted. Clicking the <Insert

Command> icon will place the new command below the line marked in the program list.

Delete Command. To delete a command, select one or several commands as described above and click the <Delete Command> icon.

Undo Delete Command. Clicking the icon reverses the last <Delete Command>.

Disable/Enable Command. To disable commands in the current script file, or enable previously disabled commands, select commands as described above and click the <Disable/Enable Command> icon.

In this editor on the left side the possible commands are visible, on the right-hand side the program listing can be seen.
A command can be chosen by clicking on it. The transfer into the script listing is done by double click on the command or by marking it and a further click on the red arrow between the both windows.

It is useful to firstly define the basic starting settings of the experimental script like „Set Meas. Light =“ until „Set Sat. Interval =“ with which also „personal settings“ can be defined. After the script has been processed the settings stay at the set values so that further changes can be done by another script or additional manual settings.

For an automated experiment after the basic settings the experimental settings can be defined:

Programm Commands:

TimeStep(s)	determines a waiting time after the last command. This command implements the time needed for the previous command (maybe 800 ms of the FvFm). Using TimeStep the distortion of a very long script can be avoided.
Wait(s)	determines a waiting time after the last command independently of the command's duration.
Call	runs another script file as sub-program, returns to the running script when the sub-script ends.
Begin of Repetition Block	initiates a loop – enter the name of the loop and the number of repetitions. Each loop produces one or more lines in the report so that also the maximum amount of lines in the report must be taken into account (max length of the report is 999 lines) – with e.g. each saturating flash a new line in the report is written.
End of Repetition Block	terminates a loop. Here the number of repetition is defined.
Paste to Comment Line	enters a remark to the comment line of the report file.
Message =	opens a message window, pauses the script till the message is confirmed by the user

Remark =	to write a remark in the script file. This remark is not stored in the report file unless the command "Paste to Comment Line" is chosen.
Spacer	provides an empty row to the script file
Exit	quits IMAGING-WIN

General Commands:

New Record	generates a new record
FvFm	determines the max quantum yield
Yield	starts one saturation pulse (SP) for the measurement of the effective quantum yield
Ft only	starts a measurement of Ft only
Abs	starts an absorptivity measurement
Measure	switches the measuring light ML on
Start Light Curve	starts a Light Curve
Start Induction Curve	starts an induction curve
Stop Induction Curve	stops the induction curve
Save pim file	saves the recorded pim file under a given name
Save NIR file	saves the recorded NIR file under a given name

Export to Tiff File= saves all images as tiff file

Export to CSV File=saves report as csv file

Select Image =	to select exportable image

Save Tiff Image = export selected image as tiff file

Save Jpeg Image = export selected image as jpeg file

General Settings:

Set Gain = sets gain:
= No to set gain to a desired value
+ or - No to raise or decrease gain by a desired number of steps

Set Damping = sets damping:
= No to set damping to a desired value
+ or - No to raise or decrease damping by a desired number of steps

Light Settings:

Set Meas. Light = sets measuring light (ML) intensity:
= No to set the measuring light intensity (ML) intensity to a desired intensity step
+ or - No to raise or decrease measuring light intensity by a desired number of steps

Set Meas. Freq. = sets the measuring light frequency

Set Act. Light = sets actinic illumination (AL) intensity:
= No to set the actinic illumination (AL) intensity to a desired intensity step (No equals intensity step of the light list)
+ or - No to raise or decrease actinic illumination intensity by a desired number of steps

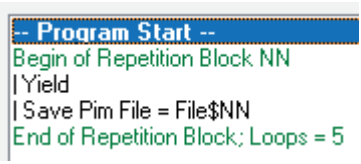
Set Act. Width = sets actinic illumination (AL) width:
= No to set the actinic illumination (AL) width to a desired duration (in seconds)
+ or - No to raise or decrease actinic illumination width by a desired number of seconds

Set Sat. Light = sets saturation pulse intensity:
= No to set the saturation pulse intensity to a desired value
+ or - No to raise or decrease saturation pulse intensity by a desired value

Following the whole procedure can be copied by marking the respective command lines in the listing window and copying with the  button. The button  transfers the copied lines to the lines under the cursor position.

The diskette symbol button saves the script file with the given name and the ending ".prg" in the data folder of the PAM instrument.

Counting of e.g. exported Images



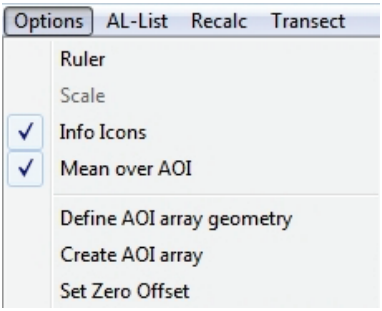
This is a new feature in the ImagingPam software. If images, files or other data shall continuously be stored by the ImagingPam software during the experiment, this option can be used. In the example on (left) the syntax can be seen. Continuous storing of e.g. images makes it necessary to count up their file names, otherwise the software would just overwrite the older file.

Generally, counting is only possible within a repetition block (which means that the “save …” command must be nested between the “Begin Repetition Block …” command and the “End Repetition Block …” command. In this case the repetition block is called NN (it is just a name for the loop variable and can freely be chosen). It must only be preceded by a $ symbol. With the sample script one would get 5 jpg files in the folder C://ImagingPAMGigE/Data_...

11.1.3 Exit

Exit quits ImagingWin.

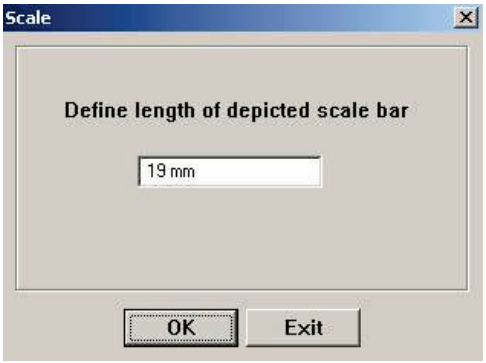
11.2 Options



11.2.1 Ruler

The Ruler, which shows numbers from 0 to 100, is placed above the false color scale ranging from 0 to 1. Hence, the ruler may help to estimate the pixel value of a particular color. For example, yellow corresponds to pixel values around 0.2.

11.2.2 Scale



After clicking this option, the Scale window is opened in which the length of the depicted scale bar can be defined by the user. In the case of the MAXI-version, for the standard working distance (18.5 cm) a standard scale width of 19 mm is proposed, which in

principle can be modified by the user. When confirmed via clicking O.K., the scale width is written underneath the Scale bar. Please note that the Scale Option can be applied in the View-mode only. Different standard scale widths are proposed when using the MINI-version (5 mm) and MICROSCOPY-version (0.1 mm). In the case of the MICROSCOPY-version the scale depends on the particular microscope and the choice of objective lens. Users are advised to determine themselves the correct scale for their optical system. The modified scale width will be saved when the program is closed.

11.2.3 Info Icons

If Info Icons is enabled, information text is available clicking the info buttons.

11.2.4 Mean over AOI

Two different modes for analysis of the pixel values within an AOI can be selected under Options.

1) When "Mean over AOI" is enabled, the "Filled" checkbox applies.
2) When "Mean over AOI" is disabled, the "Limits" checkbox applies.

"Mean over AOI" normally should be enabled with objects for which AOIs with close to uniform photosynthetic activity can be defined (e.g. leaf or well filled with algae suspension).

"Mean over AOI" should be disabled when the photosynthetically active object is "patchy", so that it is difficult or impossible to define an AOI with photosynthetic activity (e.g. patches of algae growing in a well).

Definitions:

Mean over AOI enabled: The pixel values over the whole area of the AOI are averaged and the average pixel value is displayed.

Mean over AOI disabled: Within the defined AOI only those pixel values are averaged for which Fv/Fm > 0. Hence, the displayed value corresponds to areas with photosynthetic activity only.

Filled: When enabled, the whole area of the AOI is "filled" with the color corresponding to the average pixel value, which works only when "Color" is selected under Settings/Display. When disabled, each pixel is displayed with the color corresponding to its individual pixel value.

Limits: When enabled, the pixel values in the photosynthetically active areas are averaged, whereas the non-active areas are displayed in black. In this way the limits of the photosynthetically active areas with respect to the non-active areas are emphasized.

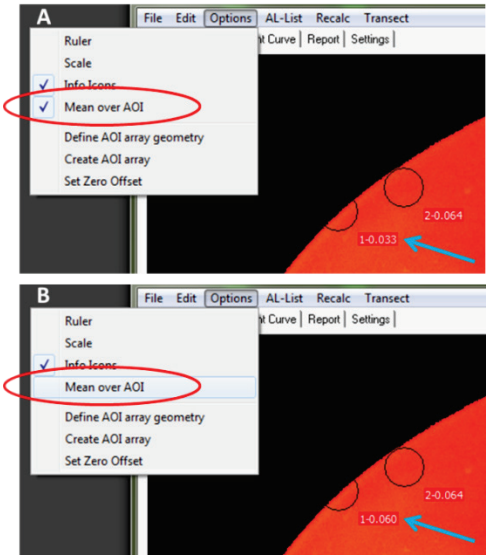
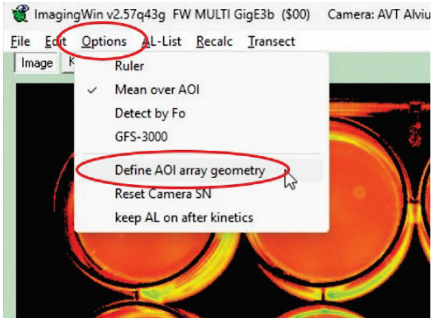


Fig. 93: Mean over AOI enabled (A) and disabled (B)

11.2.5 AOI patterns and groups

In the new software (ImagingWin release version 2.57q43h and higher), the workflow for creating AOI arrays has been redesigned. The reason for this was to improve the handling of large numbers of circular AOIs (the maximum number has been increased to 400 AOIs). The following steps still only work with circular AOIs of the same size as those used for multiwell plates.

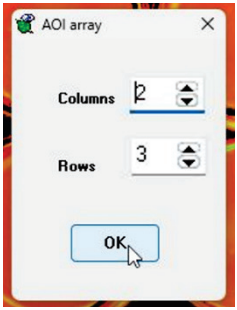
In the newer software versions, AOI patterns can now be generated independently of the position of the multiwell plate. In addition, each pattern generated is assigned to a new group. This makes it possible to activate and display entire groups of AOIs later in View Mode. This makes work easier, especially when AOI groups need to be examined in advance for differences in their behavior.



To set the first group of AOIs, proceed as follows:

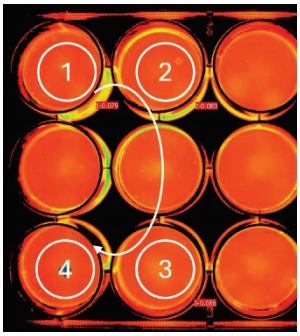
Under “Options,” select the “Define AOI Array” function with a mouse click.

A new window will open in which you can define the number of columns and rows (in the example image, we initially only want to create one AOI group on the left side of the plate, so only half of the available wells will be assigned AOIs).



Once this is done, click OK to go directly to the mode where you can manually define the positions of the corner AOIs.

Make sure to start in the upper left corner and then click on the other corners in a clockwise direction (shown in the following image).



After the last click, the missing AOIs are automatically added at equal distances. If the plate was slightly tilted when the corner points were defined, this is also taken into account.

In the example, a group with 6 AOIs was then created (this first group is then automatically annotated with “A”).

If you do the same with the other wells, a second group of AOIs is generated in another AOI array. And the window may appear as shown in the following image.

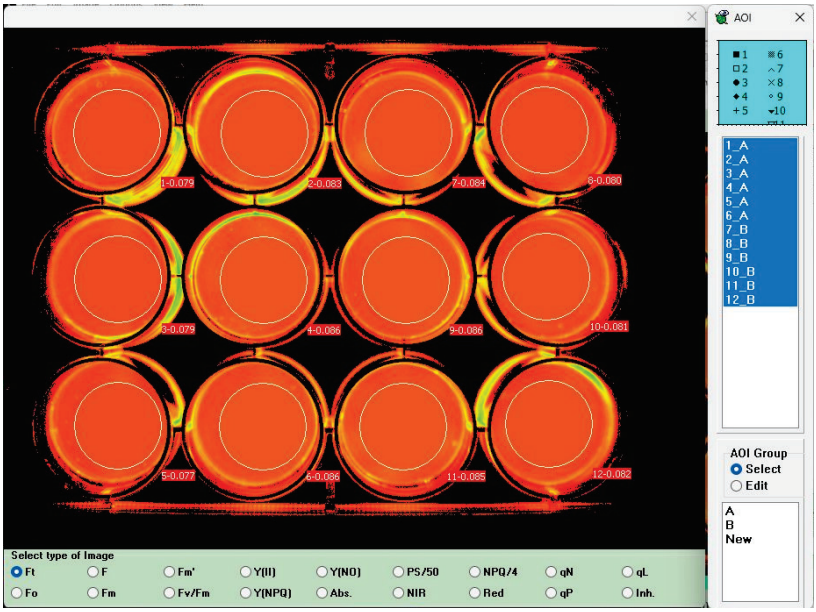
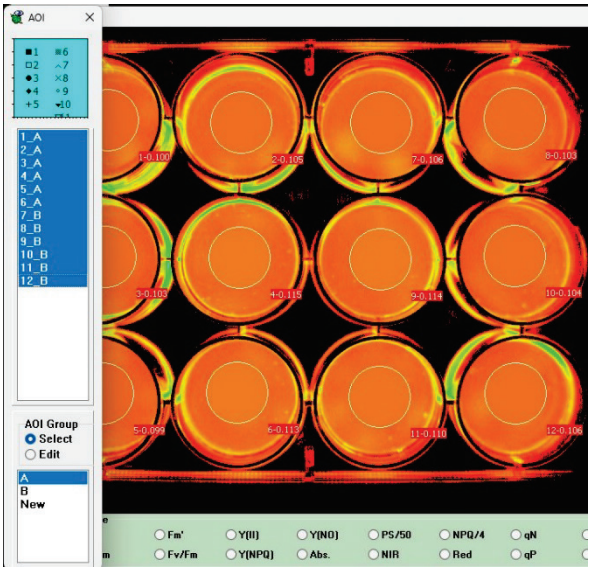


Fig. 94 Automatically generated AOI pattern consisting of AOIs assigned to two groups (A and B). The AOI window shown on the right opens after clicking the “AOI” button, which is located at the top right of the “Kinetics,” “Light Curve,” and “Report” tabs.

11.2.6 AOI window

The AOI button can be found in the upper right part of the “Kinetics,” “Light Curve,” and “Report” tab.

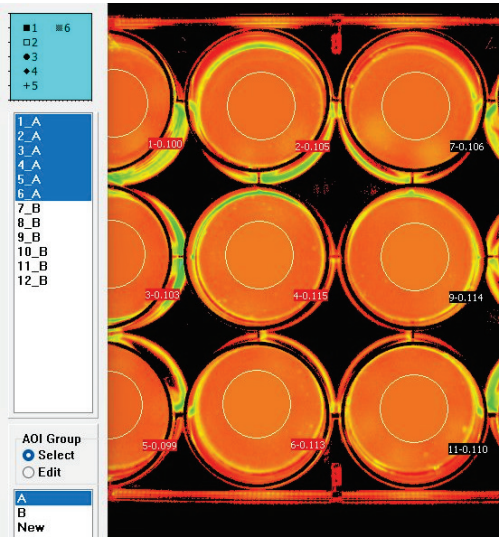
When the “AOI” button is pressed, a new window opens in which AOIs can be activated and deactivated. In the graphics windows, symbols are assigned to the AOIs. This link can be seen in this window. In addition, since software version 2.57q43, this window also shows which groups the respective AOIs are assigned to and allows you to create new AOI groups and edit existing groups. Users of older ImagingWin software versions can find a description of the older AOI window here: 10.3.



After opening the AOI window Once you have opened the AOI window, the symbols for all active AOIs will be visible in the top section. Below that, there is a list of all defined AOIs, including their serial number and group letter. AOIs that are highlighted in color are active and are also displayed in the 'Kinetics', 'Light Curve' or 'Report' tabs. This makes it easier to keep track of data, especially when there are a lot of AOIs.

Individual AOIs can now be manually set to visible or invisible in the AOI window. Alternatively, entire groups can be set to invisible in the “AOI Group” section of the window.

In the “AOI Group” area, there are as many AOI letters as there are defined groups (in the example, A and B). If one of the letters is clicked, it is highlighted in color, is active, and is therefore visible in other tabs.



By clicking on “New,” a new group (C in alphabetical order) can be created. To do this, the “Edit” function must then be activated.

In the third step, the AOI to be renamed must be clicked. This AOI is now assigned to a new group. In this way, AOIs can also be moved between existing groups. Their sequential number remains unchanged.

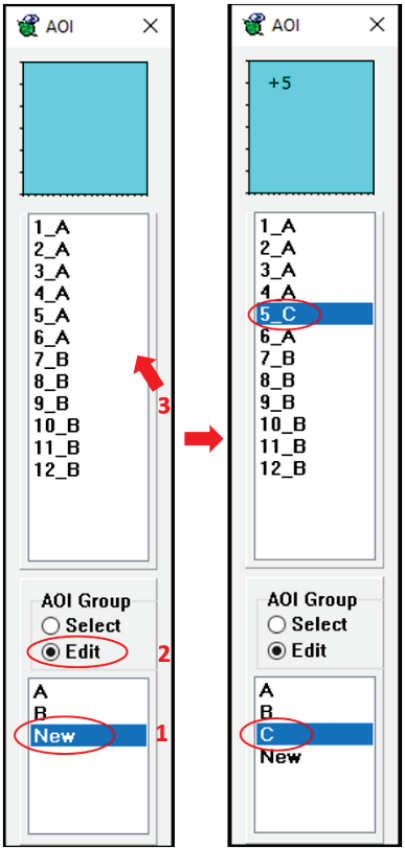
In the “Image” tab, you can also see that the formerly red flags, which display the running number of the AOI and the respective value, change their background color from red to black (when they are inactive and therefore invisible).

The “Edit” function:

To assign one or more existing AOIs to a completely new group, please select the "New" function by left clicking on the button in the lower part of the AOI window.

Now ◉ Edit can be chosen.

If you now click on an AOI from the upper AOI window area, it gets a new group name (here "C" because the groups "A" and "B" already exist, and the next letter of the alphabet is always used).



In the example shown the AOI 5 (member of group "A") shall be assigned to a completely new group "C" (shown in the image one right side. Edit stays active and the new group "C" is listed. From here on, more AOIs can be re-assigned by simply clicking them.

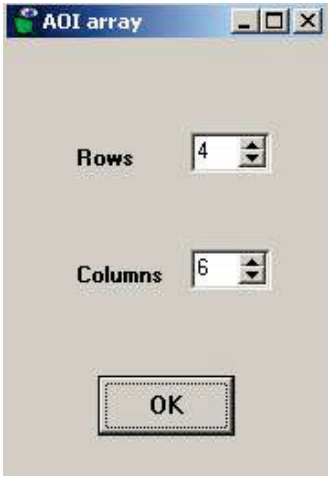
The new group can now be selected to be shown in Kinetics or Report tabs. Only one group can be selected at a time, but after highlighting a whole group some more AOIs of other groups can be highlighted manually.

For security reasons, renaming AOIs can only be done in one group. If AOIs shall be assigned to another group (e.g. by clicking "B", The software will automatically switch back to "Select" and thus the edit process has to start from the beginning.

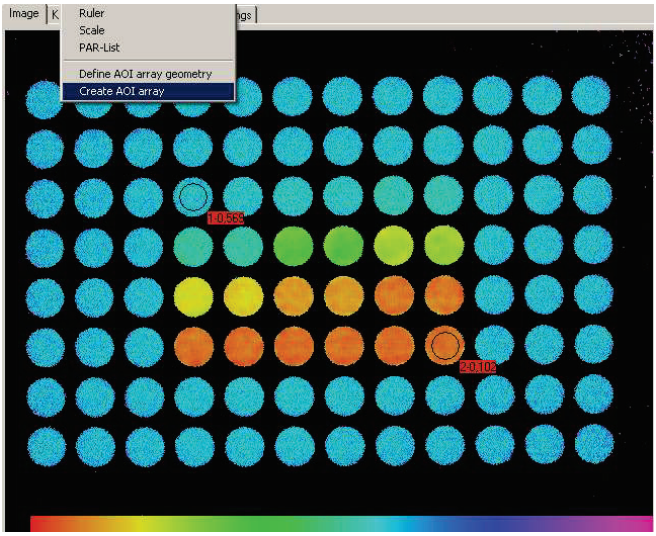
By double-clicking into the middle section of the AOI window, all AOIs can be selected.

11.2.7 AOI array definition and workflow (older software versions)

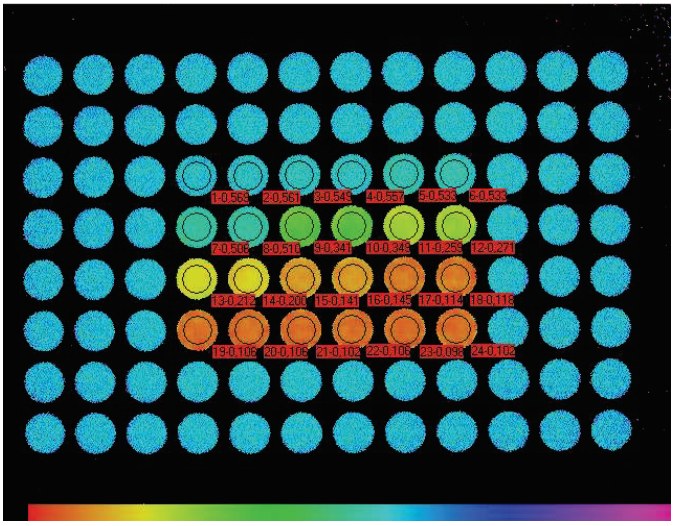
Up to software version 2.56zn, the following workflow can be used to define a group of AOIs, e.g., for the evaluation of multiwell plates. It is important to note that these AOIs are set in a regular distribution and therefore the samples to be measured must also be aligned exactly horizontally in the image. The functions described can be found under “Options” in the top menu bar. Up to 100 AOIs can be defined for such a pattern so that a whole 96 well plate can be covered.



Clicking 'Define AOI array geometry' (under “Options”) opens a window in which the number of rows and lines of the array grid can be defined. This definition will remain valid until a new one is carried out, once confirmed with 'OK'. Before an AOI array can be created, the position of the array within the overall image must be defined. This is achieved by defining the AOIs in the **top left** and **bottom right** corners of the array. After the basic setting a pattern can be placed onto the image.



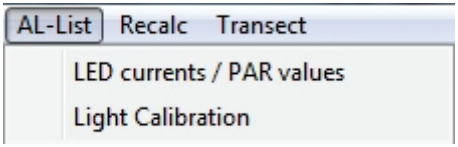
After the two corner AOIs are defined, the **Create AOI-array Option** can be carried out and the corresponding array will be displayed after some delay (calculation time).



In the above example, an array of 24 AOIs, arranged in four rows and six lines, was defined in the center of a 96-well microwell plate.

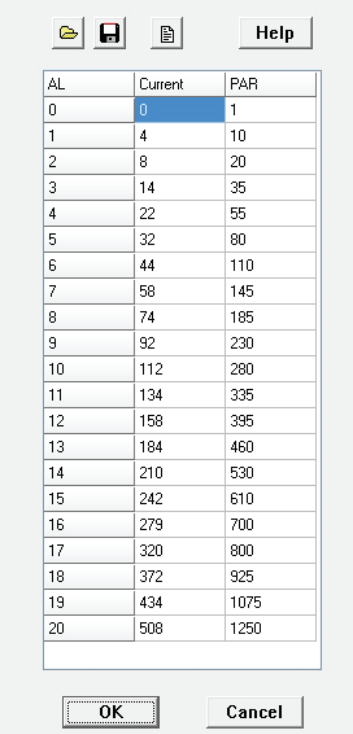
While all wells were filled with algae suspension, only the 24 wells covered by the array contained increasing concentrations of the PSII inhibitor diuron, as reflected by decreasing Y(II) values.

11.3 Al-List



In the AL-List menu actinic light lists can be loaded, viewed and edited via LED currents / PAR values. In combination with an ULM-500 Light Meter & Logger also a Light Calibration routine is available. Measured AL lists are stored in the data file of the corresponding measuring head (e.g. C:\ImagingPamGigE\Data_MAXI)

11.3.1 LED currents / PAR values



LED currents / PAR values opens up the window displayed on the left.

"Open PAR-file" loads an existing .par file.

"Save as PAR-file" saves the displayed PAR values in a .par file and gives the opportunity to save a comment file in the context of this PAR-list.

"Show comment file" displays the information stored in conjunction with the opened .par file.

The Help button provides further information.

The PAR-List shows the LED current and PAR values for the Actinic Light intensity settings 0 - 20. **LED Current as well as PAR values may be edited.** Possible Current values range from 1 to 511. Changes in LED current values result in changes in LED light intensity. PAR values are listed in **µmol quanta m^{-2} s^{-1}**. Editing the PAR values does not change the LED intensities, it changes the annotation of the actinic Light intensity. Different lists apply to the various Measuring Heads. In the case of the MAXI-version, the Default PAR-List refers to the standard working distance of 18.5 cm between LED-Array Illumination Unit and sample plane. The listed values can be edited by the user and the edited list confirmed by clicking O.K. A “default.par” PAR-List for each measuring head version, determined

at the factory under standard conditions, is stored in the corresponding Data folder.

While the **relative values** of Actinic Light intensities 0 - 20 do not vary between individual instruments, there may be some variation between instruments in terms of **absolute intensities** due to different charges of LEDs. Absolute Intensities can be determined in conjunction with an ULM-500 Light Meter & Logger by the light calibration routine described in the next chapter.

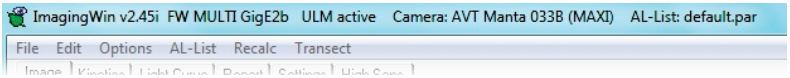
In microscopy applications the PAR does not only depend on the LED intensity, but also on the choice of objective lens. Using the RGB-Head also the LED color has to be taken into account. Calibration can be done using an ULM-500 with the micro quantum sensor (e.g. MC-MQS) or the use of relative instead of absolute PAR values may be recommended. In any case, when dealing with microscopically small organisms, there is a large difference between incident and absorbed PAR.

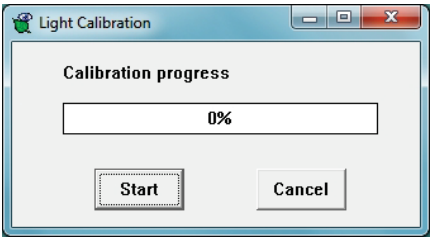
When dealing with leaves, it has to be considered that as a consequence of pronounced light gradients within the samples, the effective PAR at a particular setting does not correspond to one value, but rather to band of values with Gaussian distribution around a center value. Therefore, differences between incident and effective PAR values may be expected. On the other hand, as most of the measured fluorescence originates from the leaf surface, the observed light response will be dominated by the upper cell layers where the incident intensity is effective. These aspects have to be considered when comparing apparent electron transport rates derived from fluorescence measurements (ETR) with gas exchange rates. In contrast to chlorophyll fluorescence, the gas exchange response involves deeper cell layers with effective PAR being attenuated relative to incident PAR.

11.3.2 Light Calibration

Light Calibration requires an ULM-500 Light Meter & Logger with a micro quantum sensor e.g. LS-C for the MAXI- and MINI- version or MC-MQS for the MICROSCOPY-version.

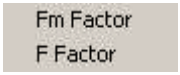
Please connect ULM-500 to the computer before starting ImagingWin. "Ulm active" appears in the window title to show enabled ULM-500 - ImagingWin communication.





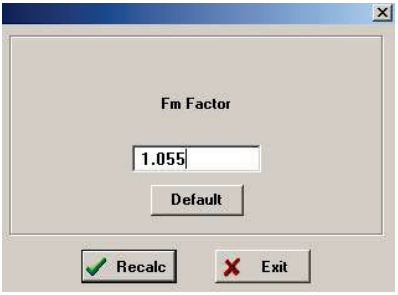
Clicking Light Calibration in the Al-List menu opens up a Light Calibration window. Please place the micro quantum sensor at the object´s position and start the automated calibration routine for the 20 light intensities by pressing Start.

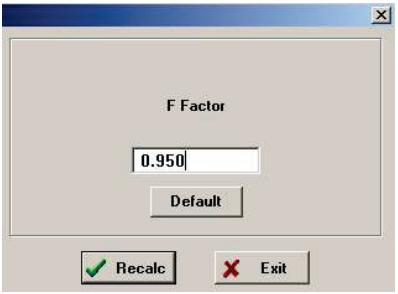
11.4 Recalc



With the help of the **Recalc Option** it is possible to recalculate the data of a given Record on the basis of correction factors for Fm (or Fm') and F as already described in the sections on **Fm Factor** (10.5.13) and **F Factor** (10.5.14). Recalculation is always for the whole Record. It can be carried out in the View- as well as in the Measure-mode. In the Measure mode, however, this definition has to be done before the first measurement (normally Fo Fm determination). If the user tries to change the F factor after Fo, Fm-determination, there is a

corresponding warning: **"For changing Fm factor start new record"** or **"For changing F factor start new record".**





Recalculation is started upon clicking the **Recalc button**. When the **Default button** is clicked, the data are recalculated on the basis of the Default value of the Fm Factor = 1.055 or the Default value of the F Factor = 0.950. As the Recalculation is carried out in the View-mode, it is irrelevant whether under Settings the Fm Factor and F Factor checkboxes are enabled or disabled.

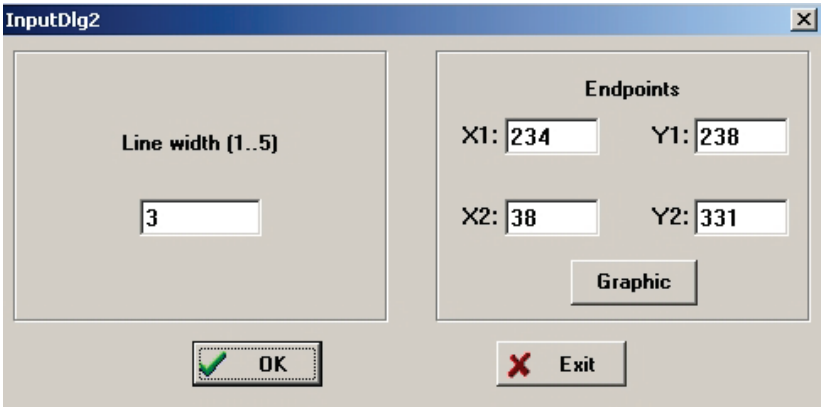
The possibility of recalculation of previously recorded data in the View-mode is particularly helpful, if at the time of the actual measurements there is uncertainty about the proper values of Fm and F Factors. In the case of Light Curves, for example, the data may be recalculated based on various Fm Factors to obtain a reasonably shaped saturation curve. A biphasic response curve without apparent saturation suggests that the applied Fm Factor is too high. If the response curve shows a maximum followed by a decline, this normally suggests that the applied Fm Factor is too low. Unless rather long illumination times at high intensity settings are applied, a genuine decline of ETR by photoinhibition is unlikely.

Application of a F Factor in conjunction with a Light Curve is problematic and cannot be recommended. As F values are increasing with PAR, the relative increase of F due to the individual ML pulses

(see 10.5.14) will decline. Therefore, application of a constant F Factor is bound to result in overestimation of ETR at high PAR. If Light Curves were recorded with a F Factor being enabled, the data can be readily recalculated with F Factor = 1.

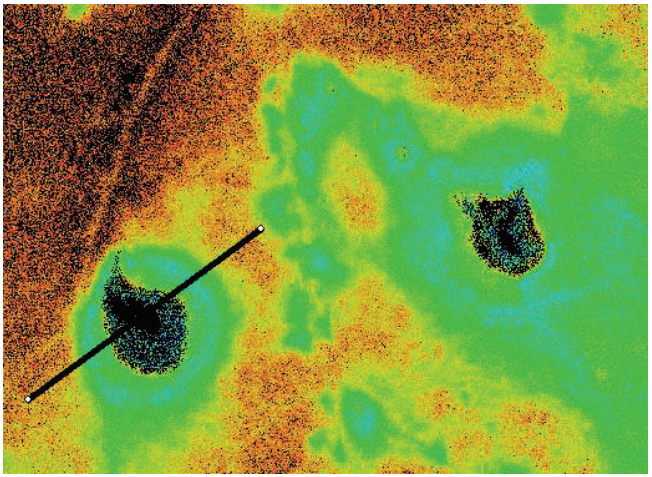
11.5 Transect

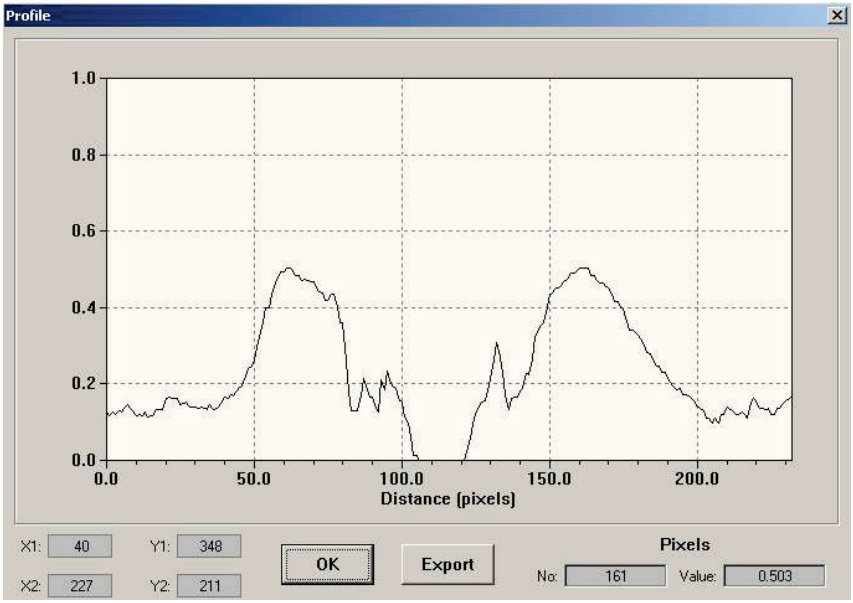
The **Transect Option** allows to plot the pixel values of an imaged parameters along a previously defined line segment. In this way a profile of this parameter across a sample can be obtained.



Upon clicking **Define Endpoints** a dialog window is opened in which the user may choose one out of 5-line width**s** and define the coordinate**s** of the two endpoints of the line segment. With increasing line width, the signal/noise ratio of the Transect plot is increased. The coordinates of endpoint 1 (X1, Y1) and endpoint 2 (X2,Y2) can be manually entered into the corresponding boxes. Then upon O.K. the Transect profile is displayed. If the coordinates of the envisaged endpoints are not known, as normally the case, the endpoints can also be defined graphically. When the **Graphic button** is clicked, in the Image window a line segment appears, one of the endpoints of which temporarily is fixed, while the other one can be freely moved

with the cursor (marked by a cross) until it is fixed by a left mouse click. Now the other end is marked by the cursor and can be freely moved to the second endpoint position, which can be fixed by another left mouse click. Then automatically the Transect profile is calculated and displayed in a separate window.





The Pixel values of the selected Image parameter are plotted as a function of Pixel number along the line segment. In the lower left corner of the window the **x-y coordinates** of the two endpoints of the transecting line segment are displayed. They may serve for reproducing an identical transect later. In the lower right corner the **Pixel value** and **Pixel number** of the point marked by the cursor are displayed. When the **Export button** is clicked, a list of all Pixel value/Pixel number couples is saved in form of the file **profile.csv** into the **Data directory** of the applied Measuring-Head, from where it can be exported into a spread sheet program, like **Excel**. If Excel is installed on the PC, the export.csv file is automatically opened under Excel. Please note that the export.csv file will be overwritten with the following data export. To avoid this it should be renamed.

12 List of key commands

The IMAGING-PAM normally is operated via the ImagingWin user surface by cursor and mouse click operations. However, also some key commands are possible which in certain instances may be used as shortcuts. All key commands require simultaneous pressing of the Ctrl-key:

Ctrl A	Actinic Light on/off
Ctrl I	Open Image window
Ctrl K	Open Kinetics window
Ctrl L	Open Light Curve window
Ctrl M	Fo, Fm determination
Ctrl Q	Switch between Measuring Light Frequency 1 and 8
Ctrl R	Open Report window
Ctrl S	Open Settings window
Ctrl V	Switch to Live Video mode
Ctrl Y	Yield determination by Saturation Pulse

13 Technical specifications

13.1 Components used in all Versions

13.1.1 Control Unit IMAG-CG

Control Unit IMAG-CG

Design:	Aluminum housing featuring large size built-in Li-ion battery, sockets for cable connections with CCD Camera IMAG-K6 or IMAG-K7, connectors for the MAXI-, MINI- MICROSCOPY-Measuring Heads, an external light source (EXT) and Battery Charger 2120-N
Microcontroller:	RISC processor
User interface:	For Windows 10 and 11 PCs with ImagingWinGigE software versions; connection is via GigE ethernet
Power supply:	Internal rechargeable Li-ion battery 14.8 V/5.2 Ah
Power consumption:	9 W (500 mA) drawn from internal Li-Ion battery
Recharging time:	approx. 4 hours (IMAGING-PAM turned off) via Battery Charger 2120-N
Operating temperature:	-5 to +45 °C
Dimensions:	25 cm x 10.5 cm x 11 cm (L x W x H)
Weight:	2.1 kg (incl. battery)

13.1.2 IMAG-K7

Design:	Black and white C-mount camera operated in 10-bit-mode at 16 frames/sec
CCD Chip size:	1/2" (640 x 480 pixels)
Interface:	GigE-Vision®
Dimensions:	8.64 cm x 4.4 cm x 2.9 cm (L x W x H) (without lens)
Weight:	< 200 g

13.1.3 IMAG-K6

Design:	Black and white C-mount camera operated in 10-bit-mode at 16 frames/sec featuring 2 x 2 pixel binning
CCD Chip size:	2/3" (1392 x 1040 pixel primary resolution resulting in 640 x 480 after binning)
Interface:	GigE-Vision®
Dimensions:	8.64 cm x 4.4 cm x 2.9 cm (L x W x H) (without lens)
Weight:	< 200 g

13.1.4 IMAG-K9

Design:	Black and white C-mount camera operated in 11-bit-mode at 16 frames/sec featuring 2 x 2 pixel binning
CCD Chip size:	2/3" (1392 x 1040 pixel primary resolution resulting in 640 x 480 after binning)
Interface:	GigE-Vision®
Dimensions:	2.9 cm x 2.9 cm x 4.1 cm (L x W x H) (without lens)
Weight:	65 g

13.1.5 Windows Software ImagingWin

Minimum PC requirements: processor 1.7 GHz, 4 GB free RAM, built-in Gigabit Ethernet (GigE), Windows Vista, Windows 7, 8 or 10

Features: Data display and instrument settings on up to 7 different windows

- Image: display of 18 different parameters
- Kinetics: time dependent changes of fluorescence parameters
- Light Curve: registration of preprogrammed light response curves
- Report: numerical lists of parameter values for selected areas of interest
- Settings: instrument settings
- High Sens.: routines for enhanced signal/noise and contrast (MAXI- and MICROSCOPY-versions)
- RGB-Fit: deconvolution of 3 different pigment types (e.g. diatoms, chlorophytes and cyanobacteria) (MICROSCOPY/RGB-version)

3D point cloud measurement (only with IMAG-3D). Combination of PSII data with surface mesh, additional separate storage of surface mesh files in Polygon File Format (ply)

13.1.6 Battery Charger 2120-N

Input: 90 to 264 V AC, 47 to 63 Hz
Output: 19 V DC, 3.7 A
Operating temperature: 0 to 40 °C
Dimensions: 15 cm x 6 cm x 3 cm (L x W x H)
Weight: 300 g

13.2 Components specifically relating to Maxi-version

13.2.1 LED-Array Illumination Unit IMAG-MAX/L

Design: LED-Array mounted on printed-circuit board in aluminum housing with central opening for CCD Cameras IMAG-K6 or IMAG-K7 with miniature ventilator and cable connections to Control Unit IMAG-CG and External 300 W Power Supply (chapter 13.2.4)

Light source for fluorescence excitation and actinic illumination: 44 royal-blue 3 W Luxeon LEDs (450 nm) equipped with individual collimating optics; standard excitation intensity 0.5 µmol/m²s PAR, modulation frequency 1-8 Hz; max. actinic intensity 2300 µmol/m²s PAR; max. saturation pulse intensity 5000 µmol/m²s PAR

Light sources for assessment of absorbed PAR: 16 red LEDs (660 nm); 16 NIR LEDs (780 nm)

Working distance: standard 18.5 cm for 9 cm x 12 cm image area; at 22.5 cm distance 11 cm x 15 cm image area (mounted independently of IMAG-MAX/GS on separate stand)

Light field properties: Vertical incidence on sample; LED distribution optimized for uniformity; at standard working distance maximal deviation from mean intensity +/- 7 %

Dimensions: 18.5 cm x 18.5 cm x 4.5 cm (L x W x H)

Weight: 1.3 kg (incl. cable 1 m long)

13.2.2 LED-Array Illumination Unit IMAG-MAX/LR

Design: LED-Array mounted on printed-circuit board in aluminum housing with central opening for CCD Cameras IMAG-K6 and IMAG-K7, filter plate IMAG-MAX/FR and miniature ventilator and cable connections to Control Unit IMAG-CG and External 300 W Power Supply (chapter 13.2.4)

Light source for fluorescence excitation and actinic illumination: 44 red 3 W Luxeon LEDs (650 nm) equipped with individual collimating optics; standard excitation intensity 0.5 µmol quanta m^{-2} s^{-1} PAR, modulation frequency 1-8 Hz; max. actinic intensity 1900 µmol/m²s PAR; max. saturation pulse intensity 3700 µmol/m²s PAR

Light sources for assessment of absorbed PAR: 16 red LEDs (660 nm); 16 NIR LEDs (780 nm)

Working distance: standard 18.5 cm for 9 cm x 12 cm image area; at 22.5 cm distance 11 cm x 15 cm image area (mounted independently of IMAG-MAX/GS on separate stand)

Light field properties: Vertical incidence on sample; LED distribution optimized for uniformity; at standard working distance maximal deviation from mean intensity +/- 7 %
Dimensions: 18.5 cm x 18.5 cm x 4.5 cm (L x W x H)
Weight: 1.3 kg (incl. cable 1 m long)

13.2.3 Optional filter plate IMAG-MAX/F (only for IMAG-MAX/L!)

Design: Black-anodized aluminum plate with 44 individual blue filters to be mounted in front of collimating optics of IMAG-MAX/L
Filter properties: 1 mm blue-green glass filters (BG 39, Schott) blocking red transmission and passing 90 % of blue light
Dimensions: 186 mm x 176 mm x 2.5 mm (L x W x H)
Weight: 180 g

13.2.4 External 300 W Power Supply

Input: 90 to 264 V AC, 50/60 Hz
Output: 43 to 57 V, 5.2 A
Operating temperature: 0 to 40 °C
Dimensions: 226 mm x 110 mm x 58 mm (L x W x H)
Weight: 1.75 kg

13.2.5 K7-MAX/Z

Design: Cosmicar-Pentax zoom lens (F1.0/f=8-48 mm) and close-up lens, extension ring, detector filter (RG645, 3 mm) and short

	pass interference filter (λ < 770 nm); adapter-frame for mounting CCD-camera IMAG-K7 at increased working distance.
Dimensions:	57 x 95 mm
Weight:	430 g (without adapter-frame)
Filter Screw Size:	φ55 mm, P=0.75 mm
Mount:	C-Mount
Focal Length:	8 to 48 mm
Max. Aperture Ratio:	1 : 1.0 (f=8 to 28) to 1.2 (f=48)
Iris Range:	F/1.0 to F/22
Temperature Range:	-20°C to +50°C

13.2.6 K7-MAX/S

Design:	Cosmicar-Pentax lens (F1.2/f=12 mm), extension ring and detector filter (RG645, 3 mm) and short-pass interference filter (λ < 770 nm).
Dimensions:	30 x 35.5 mm
Weight:	67 g
Filter Screw Size:	M27 x 0.5
Mount:	C-Mount
Focal Length:	12 mm
Iris Range:	F/1.2 to F/22
Temperature Range:	-20°C to +50°C

13.2.7 K6-MAX

Design:	Cosmicar-Pentax lens (F1.4/f=12.5 mm), detector filter (RG665, 3 mm) and short-pass interference filter (λ < 770 nm).
Dimensions:	42 x 50 mm

Weight:	135 g
Filter Screw Size:	M40.5 x 0.5
Mount:	C-Mount
Focal Length:	12.5 mm
Iris Range:	F/1.4 to C
Temperature Range:	-20°C to +50°C

13.2.8 K9-MAX

Design:	Kowa lens (F1.4/f=16 mm), detector filter (RG645, 3 mm) and short-pass interference filter (λ < 770 nm).
Dimensions:	42 x 50 mm
Weight:	155 g
Filter Screw Size:	M35,5 x P0,5
Mount:	C-Mount
Focal Length:	16 mm
Iris Range:	F/1.4 to F/16
Temperature Range:	-10°C to +50°C

13.2.9 K9-MAX/Z

Design:	Ricoh zoom lens (F1.8/f=12.5–75.0 mm), detector filter (RG645, 3 mm) and short-pass interference filter (λ < 770 nm).
Dimensions:	51 x 90 mm
Weight:	320 g
Filter Screw Size:	49 x 0,75
Mount:	C-Mount
Focal Length:	16 mm
Iris Range:	F/1.4 to F/22
Temperature Range:	-20°C to +50°C

13.2.10 K6-MAX/M and K7-MAX/M

Design: mounting set IMAG-K6 or IMAG-K7 camera on IMAG-MAX/L or IMAG-MAX/LR. Consisting of camera holder and metal rod 15 cm length, 15 mm diameter.

13.2.11 Mounting Stand with Eye Protection IMAG-MAX/GS

Design: Aluminum frame featuring two clamps for mounting LED-Array Illumination Unit IMAG-MAX/L or IMAG-MAX/LR; red Perspex sliding hood for eye protection; removable bottom part

Sample position: Detached leaves, slides or petri dishes resting on x-y stage for variable positioning; special frame for defined positioning of multiwell plates; after removal of bottom part, possibility to jack up whole Mounting Stand for study of plants in pots or trays

Dimensions: 23.5 cm x 24.5 cm x 22.5 cm (L x W x H)

Weight: 3.1 kg

13.2.12 IMAG-MAX/B

Design: Aluminum frame featuring two clamps for mounting LED-Array Illumination Unit IMAG-MAX/L or IMAG-MAX/LR

Sample position: Detached leaves, slides or petri dishes resting on x-y stage for variable

	positioning; fix working distance 18.5 cm in a plane perpendicular to the optical axis.
Dimensions:	18.8 cm x 17.8 cm x 20.4 cm (L x W x H)
Weight:	455 g

13.2.13 ST-101

Design:	Laboratory stand with wooden baseplate for mounting LED-Array Illumination Unit IMAG-MAX/L or IMAG-MAX/LR
Dimensions:	40 cm x 30 cm x 73 cm (L x W x H),
Weight:	2.8 kg

13.2.14 Transport Box IMAG-MAX/T

Design:	Aluminum box with custom foam packing for MAXI-IMAGING-PAM and accessories
Dimensions:	60 cm x 40 cm x 25 cm (L x W x H)
Weight:	5 kg

13.2.15 IMAG-MAX/GWK1

Design:	Adapter Plate with legs and eye protection for positioning IMAG-MAXI Head on 3010-GWK1
Dimensions:	18.5 cm x 20 cm 17 cm (L x W x H)
Weight:	856 g

13.3 Components specifically relating to IMAG-3D version

13.3.1 IMAG-3D

Design:	Closed aluminum housing featuring a sliding front door and side flaps. Two clamps take up LED-Array Illumination Unit IMAG-MAX/L. Takes up an LED projector on an arm mounted on rear side.
Dimensions:	42 cm x 23.5 cm x 45 cm (L x W x H w/o cables)
Weight	11.2 kg (only with directly mounted components – IMAG-MAX/L, projector, rotary disc, USB dongle, cables, projector power supply).
Computer IMAG-3D/PC:	Recent Dual- or Quad-Core Intel CPU with min 4GB RAM and NVDIA GPU
Camera:	IMAG-K6 (see also chapter 13.1.3) For 3D measurement the IMAG-K6 uses its full resolution (without binning) it switches back to 2-pixel binning mode for PSII measurements.
IMAG-3D/ANA	not available anymore

13.4 Components specifically relating to MINI-version

13.4.1 IMAG-MIN/B

Design:	12 Luxeon LEDs 460 nm with individual short pass filters and collimator optics; 16 red 650 nm and 16 NIR 780 nm LEDs for measuring PAR-absorptivity; max. actinic intensity, 3700 µmol/m²s; max. Saturation Pulse intensity, 8200 µmol/m²s; frame at

	fixed working distance (7 cm); for imaging 24 mm x 32 mm sample area; suitable for use in combination with IMAG-K6 and IMAG-K7 with camera accessories (objective lenses).
Dimensions:	11.8 cm x 9,4 cm x 8.6 cm (L x W x H)
Weight:	552 g (incl. cable)

13.4.2 IMAG-MIN/R

Design:	12 Luxeon LEDs 620 nm with individual short pass filters and collimator optics; 16 red 650 nm and 16 NIR 780 nm LEDs for measuring PAR-absorptivity; max. actinic intensity, 2800 µmol/m²s; max. Saturation Pulse intensity, 7700 µmol/m²s; frame at fixed working distance (7 cm); for imaging 24 mm x 32 mm sample area; suitable for use in combination with IMAG-K6 and IMAG-K7 with camera accessories (objective lenses).
Dimensions:	11.8 cm x 9,4 cm x 8.6 cm (L x W x H)
Weight:	552 g (incl. cable)

13.4.3 IMAG-MIN/GFP

Design:	12 Luxeon LEDs 480 nm with individual short pass filters and collimator optics; 16 red 650 nm and 16 NIR 780 nm LEDs for measuring PAR-absorptivity; max. actinic intensity; frame at fixed working distance

	(7 cm); for imaging 24 mm x 32 mm sample area; only suitable for use in combination with IMAG-K6 with camera accessories K6-MIN, K6-MIN/FS and K6-MIN/M.
Dimensions:	11.8 cm x 9,4 cm x 8.6 cm (L x W x H)
Weight:	552 g (incl. cable)

13.4.4 K7-MIN

Design:	Cosmicar-Pentax objective lens (F1.4/f=16 mm) detector filter (RG645, 3 mm) and short-pass interference filter (λ < 770 nm), extension ring 4.2 mm
Dimensions:	30 x 33 mm
Filter Screw Size:	M27 x 0.5
Mount:	C-Mount
Focal Length:	16 mm
Iris Range:	F/1.4 to 22
Temperature Range:	-20°C to +50°C
Weight:	58 g

13.4.5 K6-MIN

Design:	Cosmicar-Pentax objective lens (F1.4/f=25 mm) detector filter (RG645, 3 mm) and short-pass interference filter (λ < 770 nm), extension ring 7.2 mm
Dimensions:	30 x 37.3 mm
Filter Screw Size:	M27 x 0.5
Mount:	C-Mount

Focal Length: 25 mm
Iris Range: F/1.4 to 22
Temperature Range: -20°C to +50°C
Weight: 76 g

13.4.6 K6-MIN/FS

Design: for parallel imaging of GFP- and Chl fluorescence of identical sample areas (instead of camera objective accessory detector filter RG645, 3 mm and short-pass interference filter $\lambda < 770$ nm). Featuring alternative detector filters for green or red fluorescence (special green filter set 500 - 575 nm and red filter RG665, 3 mm with short-pass interference filter $\lambda < 770$ nm).

13.4.7 K7-MIN/M and K6-MIN/M

Design: devices for mounting camera IMAG-K6 or IMAG-K7 to the Imaging MINI-Heads

Weight: 166 g

13.4.8 IMAG-S

Design: Fine drive laboratory stand with high performance rack-and-pinion drive (50 mm traverse path) for adjustment of working distance; platform base with covered 9.3 cm central hole for mounting optional sample holders

Weight: 3.34 kg

13.4.9 IMAG-MIN/ST

Design: Fine drive tripod adapter for mounting a MINI-Head onto a tripod head with UNC 1/4-20 screw threads, High performance rack-and-pinion drive (120 mm traverse path) for adjustment of working distance

13.4.10 ST-1010

Design: Compact Tripod, for tripod heads with UNC 1/4-20 screw threads.

13.4.11 IMAG-MIN/BK

Design: Leaf clip mountable on sample frame of all MINI-Heads; MINI-Head holding grip

Weight: 184 g

13.4.12 IMAG-MIN/GFS

Design: Adapter plate with snap-on-mount for connecting IMAG-MINI Head to Standard Measuring Head 3010-S,

Dimensions: 9.5 cm x 6 cm x 1.4 cm (L x W x H)

Weight: 30 g

13.5 Components specifically relating to MICROSCOPY-versions

13.5.1 IMAG-AXIOSCOPE

Design: Modified AxioScope.5 Microscope (Zeiss) adapted for IMAGING PAM applications.

Comprises binocular phototube (30°/23 100:0/0:100), condenser 0,9/1,25H and transmitted light unit HAL 50. Detector filter RG665, dichroic mirror 420-640 nm, video adapter 60N-C 2/3" 0,5x and standard lens Fluar 20x are already mounted.

13.5.2 IMAG-L470M5

Design: Microscope LED Lamp Module 470 nm (blue) for fluorescence excitation of Chl fluorescence of most algae groups including cyanobacteria. Emission peak at 470 nm. Shipped together with a set of neutral grey filters for using the system together with higher magnifications.

13.5.3 IMAG-L625M5

Design: Microscope LED Lamp Module 625 nm (red-orange) for fluorescence excitation of Chl fluorescence of most algae groups including cyanobacteria. Emission peak at 625 nm. Shipped together with a set of neutral grey filters for using the system together with higher magnifications.

13.5.4 IMAG-RGB

Design: Red-Green-Blue Microscopy LED Lamp allowing computer-assisted deconvolution of major algae groups. Fluorescence

excitation and actinic illumination using red (620 nm), green (520 nm), blue (460 nm) or white light (mixed 620, 520 and 460 nm); featuring fluid light guide (100 cm length, 3 mm active dia.), connecting to collimator optics at excitation port of epifluorescence microscope; with cable to be connected to RGB output socket at IMAG CG; featuring printed circuit board with separate drivers for RGB LEDs;

13.5.5 IMAG-AX-REF

Design: Reflector Module with filter set for additional microscope LED lamp modules. Consisting of a beam splitter filter (420-640 nm) and a detector filter (665 nm), mounted in a Zeiss reflector module frame

Technical specificatlions are subject to change without prior notice.

14 Guarantee

14.1.1 Manufacturers Guarantee

Under this Manufacturer's Guarantee ("Guarantee"), subject to the Conditions and Instructions below, Heinz Walz GmbH, Germany ("Manufacturer"), guarantees (§443 BGB) to the end customer and user ("Customer") that all products supplied by it shall substantially conform in material respects to the Specifications for 24 months from the delivery date (date on invoice). In this Guarantee, "Specifications" means the product's features (as may be amended by Manufacturer from time to time), which are set out under the headings "specifications" and/or "technical specifications" within the product's respective brochure, data sheet, or respective tab on the Manufacturer's website for such product, and which may be included with the documents for the product when delivered. In case of an eligible guarantee claim, this Guarantee entitles the Customer to repair or replacement, at the Manufacturer's option, and this Guarantee does not include any other rights or remedies.

14.1.2 Conditions

This Guarantee shall not apply to:

- Any defects or damage directly or indirectly caused by or resulting from the use of unauthorized replacement parts and/or service performed by unauthorized personnel.
- Any product supplied by the Heinz Walz GmbH, Germany which has been subjected to misuse, abuse, abnormal use, negligence, alteration or accident.
- Damage caused from improper packaging during shipment or any acts of God.
- Batteries, cables, calibrations, fiberoptics, fuses, gas filters, lamps (halogen, LED), thermocouples, and underwater cables.

- Defects that could reasonably have been detected upon inspection of the product when received by the Customer and not promptly noticed within ten (10) days to Heinz Walz GmbH.
- Submersible parts of the DIVING-PAM or the underwater version of the MONITORING-PAM have been tested to be watertight down to the maximum operating depth indicated in the respective manual. Guarantee shall not apply for diving depths exceeding the maximum operating depth. Further, guarantee shall not apply for damage resulting from improper operation of devices, in particular, the failure to properly seal ports or sockets.

14.1.3 Instructions

- To obtain guarantee service, please follow the instructions below:
- The Walz Service Information Form available at https://www.walz.com/support/repair-service.html must be completed and returned to Heinz Walz GmbH, Germany.
- The product must be returned to Heinz Walz GmbH, Germany, within 30 days after Heinz Walz GmbH, Germany has received written notice of the defect. Duty and taxes are covered by WALZ; please instruct your forwarder and mark waybill accordingly.
- Transport costs to WALZ must be paid by the customer. In case of guarantee repairs, all further transport costs will be covered by WALZ. In case of non-guarantee repairs, the customer is responsible for all shipping charges.
- All products being returned for guarantee service must be carefully packed and sent freight prepaid.
- Heinz Walz GmbH, Germany is not responsible or liable for missing components or damage to the unit caused by handling during shipping. All claims or damage should be directed to the shipping carrier.

14.1.4 Applicable Law

This Guarantee is governed by German law. Place of jurisdiction is Bamberg, Germany.

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