

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). In order to avoid harm to your eyes, please avoid looking directly into this light, particularly during Saturation Pulses.

2 Introduction

The IMAGING-PAM Chlorophyll Fluorometer is specialized for the study of spatial heterogeneities of PSII activity. The Imaging-PAM M-series covers a wide range of applications. Large scale samples with areas exceeding multiwell plate format can be imaged as well as microscopically small samples at the level of single cells. The new IMAG-3D version additionally offers the 3D scan of a samples for further improvement of accuracy.

IMAG-3D version, MAXI-, MINI- and MICROSCOPY-versions have been issued that are operated with the same Multi Control Unit IMAG-CG. Like all PAM fluorometers, the Imaging-PAM applies pulse-amplitude-modulated measuring light for assessment of chlorophyll fluorescence yield. The same LEDs not only serve for generation of the pulse-modulated measuring light, but also for actinic illumination driving photosynthesis and for Saturation Pulses transiently saturating energy conversion at Photosystem II (PS II) reaction centers. The Saturation Pulse method provides a non-destructive means of analyzing the photosynthetic performance of plants. It allows to assess the quantum yield of energy conversion at PS II reaction centers, which is affected by numerous intrinsic and environmental parameters, like the physiological health, light conditions and various stress factors. Since the introduction of PAM fluorometry in 1985, a large amount of literature has been published on the practical use of this method in many fields of plant science. Also with all versions of the IMAGING-PAM M-series the characteristic fluorescence levels F_0 , F_m and F_m' can be assessed and quenching coefficients derived. Also, the PS II quantum yield F_v/F_m (or $\Delta F/F_m'$) can be determined and Induction Curves as well as Light Saturation Curves with quenching analysis can be measured.

The most essential new information provided by chlorophyll fluorescence imaging relates to the detection of lateral heterogeneities of fluorescence parameters which reflect physiological heterogeneities. It has been known for some time, that even physiologically healthy leaves are "patchy" with respect to stomatal opening. Furthermore, stress induced limitations, which eventually will lead to damage, are not evenly distributed over the whole leaf area. Fluorescence imaging may serve as a convenient tool for early detection of such stress induced damage. Hence, favorite fields of application of fluorescence imaging are plant stress physiology and plant pathology. An outstanding feature of the Imaging-PAM distinguishing it from standard PAM fluorometers is the possibility of parallel assessment of several samples under identical conditions. For this application the MAXI-version is particularly well suited, e.g. for the screening of mutants in plant molecular biology and for the assessment of samples in multi-well plates, e.g. 96-well plates in ecotoxicological studies. The MINI-version is particularly compact and easy to handle and therefore best suited for field applications. MINI- and MAXI-versions also can be readily adapted to the GFS-3000 Gas Exchange Measuring System for simultaneous measurements of parameters like CO₂ assimilation, Water evaporation and many more parameters.

The new 3D-version is based on the MAXI-Imaging head that has been advanced by a 3D scanner to produce a point cloud of the samples plant which are then processed to morphological data and for the correction of the measured PSII parameters.

The MICROSCOPY-versions provide the opportunity of imaging 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 types, like diatoms, chlorophytes and cyanobacteria.

The MAXI- and MINI- and 3D-versions not only measure fluorescence yield, but also provide an estimate of incident light absorptivity (not available for MICROSCOPY version). This aspect is particularly important when dealing with lateral heterogeneities of chlorophyll content often accompanying stress- or pathogen-induced damage. For assessment of absorptivity of the incident photosynthetically active radiation (PAR), the same sample is irradiated with diffuse near-infrared (NIR) and red light, the remitted parts of which are imaged with the same CCD-camera which serves for fluorescence imaging.

As the different versions of the Imaging-PAM M-series are optimized for largely different sample sizes, they apply quite different LED sources. While the same methodology applies for all versions, there are very different power requirements for appropriate intensities of measuring, actinic and saturation pulse light. The MAXI-IMAGING-PAM is equipped with an extremely powerful array of 44 high power (3 W) Luxeon LEDs, each of which is driven with currents up to 1.6 A. The same type of LEDs is also used in the MINI-IMAGING-PAM (featuring 12 LEDs illuminating 24 x 32 mm area) and various versions of the MICROSCOPY-IMAGING-PAM (with a single LED coupled to the excitation port of Zeiss AxioScope A1 Epifluorescence Microscopes). Therefore, the same Power-and-Control Unit (IMAG-CG) can be used for these different measuring heads. The new IMAG-CG also features an output for controlling a special RGB-LED-Lamp that is equipped with its own LED drivers.

As the available versions of the Imaging-PAM M-series put different demands on the sensitivity, two different CCD-cameras are employed. Two different CCD-cameras are available for the MAXI- and MINI-version. For high sensitivity applications (e.g. phytotoxicity bioassay using multiwell plate with algae suspensions) a 2/3" CCD camera (1392 x 1040 pixel with 4-pixel-binning) is

recommended, which is also generally used with the MICROSCOPY-versions. For standard applications an economical 1/2" CCD camera (640 x 480 pixel) is available, which is particularly useful in conjunction with a powerful zoom objective lens.

This manual provides some essential information on the components of the different versions of the Imaging-PAM M-series and on the ImagingWin software. While the latter in principle applies to all versions, it also offers some special features that can be used with particular versions only (e.g. RGB-Fit images using the MICROSCOPY/RGB-version or the new 3D version). As it is most frequently used and also featuring the widest range of applications/configurations, there is some emphasis on the MAXI-version in 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 multiwell plates or petri dishes) is the Filter Plate IMAG-MAX/F, which absorbs the small fraction of red light contained in the blue LED light.
- 3) CCD Cameras IMAG-K7 or IMAG-K6 with accessories and mounting sets
- 4) Mounting Stand with Eye Protection IMAG-MAX/GS, laboratory stand ST-101 or Leaf Distance Holder IMAG-MAX/B
- 5) PC with ImagingWin-software

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 Transport Box IMAG-MAX/T is provided.



In the LED-Array Illumination Unit an Adapter Ring may be inserted, which is not screwed. To avoid damage to the instrument, please do not take it out of the box by using this hole as a handle.

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 processor microcontroller, the power supply for the CCD camera and the 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). The Control Unit 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.



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



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

Fig. 2: Front and rear side views of the Control Unit IMAG-CG

POWER

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

CHARGE-LED Charge LED lights up red while battery is charged with the help of the Battery Charger 2120-N. When the battery is fully charged, the LED lights up green.

CHARGE Socket for connecting Battery Charger 2120-N. An external 12 V battery cannot recharge the internal 14.4 V Li-ion battery.



Please 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.

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- Heads as well as the IMAG-L470M or IMAG-L625M LED lamps of the MICROSCOPY-version

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

Ext. out Socket for connecting an optional external light source

When using MINI-Head, the camera can directly be mounted on the top of the Control Unit housing. For this purpose, a wing-screw is provided.

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

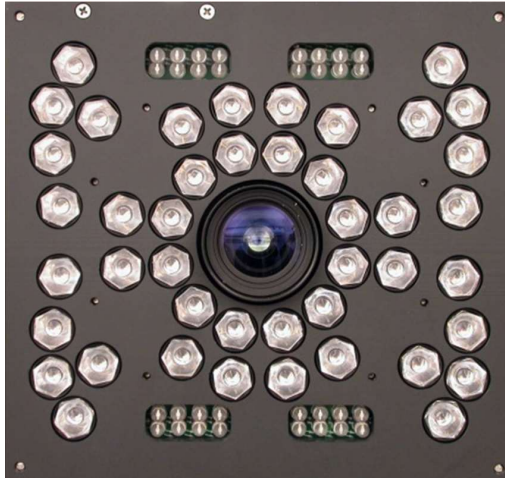


Fig. 3: Front view of IMAG MAX/L with objective lens of CCD-camera protruding through central opening

The LED-Array Illumination Unit IMAG-MAX/L features 44 high-power royal-blue (450 nm) LED-lamps equipped with collimating optics, which are arranged for maximal intensity and homogeneity at 17 - 20 cm distance to the object plane. These LED-lamps provide the pulse-modulated blue excitation light and at the same time serve for actinic illumination and Saturation Pulses. In addition, there are 4 groups of 8 LEDs providing the pulse modulated light for assessment of PAR-Absorptivity (see 10.1.1.10). These LEDs are arranged in pairs, with each pair featuring a red (660 nm) and a near-infrared (780 nm) LED. The lenses of these LEDs are removed in order to obtain homogenous illumination of the sample. While only a relatively small amount of this light is remitted from the sample to the CCD-camera, this is sufficient to give good signals,

as both wavelengths can pass the red long-pass filter in front of the CCD-chip, in contrast to the blue excitation light.

A useful accessory for measurements with mirroring samples (like multiwell plates filled with algae suspensions) is the Filter Plate IMAG-MAX/F that absorbs the small fraction of red light contained in the blue LED light. This plate can be mounted with 4 screws at the front side of the Illumination Unit. Unavoidably, the effective PAR-values are lowered by about 15% by the filtering.

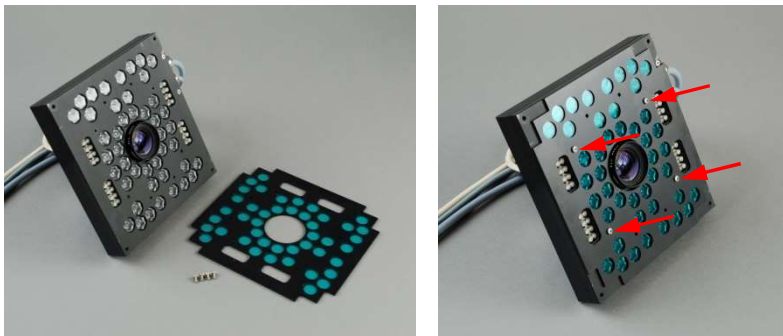


Fig. 4: Mounting of IMAG-MAX/F. Four screws are provided with the filter plate

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 .

Both Illumination Units feature two cables, which connect to the MAXI-HEAD socket at the front side of the Control Unit IMAG-CG and to the external 300 W power supply.



Fig. 5: IMAG MAX/L in conjunction with IMAG MAX/GS and mounted CCD camera IMAG MAX/K6

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 \varnothing metal bar, which may serve for mounting the Measuring Head independently of the Mounting Stand with Eye Protection (IMAG-MAX/GS) (see Fig. 6). This bar is fixed with a hex-nut screw, for which a corresponding key is provided. Please note that the nut should press against the flattened side of the bar.



Fig. 6: Measuring Head consisting of LED-Array Illumination Unit and CCD-camera mounted via 15 mm \varnothing metal bar on optional stand (Stand with Base Plate ST 101)



When used without Mounting Stand and Eye protection, the user should avoid looking directly into the LED-Array Illumination unit.

The user is urgently advised to ensure an alternative eye protection, which is effective in applications where the standard IMAG-MAX/GS cannot be applied.

When mounted independently from 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 imaged areas between 7.5 x 10 and 11 x 15 cm. Optimal light field homogeneity is obtained at the standard distance of 18.5 cm (imaged area 9 x 12 cm), with +/- 7% maximal deviation of intensity from the mean value.

A fixed standard distance of 18.5 cm is provided when the IMAG-MAX/L is mounted on the IMAG-MAX/GS (see 3.5). As the LED-intensity is defined by the ImagingWin software, the photon flux density (PAR) at this standard distance is well defined. Small variations in the PAR-distribution as well as the unavoidable vignetting effect of the camera objective lens can be measured and corrected for by software (see Image Correction in section 10.5.5). Three different Image Corrections are supported by the ImagingWin software for three different distances: Type 1, Type 2 and MAXI (chapter 10.5.5).

Note:



In the LED-Array Illumination Unit an Adapter Ring may be inserted, which is not screwed. To avoid damage to the instrument, please do not pull the IMAG-MAX/L it out of the box by using this hole as a handle.

3.3 CCD Camera IMAG-K6 and objective K6-MAX



Fig. 7: CCD Camera IMAG-K6

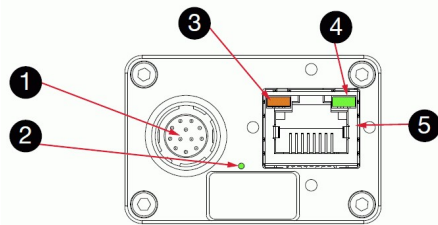


Fig. 8: CCD Camera IMAG-K6: 1 Hirose I/O port; 2 LED #3; 3 LED #1; 4 LED #4; 5 GigE interface

The CCD Camera IMAG-K6 (Allied Vision Technologies) features a 2/3" chip with 1392 x 1040 pixel. The data are digitized within the camera and transferred via ethernet interface (GigE-Vision®) to the PC. The sockets for connecting the GigE-cable as

well as the camera-control-cable (round-shaped connector) are located at the rear side of the camera.

As the CCD-chip features 1392 x 1040 pixel in contrast to the resulting 640 x 480 pixel of the ImagingWin fluorescence images, the signals of 4 pixel are combined (2 x 2 pixel binning), thus providing outstanding sensitivity. This feature is particularly useful for reliable assessment of the "dark fluorescence" parameters F_o , F_m and F_v/F_m .



The aperture of the IMAG-K6 standard lens can be closed. This increases the depth of focus but decreases the signal intensity. For plane objects, the setting of the aperture should always be completely open.

The camera is equipped with a metal angle bar (black anodized), which serves for mounting it on the corresponding adapter on the IMAG-MAX/L Illumination Unit. Mounting the IMAG-K6 camera for use in conjunction with MINI- or MICROSCOPY- Version are described in the corresponding chapters. If the camera is shared between different M-series instruments and setups, it might be necessary to adapt camera objective and the distance ring between objective and the CCD chip.

A Cosmincar-Pentax objective with 12.5 mm focal length ($F=1.4$) is used in conjunction with the LED-Array Illumination Unit IMAG-MAX/L or IMAG-MAX/LR (Fig. 9). A 3 mm RG 665 (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 - 10.1.1.14). 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 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 MICROSCOPY-Version and the GFP MINI-Version this filter has to be removed before mounting the camera.



Fig. 9: Cosmicar-Pentax objective 12.5 mm focal length, short pass filter and mounting device (left), distance ring (middle)

3.4 CCD Camera **IMAG-K7** and objectives **K7-MAX/Z** and **K7-MAX/S**



Also, the CCD Camera **IMAG-K7** (Allied Vision Technologies) can be used, which 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 the IMAG-K6 camera the sockets for connecting the ethernet cable and the camera-power-supply cable (round-shaped connector) are located at the rear side of the camera.

The camera can be equipped either with a **standard objective lens** featuring 12 mm focal length ($F=1.2 / f=12\text{mm}$ - K7-MAX/S) or a **Zoom objective lens** with 8 – 48 mm focal length ($F=1.0$ - K7-MAX/Z Fig. 11). Different mounting angles The adapter ring in the LED-Array Illumination Unit (IMAG-MAX/L) has to be detached for using the Zoom objective.



Fig. 10: Adapter Ring of the LED-Array Illumination Unit

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 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, on the other hand the applied objective lenses display a higher aperture. Please note that with the Zoom objective the aperture and consequently image

intensity drop at focal lengths exceeding 30 mm. When properly adjusted (by provided distance ring and close-up lens) the image remains focused when changing focal length (magnification). Minor adjustment by the focusing ring may be required.



Fig. 11: CCD Camera with Zoom objective

3.5 Mounting Stand with Eye Protection IMAG-MAX/GS

The Mounting Stand IMAG-MAX/GS provides the standard means for mounting the powerful LED-Array Illumination Unit at defined distance to the object, assuring full eye protection of the user. It features a red Perspex hood that absorbs the strong blue light emitted from the LED-array and at the same time allows to view the red chlorophyll fluorescence of the sample with bare eyes. The Measuring Head, consisting of the LED-Array Illumination Unit and the CCD-camera, is mounted with the help of two clamps on the top of the Mounting Stand IMAG-MAX/GS.



Fig. 12 IMAG-MAX/GS with mounted LED-Array Illumination Unit
IMAG-MAX/L and CCD-Camera

Using this Mounting Stand, the Illumination Unit and the CCD-camera are coupled with each other at a fixed working distance of 18.5 cm between LED-Array and object plane, resulting in homogenous 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. In principal, 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. 6). In this case, however, the homogeneity of the light field may be suboptimal, and the user has to take care about protecting the eyes against excessive light, particularly during saturation pulses.

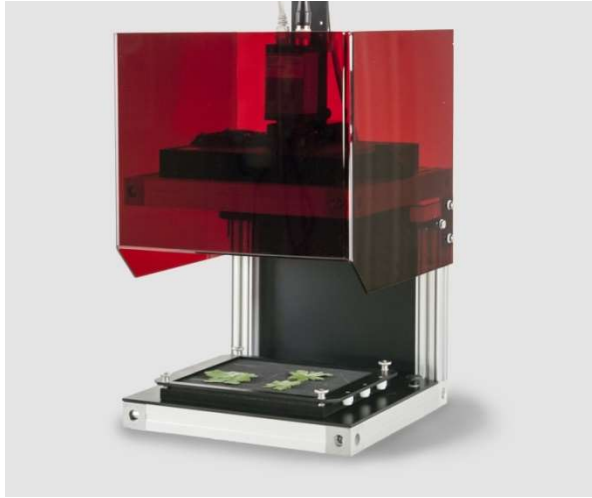


Fig. 13: IMAG MAX/GS with lifted red-Perspex hood showing leaf sample resting on x-y stage

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 has to be removed** from the sample compartment of IMAG-MAX/GS and packed separately within the Transport Box IMAG-MAX/T to avoid damages.

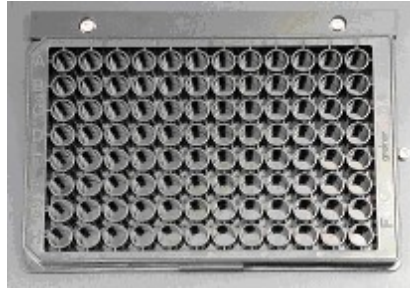


Fig. 14: Bottom part of IMAG MAX/GS with black 96-well microtiter plate in defined centered sample position

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 phenomenon is due to the fact the blue LEDs emit some red light, which can be mirrored from the multiwell plate and/or the water surface of suspension samples via the camera objective lens onto the CCD chip. If this causes a problem, the red emission can be removed with the help of the optional Filter Plate IMAG-MAX/F, which can be mounted in front of the LED-Array Illumination Unit. Now also clear multiwell plates can be used. Fluorescence imaging of algae suspensions in 96-well plates can serve as a powerful tool in ecotoxicology and plant molecular biology (e.g. screening for mutants).



Fig. 15: IMAG MAX/GS, with bottom part being removed, resting on two spacers to give room for potted plant

Alternatively, it is also possible to remove the bottom part of the IMAG-MAX/GS for fluorescence imaging of larger samples, like potted plants. 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. 15). For this purpose, with the Mounting Stand being turned upside down, the four nuts first have to be put into the grooves at the bottom corners. Standard legs with 20 cm length (including mounting screws and nuts) are delivered with the instrument. With the thus increased distance to the bottom, the use of a **Screw Jack** is advantageous, which allows to move a sample (e.g. potted plant) up/down for focusing the image at the standard working distance of 18.5 cm. In this case, the focus of the objective lens should be set for 18.5 cm working distance (between front of LED-Array and sample) and then remain unchanged.

3.6 Leaf Distance Holder IMAG-MAX/B



Fig. 16: Leaf Distance Holder IMAG-MAX/B with IMAG-MAX/L and IMAG-K6

Using the Leaf Distance Holder IMAG-MAX/B the illumination unit with CCD-camera are mounted at standard working distance of 18.5, resulting in homogenous illumination of an imaged area of 10 x 13 cm of the x-y stage plate.

The assembly of this light weight device is simple. The four black legs are screwed to the provided screw holes in the bottom corner of the LED array as well as to the base containing the x-y stage plate. For convenient sample preparation the x-y stage plate can be taken off the base by unscrewing the knurled head screws.



IMAG-MAX/B does not provide eye protection! The user should avoid looking directly into the LED-Array Illumination unit.

3.7 Notebook PC IMAG-PC

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

- processor 1.7 GHz
- RAM memory 4 GB
- built-in USB interface
- built-in Ethernet interface GigE-Vision[®]
- built-in DVD/CD-RW drive
- operating system Vista, Windows 7 32- or 64-Bit, Win 8 and 10

The **Notebook PC IMAG-PC** is delivered with fully installed software. Depending on the market situation, best value brand-name devices are chosen which have proven well suited for use in conjunction with the IMAGING-PAM.

If the instrument has been purchased without PC, the user first has to install the software (see chapter 7.2)

3.8 Adapter IMAG-MAX/GWK

The combined application of IMAGING-PAM and gas exchange measurements by GFS-3000 provides comprehensive analysis options. For examples physiological heterogeneities or differences in genotypes can be visualized in fluorescence images under changing CO₂, O₂ or temperature conditions applied by the gas exchange system. The adapter IMAG-MAX/GWK positions the MAXI-IMAGING PAM on top of the large gas exchange cuvette 3010-

GWK providing eye protection during the merge of imaging and gas exchange analysis over a measuring area of 10 x 13 cm.

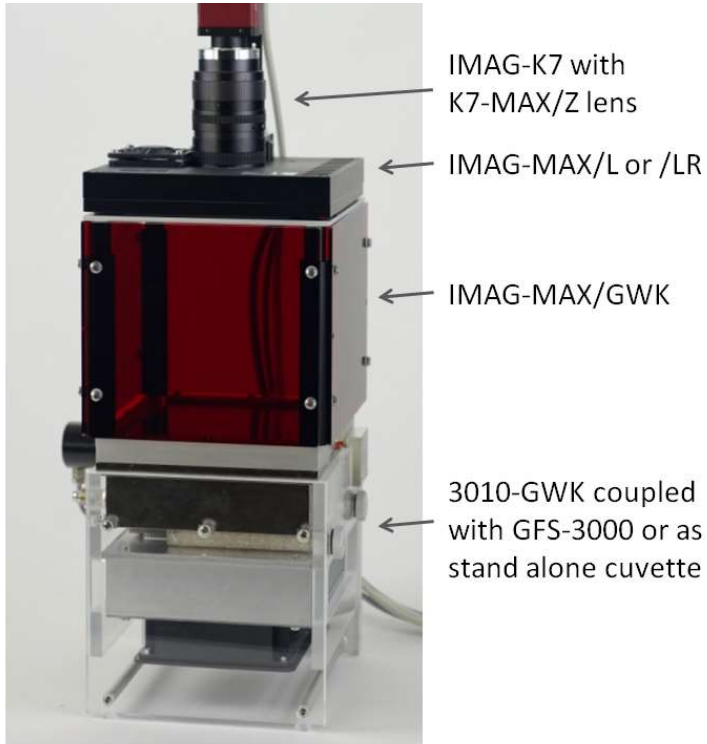


Fig. 17: IMAG-MAX/GWK on top of 3010-GWK gas exchange cuvette, equipped with Imaging head MAXI version.

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

A useful accessory for leaf measurements is the Leaf Holder IMAG-MIN/BK. For simultaneous measurements of gas exchange with the GFS-3000 the Adapter IMAG-MIN/GFS is available. Also available for convenient use is a laboratory stands as well as a tripod and a fine drive tripod adapter for outdoor applications.

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 already described in section 3.1.1. in conjunction with the MAXI-version. The LED-Array of the MINI-Head is plugged into to the MINI-Head connector on the rear side of the IMAG-CG unit. The Camera cable connector is located on the front side of IMAG-CG.

The MINI-Head with mounted camera can directly be attached on top of the Control Unit housing IMAG-CG. For this purpose, a wing-

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

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



Fig. 18: 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 except for the color of the LEDs and the LED-filters. As blue and red LEDs display different intensity-current relationships, different PAR-lists apply, that are incorporated in two different versions of the ImagingWin program (see chapter 11).

The LED-array of the MINI-Head features 12 high-power LED-lamps each equipped with collimating optics, which are arranged in 4 groups of 3 LEDs. Each group is equipped with a short-pass filter eliminating red light that otherwise would pass the long-pass filter in front of the CCD-camera and overlap with chlorophyll fluorescence. In the blue version (IMAG-MIN/B emission peak 460 nm) a blue-green glass filter (Schott BG39) is used. In the red version (IMAG-MIN/R emission peak 620 nm) a special short-pass interference filter

(< 645 nm) is used. The LEDs are mounted at an angle that is optimized for obtaining a homogenous light field at the given working distance. These LEDs provide the pulse-modulated excitation light and at the same time serve for actinic illumination and Saturation Pulses. In addition, there are four groups of LEDs (2x6 and 2x4) providing the pulse modulated light for assessment of 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. The lenses of these LEDs are removed in order to obtain homogenous illumination of the sample. While only a relatively small amount of this light is remitted from the sample to the CCD-camera, this is sufficient to give good signals, as both wavelengths can pass the red long-pass filter in front of the CCD-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.



Please avoid looking directly into the LED-Array Illumination unit to prevent Eye-damages!

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

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

The MINI-Head LED-Array IMAG-MIN/GFP is described in chapter 4.4.

4.3 CCD Camera IMAG-K6 or IMAG-K7

The CCD Cameras IMAG-K6 or IMAG-K7 are described in chapter 3.4 for the imaging MAXI-version. Both 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 chapter 4.4.)

For usage in the Imaging MINI-version these CCD 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 an 4.2 mm distance ring. For both 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 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).



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



Fig. 19: 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. 20 A)

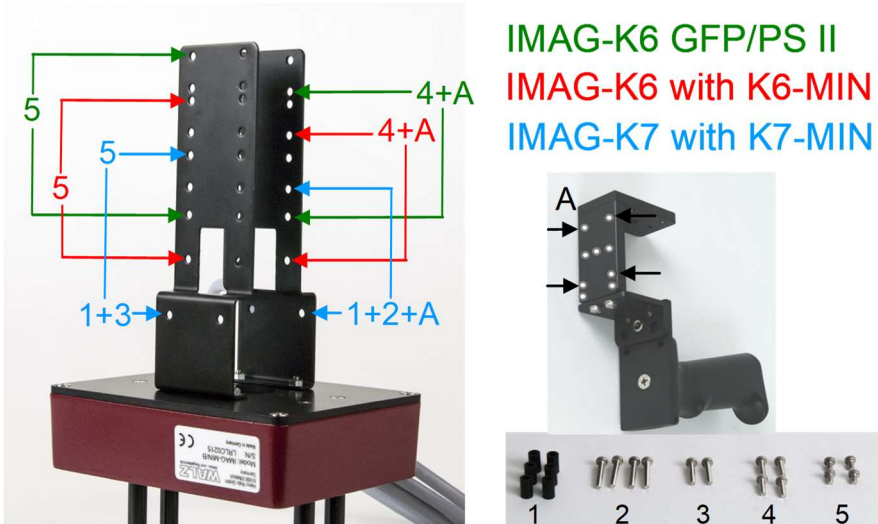


Fig. 20: Screw indication and camera mounting positions, metal angle bar including grip of Leaf Holder IMAG-MIN/BK, screw annotation

The metal angle bar of the mounting set K6 and K7-MIN/M (Fig. 20 A) serves for mounting the MINI-Head with camera onto the IMAG-CG Control Unit, to the grip 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. 21)



Fig. 21: Perspex device to unplug GigE cable from a mounted camera

4.4 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. 22 .



Fig. 22: 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 (e.g. Fig. 9 left) 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. 20).

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.

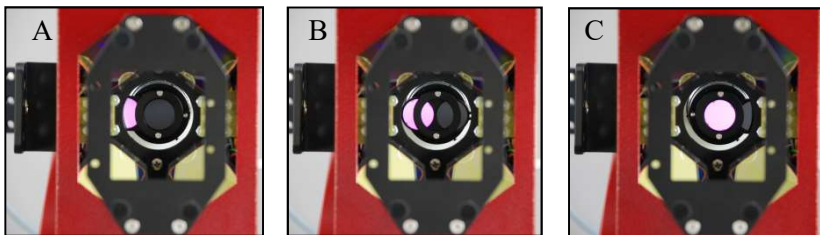


Fig. 23: switching of the filter slider between two positions (A – PSII, C – GFP position)

The view into the GFP Mini-Head from the sample side (Fig. 23) 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. 23 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.

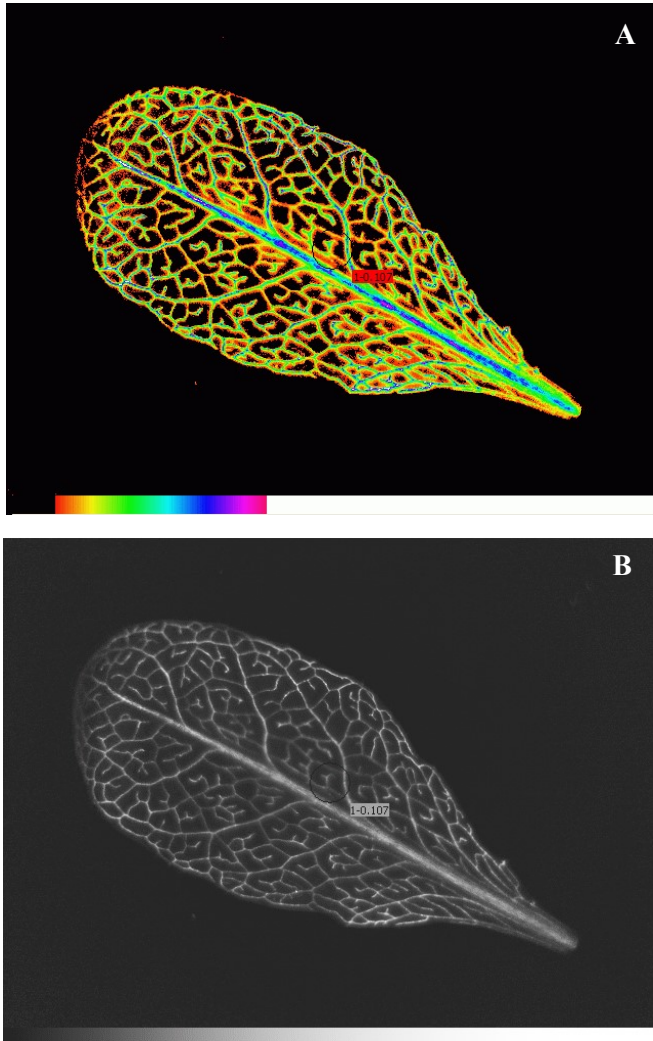


Fig. 24: possible color modes for the GFP images

There are different colour modes reasonable for the GFP measurements. Fig. 24 A shows an image in the false colour scale mode of the “Analysis” function (see also chapter 10.1.2.6). Another option is the black and white mode as in Fig. 24 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.

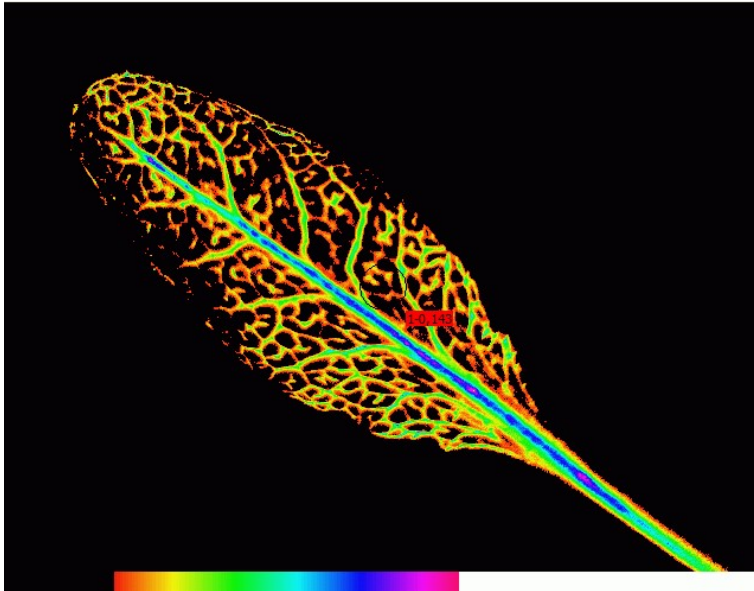


Fig. 25: “Expanded Color” setting for enhancing GFP images

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.

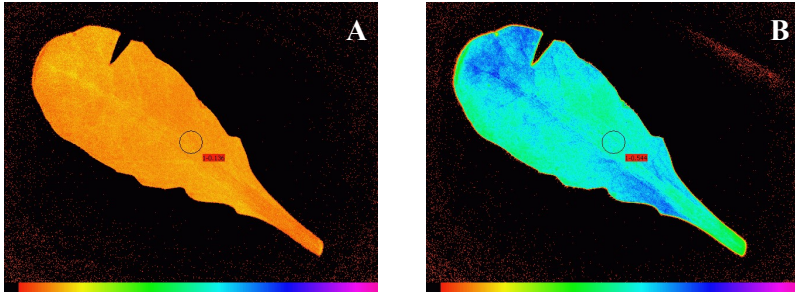


Fig. 26: Fo image in the PSII mode (A) vs. Fm image (B)

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.

4.5 Leaf Holder IMAG-MIN/BK with Grip Holder



Fig. 27: MINI-Head with CCD camera and grip holder

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. 28).



Fig. 28: Mini-Head with grip holder 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. 29).



Fig. 29: Mini-Head with Leaf Holder mounted on a tripod

When the Leaf Holder is ordered separately from the MINI-Head, it must be assembled by the user. Fig. 30 shows the components of the Leaf Holder. The following figures (Fig. 31 - Fig. 33) may help to put the various parts together.



Fig. 30: 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. 31 shows the mounted clip from the bottom (camera) side, Fig. 32 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. 31 and Fig. 32). The O-rings may age and then have to 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. 33.

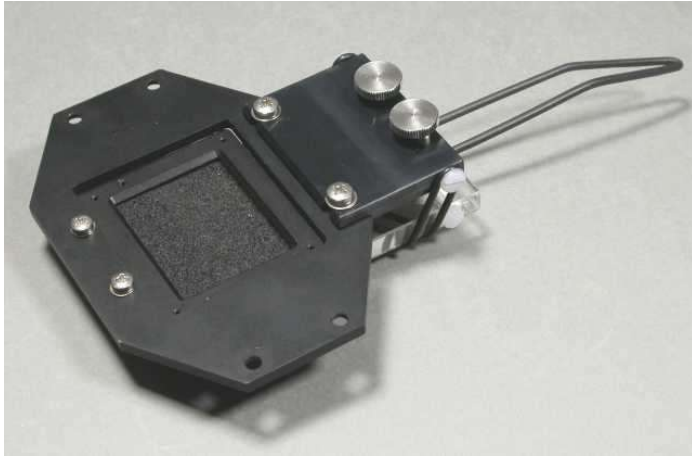


Fig. 31: Leaf clip mounted on sample platform of MINI-Head viewed from camera side

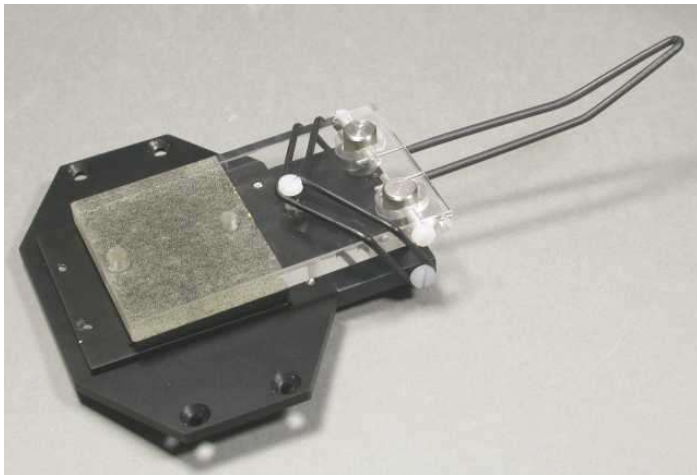


Fig. 32: Leaf clip mounted on sample platform of MINI-Head viewed from top side

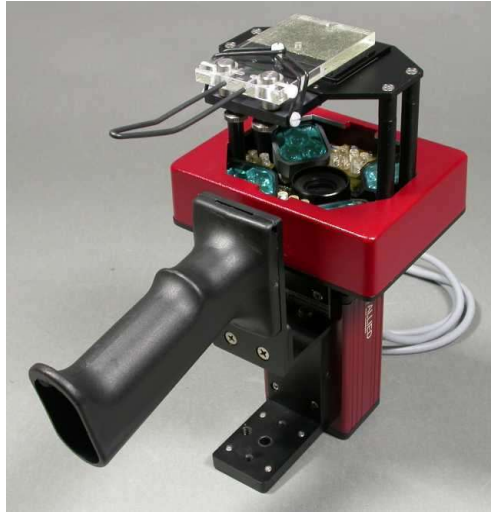


Fig. 33: Leaf clip and grip holder mounted on MINI-Head

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

The MINI-version of the IMAGING-PAM M-Series can be applied for simultaneous measurements of CO₂ 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 allow 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. 34: 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.

4.7 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.

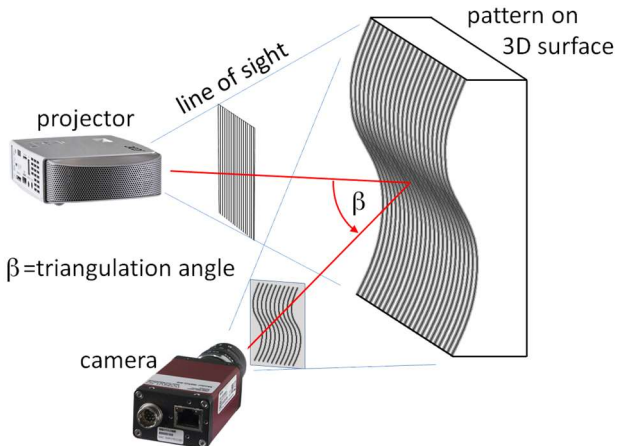


Fig. 35: Operation principle of a fringe light scanner

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.

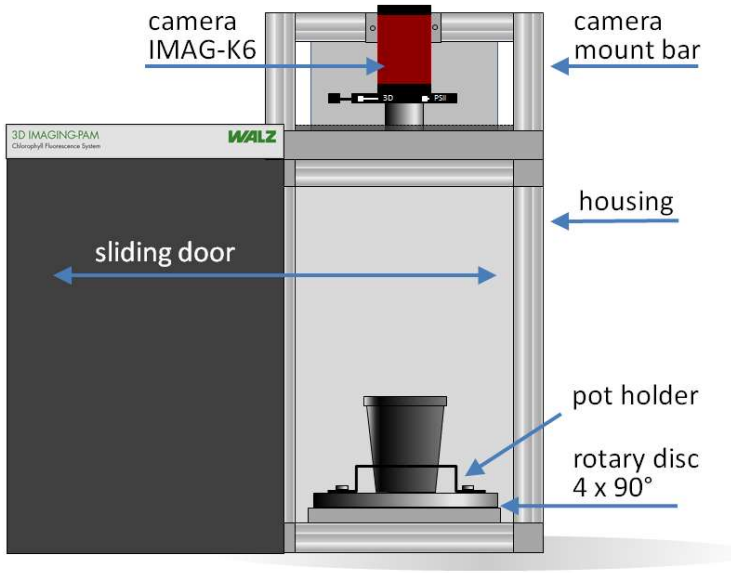


Fig. 36: Front view of IMAG-3D

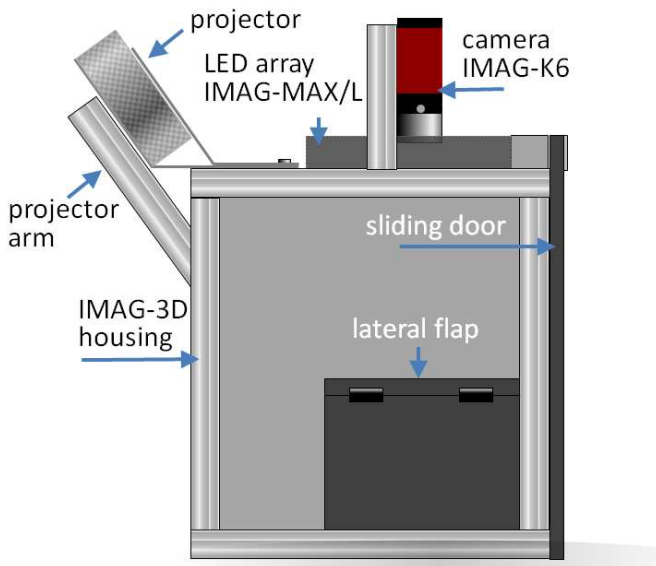


Fig. 37: Side face of IMAG-3D

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 ($4 \times 90^\circ$ 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. 38: 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 (RJ45 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. 39: 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. 40: 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.

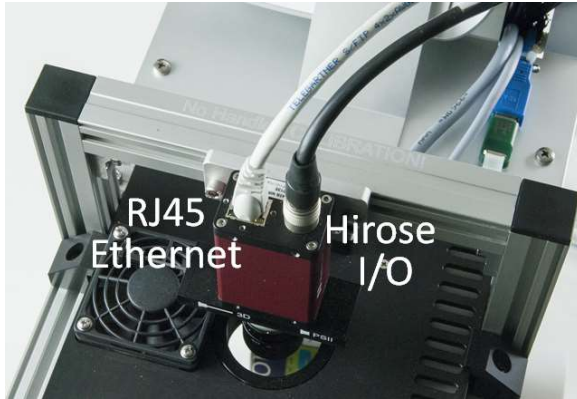


Fig. 41: Connections of camera

Furthermore, some power supplies for the components have to 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 has to be connected:



Fig. 42 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 pot holder 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
- 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

...

5.3 3D-Survey of a sample

The pot holder is designed for rectangular pots (see chapter 5) using these pots a stable and reproducible sample position can be assured. It is recommended to use a lid of a petri dish below the pots to avoid dirt or water reaching the turntable mechanics.

The IMAG-3D is normally set to a focus level slightly higher than the upper brim of the recommended flower pots, so that the user can immediately start measuring when a plant has been growing or

moved or replanted to such a pot. On demand we can also provide holder frames for special pots (please inquire at info@walz.com).

The IMAG-3D should be wired according to the previous chapters and ImagingWin3D started. Also, for the later evaluation of data the hard lock (mounted on top of the IMAG-3D) is needed, otherwise ImagingWin3D software refuses 3D functionality. The resulting meshes of previous measurements and related PSII data are available also without the hard lock. For an additional evaluation working place further dongles can be purchased (IMAG-3D/ANA).

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
- Computer

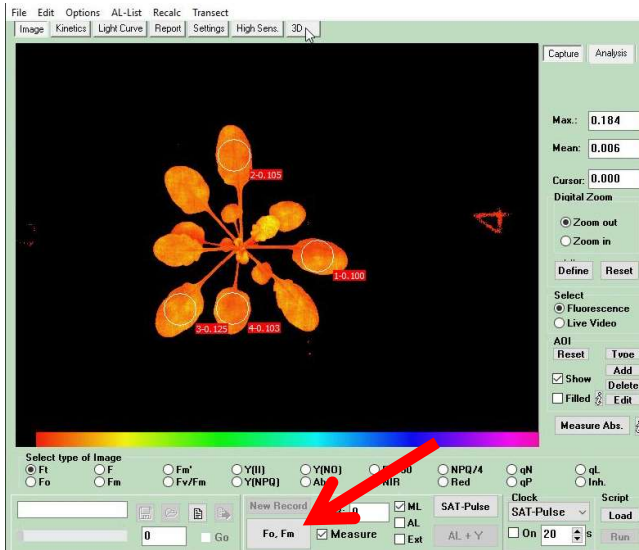
The filter slider already moves to its initial position when IMAG-CG central control unit is switched on.

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).

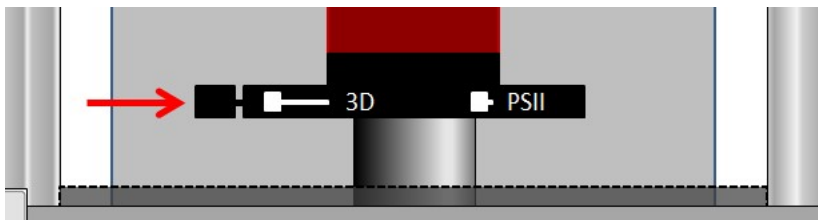
Place a sample into pot holder frame and make sure that it cannot move during the measurement. To prevent dirt or moisture reaching the rotary table mechanics it is recommended to you a Petri dish or similar underneath flower pots during the measurement.

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 has to 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 as long as possible, do not touch or scratch the surface. Clean only with water and a soft cloth if necessary.

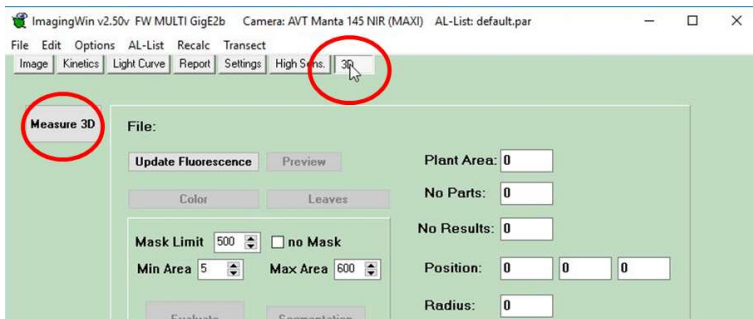
The calibration stand (phantom) is used in three different configurations (horizontal, tilted right 30° or tilted left 10°) with two different calibration patterns. For a successful calibration it is essential that the images taken are within the contrast range of the camera and that all dots of the calibration patterns are visible (for the camera) throughout the whole procedure. Hence the configuration of the setup is fixed. In case some of the dots cannot be seen by the camera in one or more images a message (“no pattern found”) will appear and the calibration will have to be repeated. The error might be caused by a shifted or wrong calibration pattern or excess light falling onto the calibration pattern.

In each position of the calibration phantom a sequence of images will be displayed by the projector. Each single image is important and opening of the door during the calibration sequence might lead to errors in the pattern recognition which results in an error.

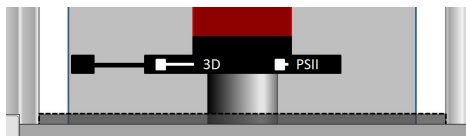
1. Mount the calibration phantom with large calibration pattern so that the “1” on its foot aligns with position “1” of the rotary disk. Use the large calibration pattern (make sure that the larger spot is located in the rear left edge. Orientation is horizontally in the beginning.



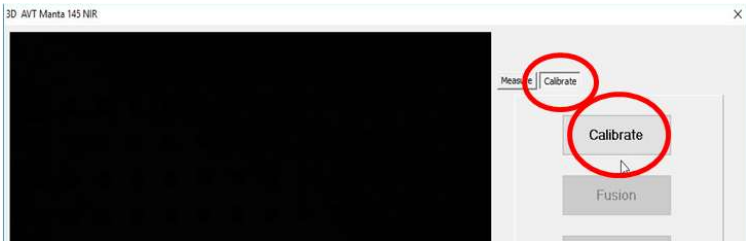
2. Switch to “3D” and afterwards activate “Measure 3D”



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

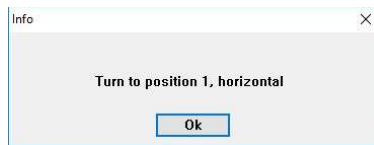


- Now click “Calibrate” in the newly appearing window (upper button next to “Measure”, afterwards start calibration by clicking the lower “Calibrate” button. A



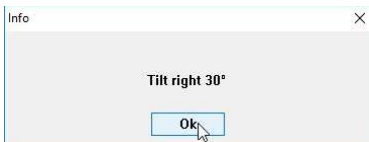
A message appears:

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

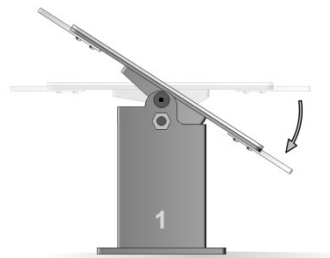


Before clicking “OK” the slider door shall be closed to avoid interfering light on the calibration pattern.

- A sequence of images is projected onto the calibration pattern. They can be judged in the software preview window
- For the next sequence the table has to be tilted to the right (30°)

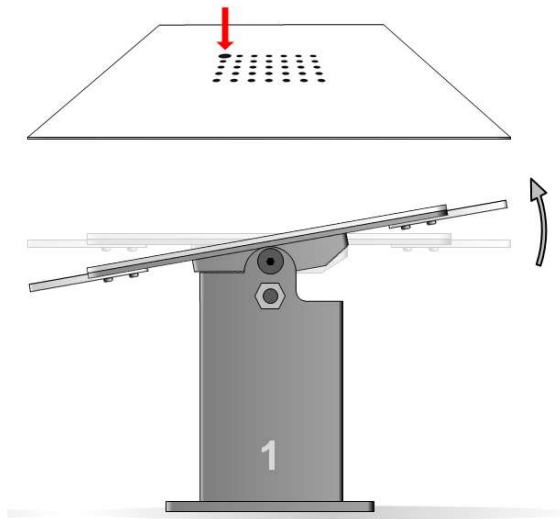


and click OK

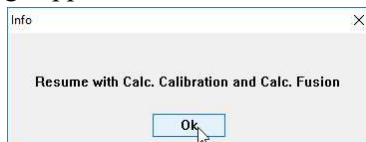


The after the first sequence of images the table automatically turns by 180° (from position 1 directly to position 3 and back to position 1).

7. The following calibration step is the “Fusion”. Now the small calibration pattern must be used. Detach the large pattrer 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 has to 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 pot holder can be mounted again.

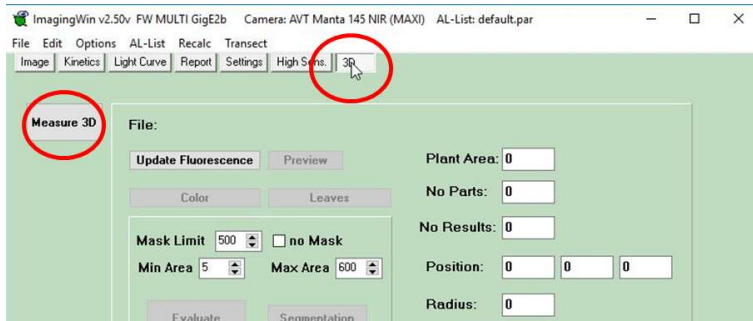


Viewer commands. Please refer to chapter 5.3.2.1 for details about the 3D viewer

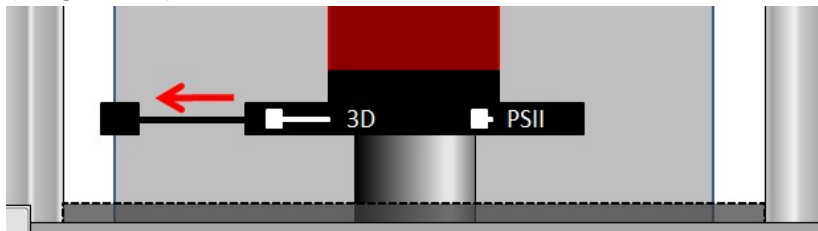
5.3.2 3D measuring workflow with IMAG-3D

For accurate discrimination between background and plant sample the ImagingWin-3D software need a fluorescence image. This means that prior to the 3D scan (pressing “Measure 3D” button) an Fo Fm measurement should be done regardless if chlorophyll fluorescence shall be used later on or not.

For the first steps it should be resumed as described for 3D calibration on the pages before.

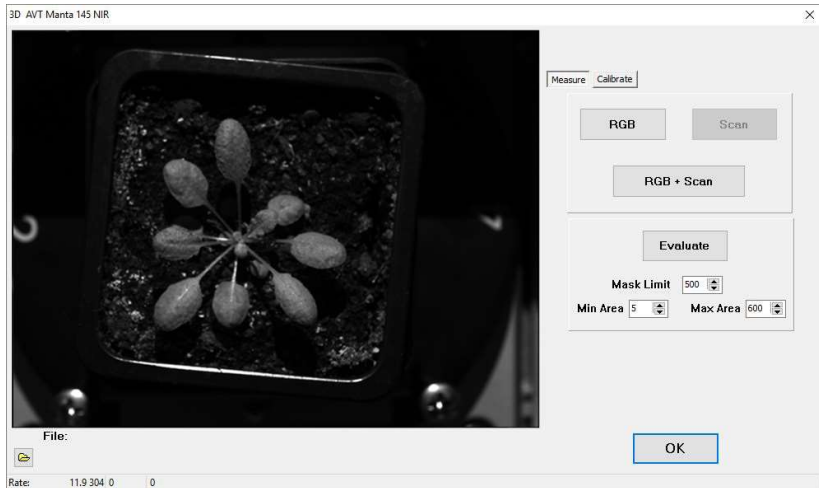


The following steps need a neutral grey filter replacing the PSII detector filter used in the prior steps. This is done by the software automatically if you are using an automated version and in the manual version the filter slider has to be pulled into “3D” position (image below).



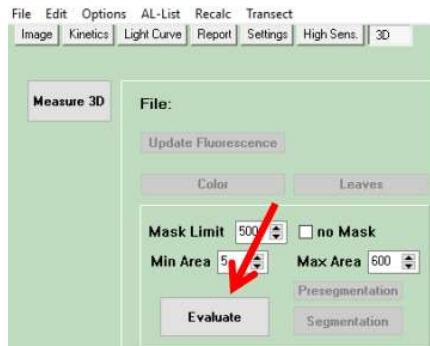
As next step the 3D tab can be clicked in ImagingWin and afterwards the button “Measure 3D”. While being in 3D mode ImagingPAM measuring light is switched off.

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 button “Scan” is inactive initially. Alternatively, also the complete scan including 3D scan can be chosen “RGB + Scan”. The resulting RGB image will later on be used as texture for the merged surface meshes.

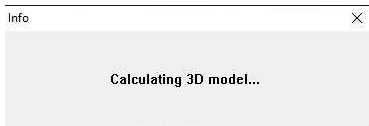
After completion of the RGB image and Scan the extra window shown above closes and brings you back to the 3D tab of ImagingWin software.



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.



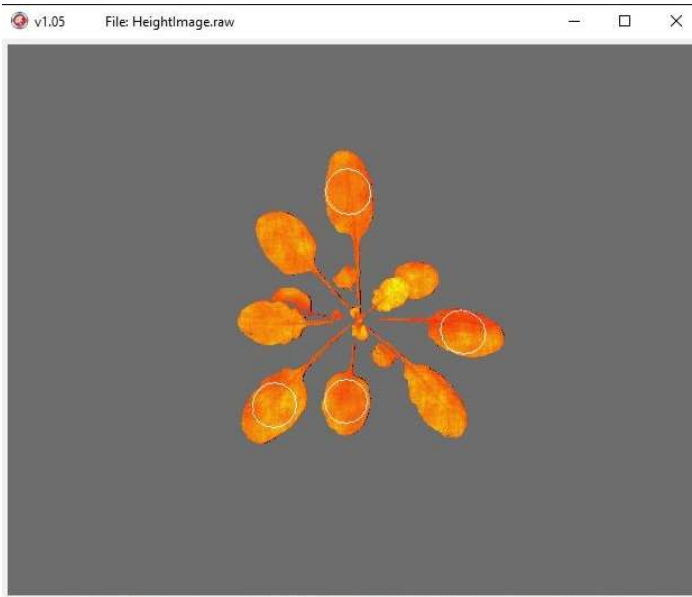
At the end some computing time is needed to finish a 3D model. During this time the computer might not be responsive since its

CPU is completely busy.

In case the 3D model is blemished 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

ImagingWin v2.51x FW MULTI GigE3 Camera: AVT Manta 145 NIR (MAXI) AL-List: default.par

File Edit Options AL-List Recalc Transect

Image Kinetics Light Curve Report Settings High Sens. 3D

Measure 3D

File:

Update Fluorescence Preview

Color Leaves

Mask Limit 500 no Mask

Min Area 5 Max Area 600

Evaluate Presegmentation Segmentation

Plant Area: 184.1

No Parts: 9

No Results: 9

Position: -44.0 25.4 1.3

Radius: 20.9

Plot Table Export Table

Parts	Position /	Area	Angle	Normal X	Normal Y	Normal Z	Center X	Center Y
1.0	5.9	22.5	0.9	0.00765	0.01277	0.99989	-45.0	16.2
1.0	44.8	22.2	2.0	0.02419	0.02620	0.99936	-56.9	12.4
1.0	83.5	21.3	1.7	0.01597	0.02398	0.99958	-56.8	24.0
1.0	135.4	18.1	1.0	0.01192	0.01211	0.99986	-55.3	36.9
1.0	165.4	18.0	0.3	0.00523	-0.00107	0.99999	-48.1	41.0
1.0	212.2	18.6	2.2	0.03738	0.00474	0.99929	-34.3	40.8
1.0	224.0	20.5	1.6	-0.00147	0.02780	0.99961	-39.6	30.0
1.0	278.6	21.0	1.3	0.00424	-0.02142	0.99976	-26.1	22.7
1.0	340.8	21.8	2.3	0.02171	0.03431	0.99918	-38.5	9.7

New Record PAR: 0 ML SAT-Pulse Clock SAT-Pulse

Fo. Fm Measure AL Ext AL + Y On 20 Script Load Run

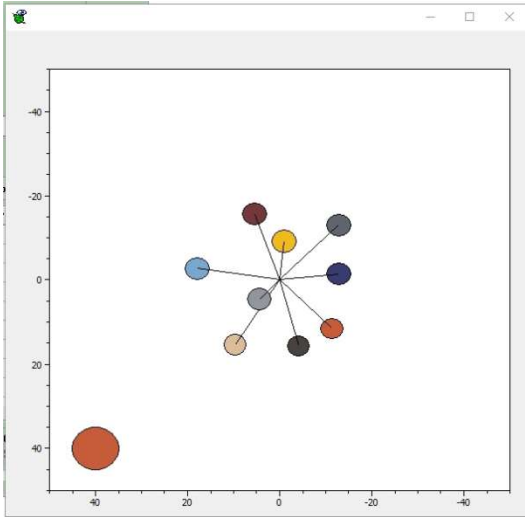
After performing a 3D measurement and closing of viewer windows further 3D options and evaluations on the basis of the previously measured sample are shown.

On the upper right quadrant of the 3D tab parameters like “plant area” [mm²], 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.

Plot Table

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).

Export Table

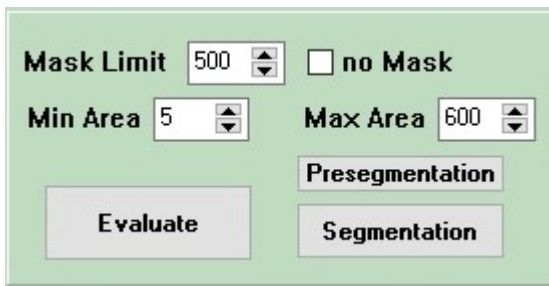
Exports evaluated morphological data, shown as table in the 3D tab, into a CSV file that is saved. Additionally a tiff file is stored containing a “realcolor” image taken from the sample.

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 long 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]).

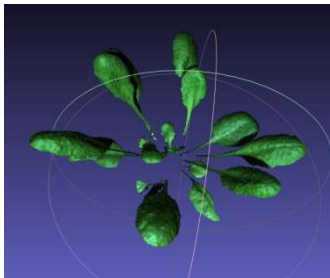
Further content is the complete set of images that have been taken for 3D evaluation and the calibration folder of the instrument that has been used for the experiment. The dataset itself is quite large since it contains all the calibration data also but it is recommended to keep

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.

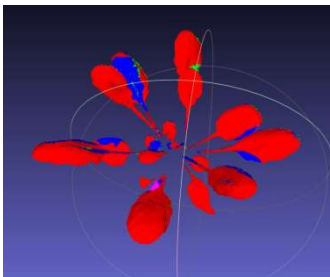
Most of 3D related files need a hard lock to be used in ImagingWin3D, but some of the 3D files are stored in PLY format so that they can be opened in 3D dedicated software like e.g. Meshlab (which is a quite powerful freeware). These PLY files have always the same name (like “Reconstruction_triangulation_color.ply”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

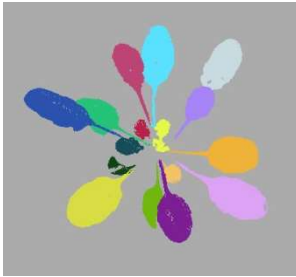
Reconstruction_triangulation_fluorescence.ply carries information



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

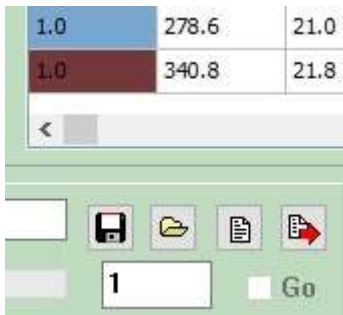


sample mesh with an overlay of the segmentation image. It can be useful to analyze problems with the segmentation algorithm.

5.3.2.3 Opening of a 3D dataset in ImagingWin3D

In the previous chapter data structure of stored data already has been discussed a little. In contrast to a single PSII measurement that is stored as PIM file in the data folder of ImagingWin, with a 3D system a lot more data have to be stored in a folder carrying the name of the experiment. Within this folder PIM data as well as the 3D data can be found so that opening an experiment in ImagingWin3D is not like opening a single file, it is the selection of a folder in which all needed information is collected.

As mentioned before PIM files and PLY files can be opened in ImagingWin or 3D software also without a hard lock, but for opening



a 3D folder with full data access in ImagingWin3D the dongle is necessary to use 3D software modules that have been programmed by Fraunhofer IIS (Fürth, Germany).

In case an additional workplace for data evaluation shall be built, it is possible to purchase additional dongles.

Generally 3D data are opened by clicking the folder button in ImagingWin3D. Afterwards a window pops up in which a folder can be selected. *The files comprised are not shown!* Just select a folder and click “open”. ImagingWin3D will open up with the image window as it would do in the 2D version of the software also, but the 3D viewer can be started by double clicking on the fluorescence image.

It is also possible to scroll through an experiment, select another image with the parameter desired and after a double-click on the image window the viewer will open with the PSII / parameter image as overlay of merged mesh. Also AOI borders are shown if selected.

After closing the viewer window again, reevaluation of data can be done via the known PSII functions of 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 A1
- 4) LED Modules as:

Microscopy LED Lamp (blue) IMAG-L470M or

Microscopy LED Lamp (red-orange) IMAG-L625M 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-L470M or IMAG-L625M 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. 43).



Fig. 43: Camera adapter for connecting Axio Scope.A1 with IMAG K6



Fig. 44: 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. 44. 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.



Fig. 45: Pathway switch

The user should get familiar with the switch which allows switching between binocular or phototube / photomultiplier pathways (see Fig. 45).

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

6.3 Axio ScopeA.1 Epifluorescence Microscope



Fig. 46: MICROSCOPY-IMAGING-PAM based on Zeiss ScopeA.1 Epifluorescence Microscope

The MICROSCOPY-IMAGING-PAM is readily adapted to the **Zeiss Axio ScopeA.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 Axio ScopeA.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 Axio Scope.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 Axio Scope.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. In view of the fact that 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 Axio Scope.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

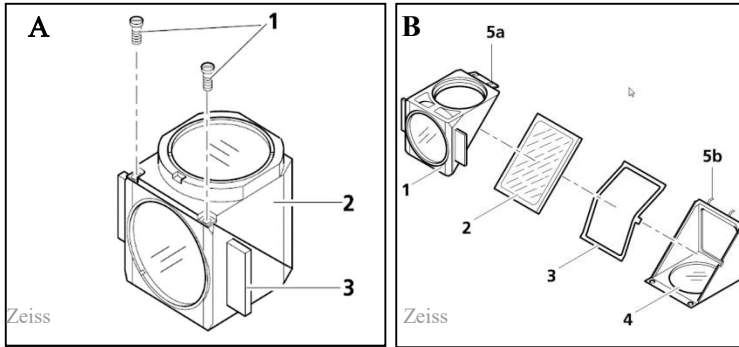


Fig. 47: The reflector module – mounting the beam splitter

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.

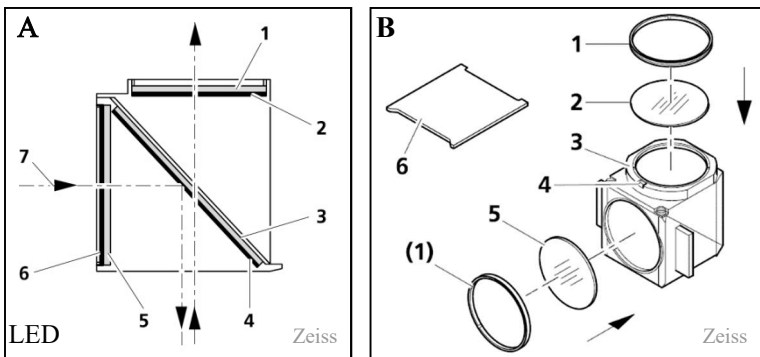


Fig. 48: Reflector module with filter configuration

A) The arrows in Fig. 48/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. 48/B).

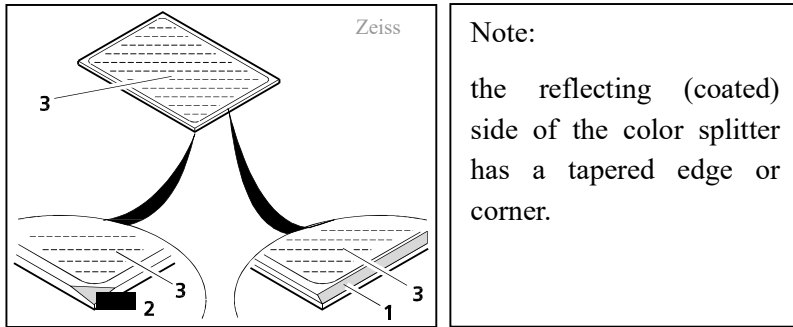


Fig. 49: Labeling the color splitter

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. 48/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. 48/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. 48/B5 – not used in the standard setup).

6.3.3 Mounting of the reflector module

The mounting of the reflector module into the Zeiss Axio Scope.A1 is described in Fig. 50.

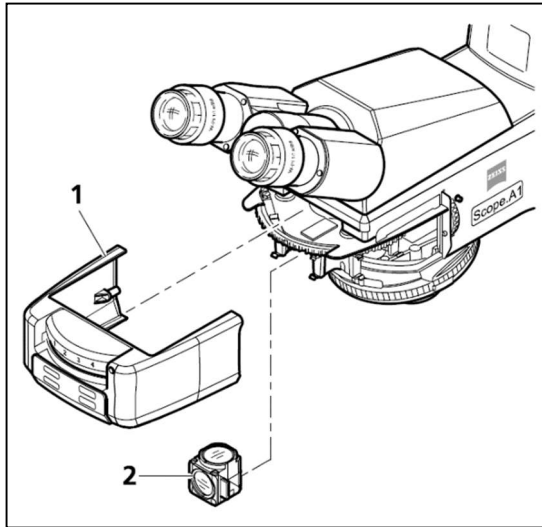


Fig. 50: Changing the reflector module in the upper stand FL-LED

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. 50) in front of the filter turret to get access to the reflector module ports.



Fig. 51: 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. 48/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 Axio Scope.A1.

A grey circle (a) in Fig. 51 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 6.4.3).

6.4 LED Modules

There is a wide choice of wavelengths available for the Zeiss LED modules so that experienced users can easily modify their epifluorescence system by additional light sources with various wavelengths. LED modules might need to be adapted as described in this chapter. Also the filters used in the reflector module might be necessary to adapt (see chapter 6.3.3)

6.4.1 Adjustment of brightness by grey filters

Since the original LED modules from Zeiss are far too bright for PAM purposes (especially for the measuring light) neutral grey filters have to be used.

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



Fig. 52: LED-module filter tool

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 front of the LED module by using the provided tool shown in Fig. 52. The side pointing upwards is used for mounting the filters of the LED modules.

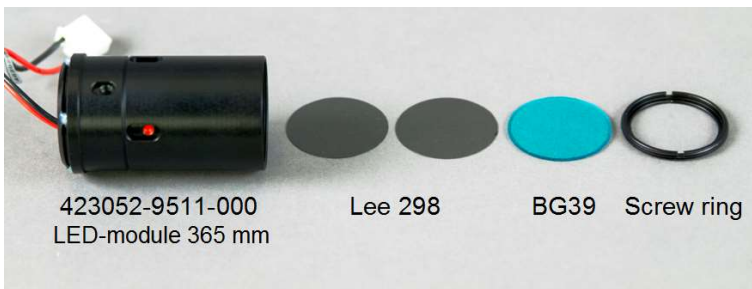
Zeiss LED modules are shipped together with a set of filters:



The red LED module IMAG-L625M 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-L470M 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 colorsplitter modules.***

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

6.4.2 Integration of LED modules into Axio Scope.A1

The integration of LED modules in the Axio Scope.A1 is shown in Fig. 53.

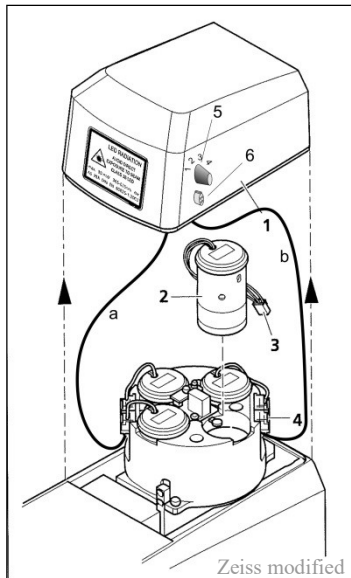


Fig. 53: Changing the LED module in the upper stand part FL-LED

- Lift the covering cap (Fig. 52 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. 52) 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 Axio Scope.A1 shall also be used for further epifluorescence applications beside PAM measurements, the electrical connections shown in Fig. 53 (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. 53 are connected the switch for the epifluorescence lamps on the right side of the microscope is not active (Fig. 51 grey circled a).

6.4.3 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. 53 number 6) on the cover cap of the Zeiss Axio Scope.A1 and with the 6-pin “MINI-Head” connector on the IMAG-CG control unit.

6.4.4 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. 52 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.

6.4.5 IMAG-RGB



Fig. 54: IMAG-RGB Microscopy LED Lamp

The Red-Green-Blue Microscopy LED Lamp (in contrast to the IMAG-L470M or IMAG-L625M) 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 with the epifluorescence microscope via a fluid light guide (100 cm length, 3 mm \varnothing). 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 \varnothing plastic optical fibers. The fibers of 7 LEDs (2x Red, 3x Green, 2x Blue) are put together resulting in a 3 mm \varnothing bundle, to which the 3 mm \varnothing 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.

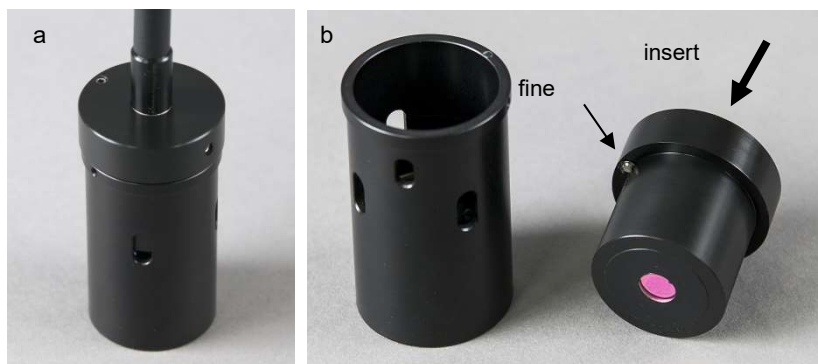


Fig. 55: Fluid Light Guide with Adapter Endpiece for connecting to excitation entrance port of Epifluorescence Microscope

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 Axio Scope.A1

- Lift the covering cap (Fig. 52 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. 52) 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.

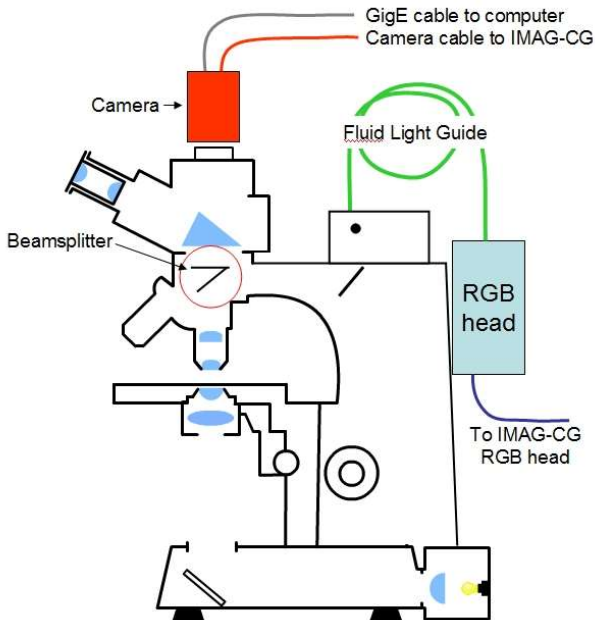


Fig. 56: Schematic presentation IMAGING-PAM MICROSCOPY-version with IMAG-RGB

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-L625M and IMAG-L470M is connected via MINI-Head cable from the 3-pin connector (Fig. 53 number 6) on the cover cap of the Zeiss Axio

Scope.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

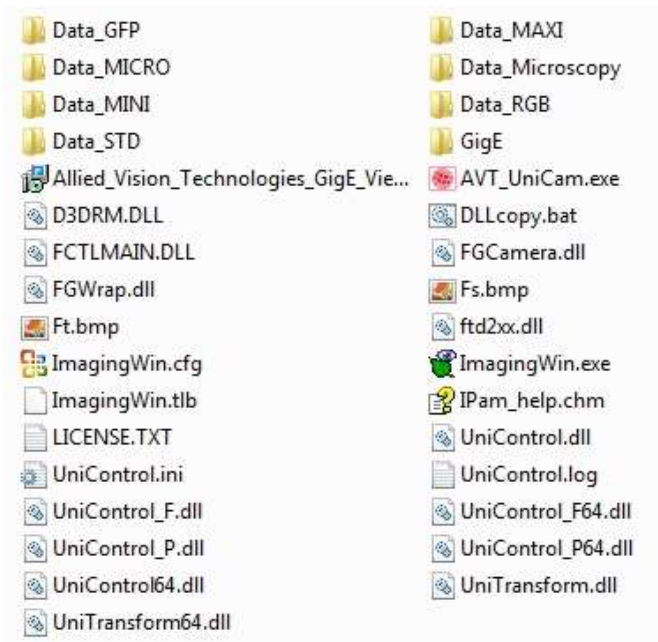
All software required for operating the IMAGING-PAM already is installed, when the Notebook PC IMAG-PC was purchased together with the instrument. Otherwise the user can install the required software as outlined in the following sub-sections.

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.



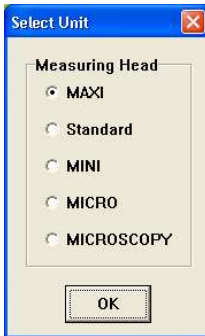
For updates of the ImagingWinGigE software update setup files can be downloaded from the Walz website (www.walz.com). Please note that the Data directories and all system settings are not affected by the Update. After clicking "Install" the Windows "Hardware Installation" may give a warning which, however,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

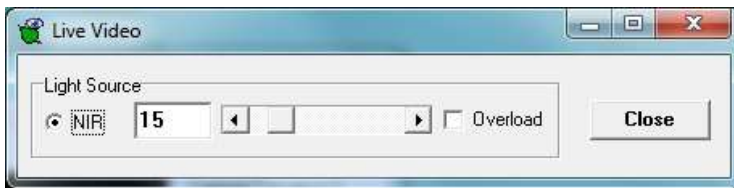


After the IMAGING-PAM is set up, all cables are connected and all software is installed (see 7.2), first measurements can be carried out in order to become acquainted with the instrument. In the following description, use of the **MAXI Measuring Head** with the LED-Array Illumination Unit IMAG-MAX/L in conjunction with the Mounting-Stand IMAG-MAX/GS is assumed. Before starting the program, power should be switched on at the Control Unit IMAG-CG (via **POWER**-switch on the front), as well as at the External 300 W Power Supply (via switch at the rear side). When the program is started by clicking the **ImagingWinGigE.exe start icon**, a selection window appears.

If one of the other Imaging systems (MICROSCOPY- or MINI-version) is used you will be asked to make choice for, for example, the light color. It is important to choose the right color and head, because the LEDs may be damaged otherwise.

After selection of the Measuring Head and confirmation by OK, the Maxi button should be checked and confirmed by O.K. Then the pulse-modulated fluorescence measuring light is automatically

switched on. Fluorescence is measured with relatively weak measuring light pulses at low repetition rate (ca. 1 Hz). This weak measuring light does not cause significant changes in the given state of a leaf sample. The PC monitor screen shows the **Image window** on which after program start the image of the **fluorescence parameter Ft** is displayed. The Ft-image is black as long as no leaf sample is present. When a leaf sample is placed on the x-y stage plate, at the given slow rate of measuring light pulses the **Ft-image** slowly appears on the screen. In order to arrange a defined position of the sample within the field of view and to focus the image, it often is advantageous to switch from Fluorescence-imaging to **near-infrared (NIR)-imaging** by selecting **Live Video** (at the right hand side of the Image window). Now NIR-measuring light pulses with which image changes can be followed. The NIR light pulse frequency is adjustable in the Live Video window.



The image can be **focused** by turning the **adjustment ring** of the **objective lens**. After having focused the image using NIR-light, the Live Video window must be quit by clicking "Close" or the exit box in the upper right corner. Then the system returns to **the Fluorescence measuring mode**, displaying the focused Ft-image. Fig. 57 shows a PC screen shot of a typical fluorescence image of a leaf under the described conditions.

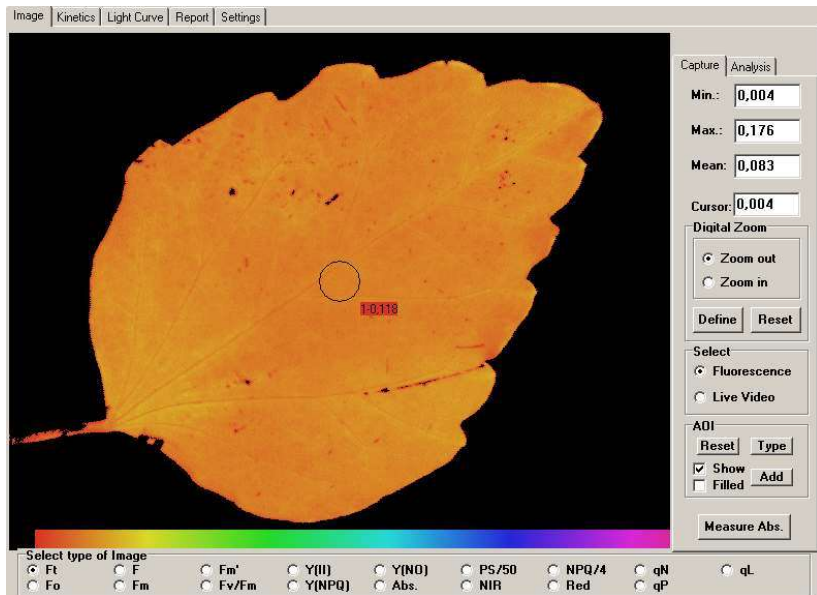


Fig. 57: PC screen shot of Ft-image of leaf

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. Actually, 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 **F_o, F_m** measurement.



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

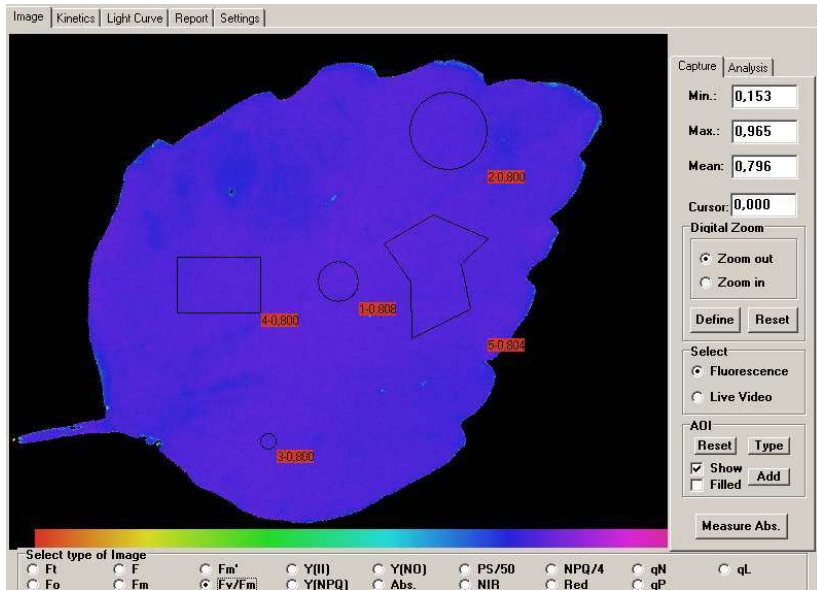


Fig. 58: PC screen shot of Image window following F_o , F_m determination with the F_v/F_m image being selected and various types of AOI being defined

In Fig. 58 the Image window following F_o , F_m determination is shown, when the **F_v/F_m -image** is selected. F_v/F_m reflects the maximal PS II quantum yield of a dark-adapted sample. With the given leaf sample, F_v/F_m is distributed quite homogeneously over the whole leaf. It may be noted that after F_o , F_m determination the F_o , F_m button is not accessible anymore and that instead the **New Record button** has become accessible. The F_o , F_m determination will remain valid until a New Record is started. All F and F_m' values measured in conjunction with the help of Saturation Pulses (triggered via the **SAT-Pulse button**) are compared with F_o and F_m 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, F_m' , Yield, q_P and q_N) change with preillumination.

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. 59 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 $81 \mu\text{mol quanta m}^{-2}\text{s}^{-1}$.

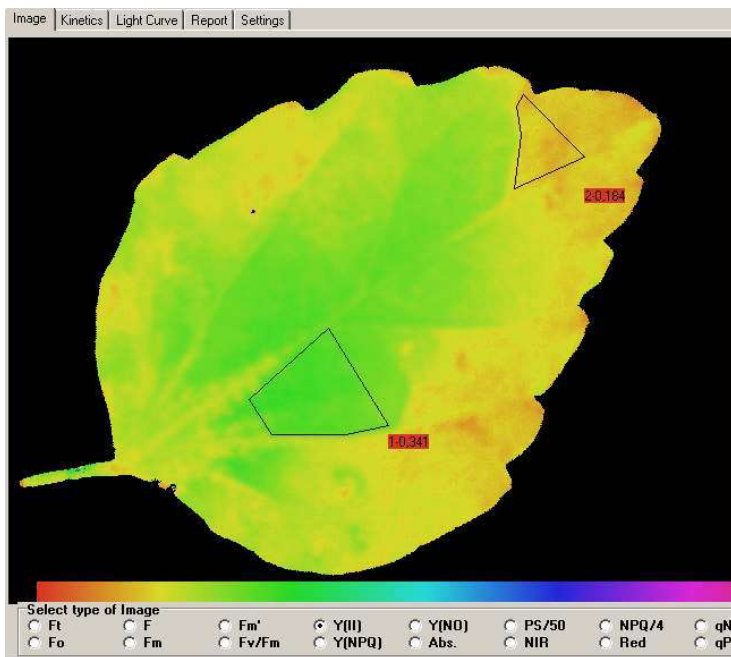


Fig. 59: $Y(II)$ image assessed after 2 min illumination at $81 \mu\text{mol quanta m}^{-2}\text{s}^{-1}$

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 q_N , see also 10.1.1.15) as illustrated in Fig. 60

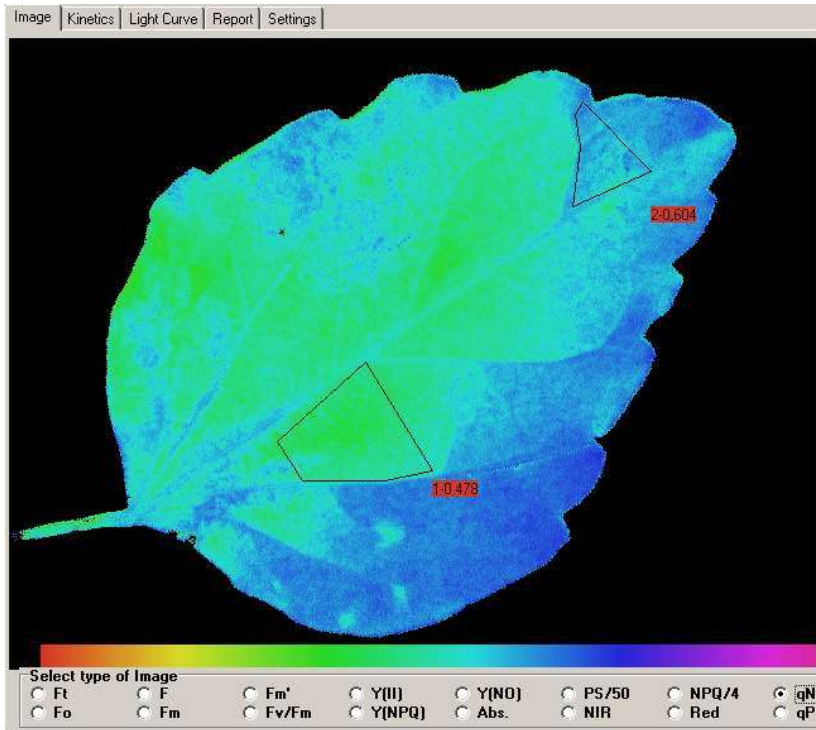


Fig. 60: Image of the coefficient of nonphotochemical quenching q_N measured 2 min after onset of illumination at $81 \mu\text{mol quanta m}^{-2}\text{s}^{-1}$

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”).

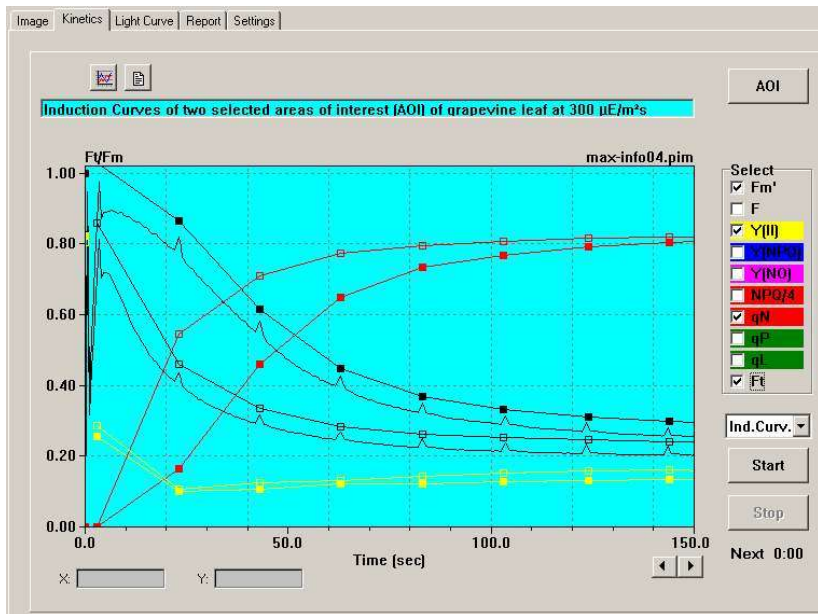


Fig. 61: Kinetics window showing Induction Curve. Two AOIs are selected, for which the averaged pixel values of F_m' , $Y(II)$, qN and F_t are displayed.

Under ImagingWin the recording of an Induction Curve constitutes a **New Record**, which is first stored in a **buffer memory**. It later can be permanently saved on hard disk. An Induction Curve recording normally starts with an **F₀, F_m measurement**, on the basis of which the quenching coefficients are calculated. When the

recording of the Induction Curve is terminated, ImagingWin quits the **Measure-mode** (green check box inactive) and enters the **View-mode**, which allows to look at the recorded data. During the course of an Induction Curve a vast amount of information was stored, which can be analyzed at any time after the recording. Analysis is also possible in the **Off-line mode**, i.e. without the IMAGING-PAM being connected to the PC. For each Saturation Pulse the images of the various fluorescence parameters were captured. 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 shown like in a movie, starting with the data set corresponding to the F_0 , F_m determination. The **Go Speed** can be modified under **Settings** (click the corresponding register card). Images can also be selected manually after deactivating **Go** and clicking with the cursor into 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 the View-mode the data can be stored in form of a so-called **PAM Imaging (pim) file** on hard disk. Individual images can be also exported in form of **TIFF** or **JPEG files**.

Dark-light Induction Curves give important information on various steps of the complex photosynthetic process and allow to identify the site of a possible limitation, e.g. induced by a stress parameter. The IMAGING-PAM allows to apply this tool with high reproducibility using **pre-programmed Standard Induction Curves**. The **Induction Curve parameters**, like Actinic Light Intensity, time interval between Saturation Pulses and duration of illumination can be defined by the user under **Settings**.

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 F_o , F_m 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 F_o , F_m values may be retained, provided there was no change in the position of the sample.

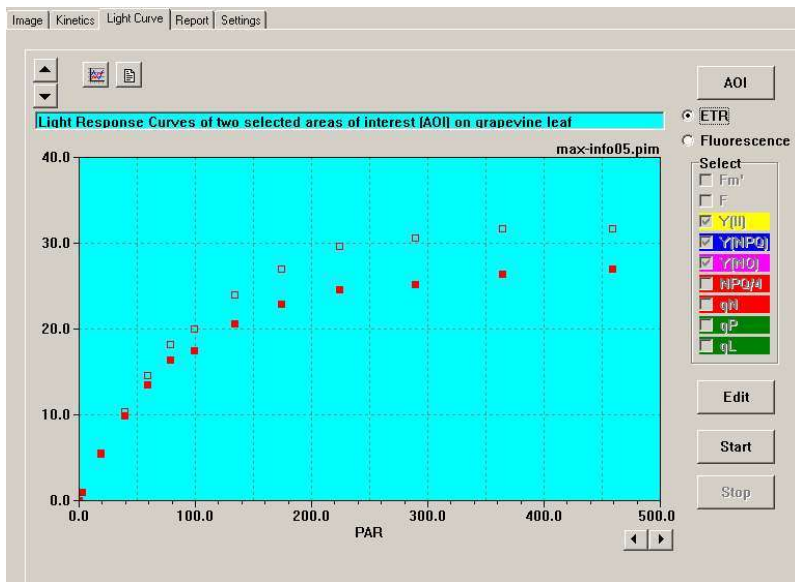


Fig. 62: Light Curve window showing the Light Curves of two AOIs, for which the averaged values of the ETR parameter (relative apparent electron transport rate) are displayed.

In Fig. 62 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:

$$\text{ETR} = \text{Yield} \times \text{PAR} \times 0.5 \times \text{Absorptivity}$$

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 functions surprisingly well, provided the intensity of the red light was appropriately adjusted with respect to the NIR light. A PAR-Absorptivity measurement is started by 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 $\text{Abs.} = 1 - \text{R/NIR}$ and the Abs.-parameter is displayed on the Image-window, as illustrated in Fig. 63

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.

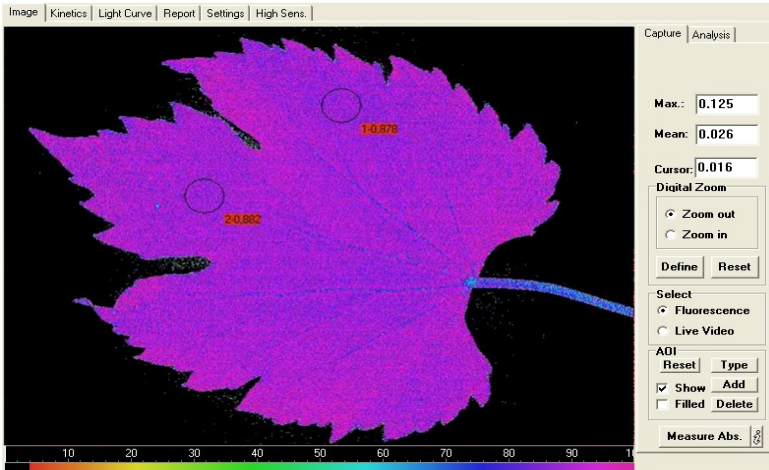


Fig. 63: Image of PAR-Absorptivity determined by the Measure Abs. routine

In the example of Fig. 63 Absorptivity is distributed quite homogeneously (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:

$$\text{PS} = \text{Yield} \times \text{PAR} \times 0.5 \times \text{Abs.}$$

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.

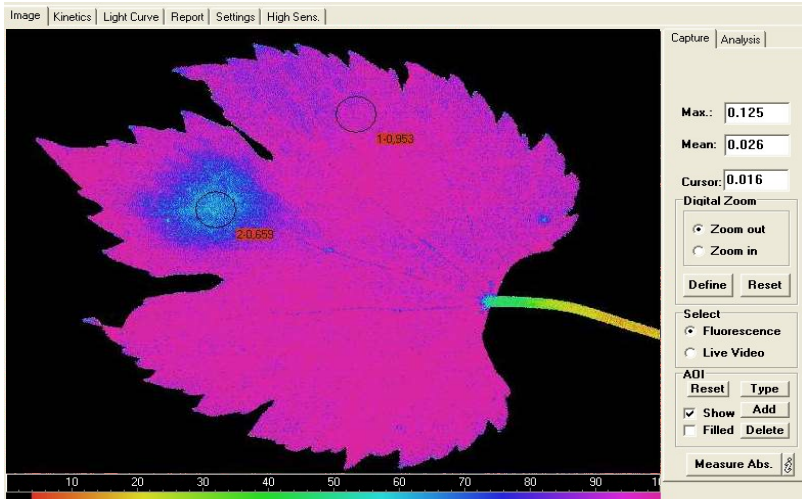


Fig. 64: Image of the relative apparent rate of photosynthetic electron transport as measured by the PS/50 parameter

The PS/50 image displayed in Fig. 64 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. 65 shows the user surface of ImagingWinGigE after start of the program, as seen on the PC monitor screen.

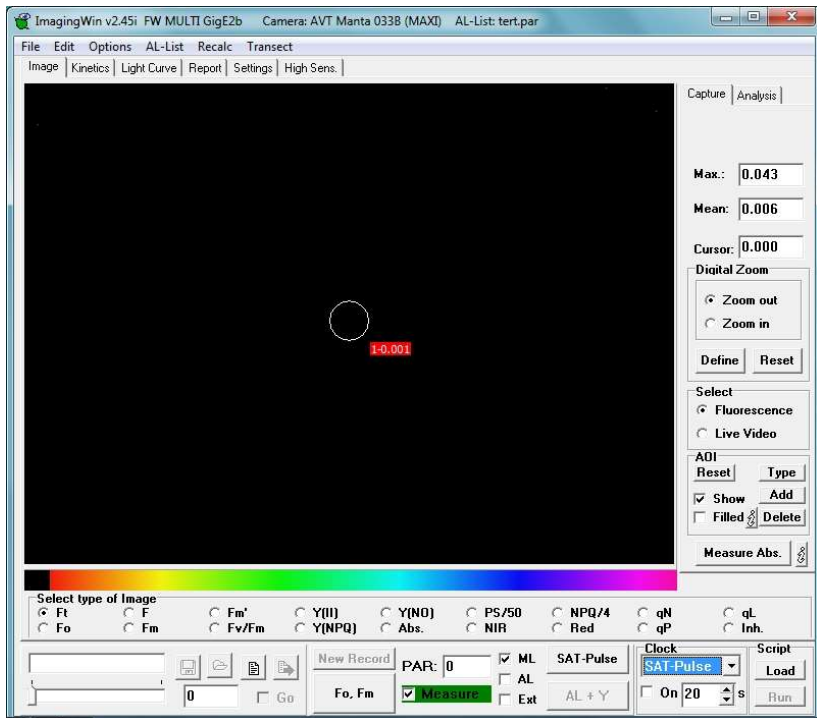


Fig. 65: User surface of ImagingWinGigE after start of the program

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 Fo, Fm-determination is of central importance for recordings with the IMAGING-PAM. Only after appropriate determination of Fo and Fm with a more or less dark-adapted sample, the consequently measured values of the fluorescence parameters q_P , q_N and NPQ will be meaningful. All data recorded after an Fo, Fm-determination are stored as one "Record" in a current buffer memory (see below) and eventually may be saved as a PAM Image (PIM)-file. Please note that upon Fo, Fm determination all data previously stored in buffer memory will be erased. Therefore, the user is asked: **"Save previous Record?"** If this question is answered with "No", the previously recorded data irrevocably are gone. Upon start of a Kinetics recording (see 10.2) or Light Curve recording (see 10.3) the user is asked : **"Do you want to keep the previously recorded Fo, Fm?"** During a running Record no Fo,Fm-determination is possible. Fo- and Fm-images, which can be selected on the Image-window, are prerequisite for calculation of images of Fv/Fm and of the quenching coefficients q_P , q_N and NPQ. The Fv/Fm image not only defines the maximal PS II quantum yield, but also serves for definition of the sample limits. This definition is applied for noise suppression outside the sample limits in Y(II) images.

9.1.2 New Record



Upon start of a New Record previously recorded data stored in the buffer memory are erased in order 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 irrevocably are gone. While normally, a New Record is 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 by application of Saturation Pulses without previous Fo, Fm determination. In this case, however, no quenching coefficients can be calculated and also the noise suppression based on the Fv/Fm image (see above) does not work. A later Fo, Fm-determination within a running Record is not possible. 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

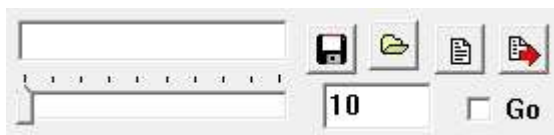


With the help of the Measure-checkbox it is possible to switch between the Measure- and the View-modes. In the **Measure-mode** only the images recorded during the last measurement (last Saturation Pulse) are displayed, whereas in the **View-mode** all previously recorded data of a record can be viewed. In the View-mode the functions located in the box at the left apply. 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. After the data are saved, the Measure-mode is automatically reinstalled, such that the running Record can be continued. Hence, data can be saved successively in the course of a Record.

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).



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.

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 comment (.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 **JPEG-** or **TIFF-files**. A JPEG-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”.

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 Checkbox for switching **Actinic Light on/off** or to start a period of actinic illumination, the duration of which is defined under Settings. When AL is switched on, ML frequency automatically is switched to maximal 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 $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$, which 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).

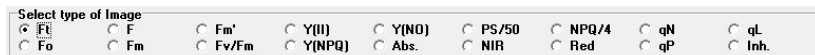
10 ImagingWin - Register Cards

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 F_t) in conjunction with the application of a Saturation Pulse. When a Saturation Pulse is triggered, the current F_t 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, F_m

Maximal fluorescence yield, F_m , can be assessed after dark adaptation using the F_o, F_m -key. The F_m -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 F_m 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 F_m at substantially enhanced sensitivity (see 10.6.1).

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

10.1.1.5 Maximum fluorescence yield, F_m'

In illuminated samples, the maximum fluorescence yield, F_m' , is observed, which normally is lowered with respect to F_m 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 F_m' 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 F_m' at substantially enhanced sensitivity (see 10.6.1).

A given sample can show an infinity of different F_m' -images, depending on the state of illumination at the very moment when the Saturation Pulse is applied. On the other hand, the same sample is characterized by unique F_o - and F_m -images, which are determined with a dark adapted sample (see 10.1.1.4 and 10.1.1.2).

10.1.1.6 Maximal PS II quantum yield, F_v/F_m

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

$$F_v/F_m = (F_m - F_o)/F_m$$

After dark adaptation normally all PS II reaction centers are open ($F = F_o$) and non-photochemical energy dissipation is minimal ($q_N = NPQ = 0$) and maximal fluorescence yield, F_m , is reached during a Saturation Pulse. In this state the fluorescence increase induced by a Saturation Pulse (variable fluorescence, F_v) as well as the PS II quantum yield ($\Delta F/F_m = F_v/F_m$) are maximal. The F_v/F_m image is measured in conjunction with an **F_o , F_m -determination**. It remains unchanged until the next F_o , F_m -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: $F_v/F_m = 0$ if $F_m < 0.048$. All pixel for which this limit is not reached, are displayed in black in F_v/F_m 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:

$$Y(NPQ) = 1 - Y(II) - 1/(NPQ+1+qL(F_m/F_o-1))$$

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 $F_v/F_m = 0$ ("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:

$$Y(NO) = 1/(NPQ+1+qL(F_m/F_0-1))$$

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 NIR LEDs that are integrated in the LED-arrays of the MAXI- and MINI-Heads in the **blue measuring light version**. 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 the incident Red-light which is absorbed by the leaf sample. It is recorded with the help of the "**Measure Abs.**" function (see 10.1.2.1). When the Measure Abs. key is pressed, automatically the sample is first illuminated with Red and then with NIR light and the **R-** and **NIR-images** are recorded. The apparent Absorptivity is calculated pixel by pixel from the R- and NIR-images using the formula.

$$\text{Abs.} = 1 - R/NIR.$$

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 $\text{Abs.} = 1 - R/NIR$ 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 Abs.-value does not necessarily correlate with chlorophyll content per leaf area (as commonly determined by extraction). For example, if the leaf surface is covered by reflecting hairs (pubescence) this will strongly affect PAR-Absorptivity, thus lowering the measured Abs.-values, without affecting leaf chlorophyll content. The same leaf normally shows considerably lower Abs.-values at the lower side compared to 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.):

$$\text{PS} = 0.5 \times \text{Y(II)} \times \text{PAR} \times \text{Abs. } \mu\text{equivalents m}^{-2} \text{ s}^{-1}$$

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:

$$\text{ETR} = 0.5 \times \text{Yield} \times \text{PAR} \times 0.84 \mu\text{equivalents m}^{-2} \text{ s}^{-1}$$

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 function "Measure Abs." (see 10.1.2.1). It provides a measure for the remission of light that is not absorbed by photosynthetically active pigments. For this purpose 780 nm light is applied. The remission includes both reflection and backscattering of the 780 nm light received by the CCD detector. The reflection mainly occurs at the leaf surface, i.e. before the incident light has reached the cell layers containing photosynthetic pigments, and therefore should be similar for 660 nm and 780 nm light. On the other hand, the backscattering is very different for 660 nm and 780 nm light, as most of the 660 nm light in contrast to the 780 nm light is absorbed by the photosynthetic pigments. In this respect, backscattering is similar to transmission, which in a leaf is strongly influenced by light scattering that causes a considerable increase of 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 in conjunction with the function "**Measure Abs.**" (see 10.1.2.1). It is an inverse measure of the absorption of photosynthetically active radiation (PAR) by the investigated sample. The 660 nm light emitted by the red LEDs in the LED-Ring-Array is strongly absorbed by chlorophyll. Hence, only a small fraction of the incident red light is remitted (i.e. reflected and backscattered) from the sample. The same is true for the 450 nm light serving for fluorescence excitation and actinic illumination. However, in contrast to 660 nm, 450 nm cannot penetrate to the CCD-detector, which is protected by a red filter. The intensity of the red light is adjusted such that a highly scattering white sample (white piece of paper or white flower petal) gives the same signal as with the near-infrared (780 nm) reference light. The 780 nm light is emitted by LEDs adjacent to the 660 nm LEDs in the LED-Ring-Array (3.2). Both types of LEDs emit rather diffuse light, thus giving very homogenous illumination of the sample. In contrast to the 660 nm light, the 780 nm light is not absorbed by leaves, as chlorophyll absorption does not extend into this wavelength range. Please note that the R-image is not corrected for heterogeneity of intensity distribution, which in this case mostly consists in a decrease of brightness at the fringes of the image caused by the vignetting effect of the objective lens. As essentially the same effect is observed in the NIR-image, it does not affect the ratio R/NIR and the derived Absorptivity parameter $Abs. = 1 - R/NIR$.

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 q_N requires previous F_o , F_m -determination with the same sample after dark adaptation, i.e. when $q_N = 0$ per definition.

10.1.1.16 Coefficient of photochemical quenching, q_P

The coefficient of photochemical quenching, q_P , 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 q_P requires knowledge of the fluorescence parameter F_o' (minimal fluorescence yield of illuminated sample, which is lowered with respect to F_o by non-photochemical quenching):

$$q_P = (F_m' - F) / (F_m' - F_o')$$

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

$$F_o' = F_o / (F_v / F_m + F_o / F_m')$$

Assessment of q_P requires previous F_o - F_m determination with the same sample after dark adaptation, i.e. when $q_P = 1$ per definition.

While the definition of q_P 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 q_P . The fraction of open PS II centers estimated on the basis of the "lake model" is described by the quenching coefficient q_L (see below).

10.1.1.17 Coefficient of photochemical quenching, q_L

The coefficient of photochemical quenching, q_L , 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 q_L requires previous determination of the fluorescence parameter F_o' (minimal fluorescence yield of illuminated sample, which is lowered with respect to F_o by nonphotochemical quenching):

$$q_L = (F_m' - F) / (F_m' - F_o') \times F_o' / F = q_P \times F_o' / F$$

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

$$F_o' = F_o / (F_v / F_m + F_o / F_m')$$

Assessment of q_L requires previous F_o - F_m determination with the same sample after dark adaptation, i.e. when $q_L = 1$ per definition.

When during illumination nonphotochemical quenching is generated, generally $F_o' < F_o$ and, therefore, also $q_L < q_P$. 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:

$$\text{Inh.} = (\mathbf{Y}_{\text{control}} - \mathbf{Y}_{\text{sample}}) / \mathbf{Y}_{\text{control}}$$

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:

$$\text{Abs.} = 1 - R/\text{NIR.}$$

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

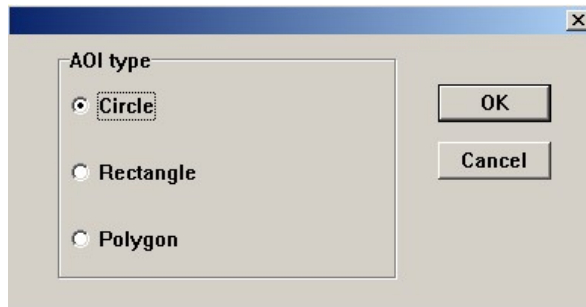
The displayed image is composed of 640 x 480 (i.e. 307200) pixels. Each pixel captures specific fluorescence information, such that in principle e.g. 307200 Light Response Curves could be recorded. In practice, however, it is necessary to reduce this vast amount of information. For this purpose, special **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.



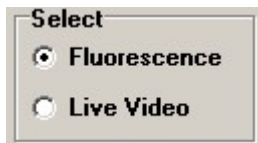
The **AOI Type** has to be selected before clicking "Add"; after clicking "Add" the Type-selection is not accessible.

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-array**, 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)

10.1.2.3 Select: Fluorescence or Live Video



After start of the program the measuring system is in the **Fluorescence Mode**. This means that the fluorescence yield is assessed by the pulse modulated **blue measuring light**. In this way the momentary Ft image is continuously captured. A rather low pulse frequency is applied in order to avoid an actinic effect of the measuring light. Consequently changes of fluorescence images are strongly damped and it is difficult to follow the fluorescence image while moving the sample or trying to focus the image.

When the **Live Video Mode** is selected, the blue fluorescence measuring light is switched off and instead the **NIR measuring light** is switched on, which is neither seen by human eyes nor sensed by the plant. However, the NIR is detected by the CCD camera and thus can serve **for imaging the leaf (monochrome)** using a relatively high measuring pulse frequency, without causing preillumination of the photosynthetic apparatus. Hence the Live Video Mode is useful for positioning the leaf sample in the field of view and for **focusing the image**.



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

When the **Overload box** on the Live Video window is activated, this indicates that the overall amount of light seen by the CCD camera is too high and, hence, the measured NIR image is likely to be disturbed. The overall signal consists of the ambient background light (e.g. from daylight) and the remitted NIR light. Hence, in the case of Overload, either the incidence of ambient light should be reduced (recommended) or the NIR intensity decreased.

Live Video images can be also obtained with continuous light of any external light source, if this light can pass the filters in front of the CCD camera. This feature is particularly important in **Microscopy**-applications using the standard through-light **condenser-illuminator** of the microscope. In this way samples can be readily centered and focused using the PC monitor screen.

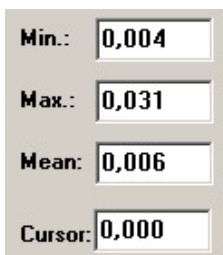
In order to quit the Live Video Mode and to return to the Fluorescence Mode click the **Close** button on the right hand side or the **Exit button** in the upper right corner of the **NIR intensity** adjustment window.

10.1.2.4 Zoom



Standard display of images (**Zoom out**) involves all 640 x 480 (i.e. 307200) pixels. While without Zoom single pixels cannot be seen, they become more and more visible, when the Zoom factor is increased. The standard Zoom factor 2 is applied when **Zoom in** is clicked. Then the 1/4 area in the center is displayed. At that magnification the individual pixels are just visible. The Zoom factor can be defined by the user after clicking the **Define Zoom button**, which is possible only in the Zoom out position. After Define Zoom is clicked, the mouse cursor arrow tip has to be moved to one of the corners of the envisaged Zoom image. This corner is fixed by a mouse click (left or right). Then the desired size of the Zoom image can be adjusted by movement of the diagonal arrow into the diagonally opposite corner of the image rectangle. The final state is fixed by another mouse click. In order to change the Zoom factor, always first **Zoom out** must be selected, before Define Zoom can be clicked again. The **Reset** button serves for reset of the standard Zoom with Zoom factor 2 (display of central quarter).

10.1.2.5 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:

Min. : the minimal value of all pixels

Max. : the maximal value of all pixels

Mean : the mean value of all pixels

10.1.2.6 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. 66 shows a Y(II)-image in normal Color display of a leaf in which the veins display lowered Yield-values. In Fig. 67 the Analysis-image and the settings of the Low-High limits are displayed. Fig. 68 shows the same image with Expanded Color display under Capture.

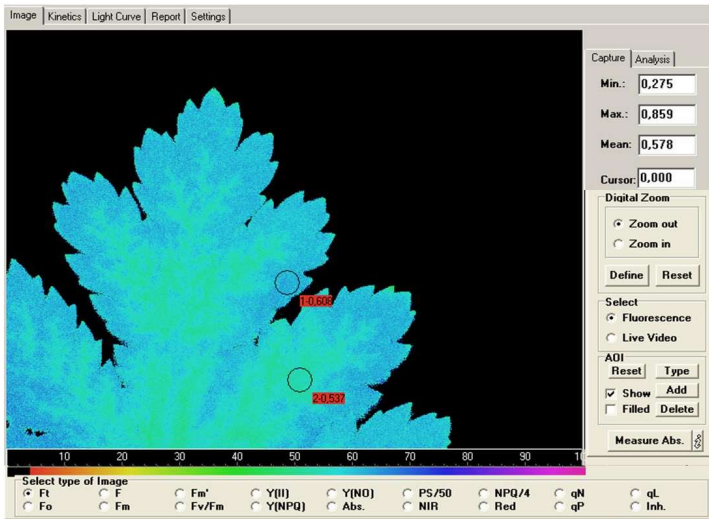


Fig. 66: Normal color display (under capture)

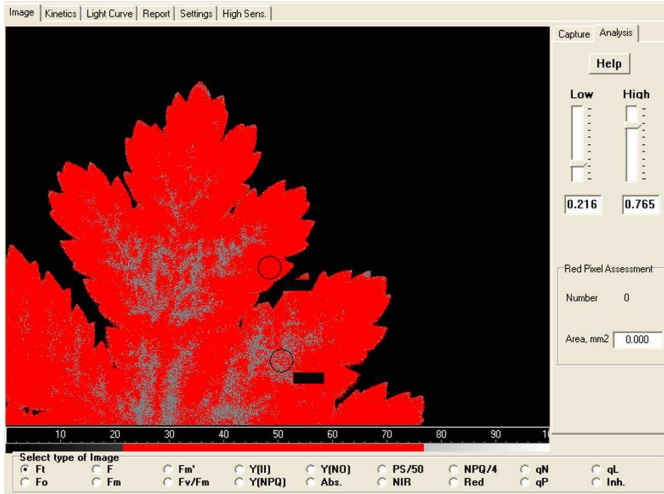


Fig. 67: Display under Analysis with particular settings of Low-High limits

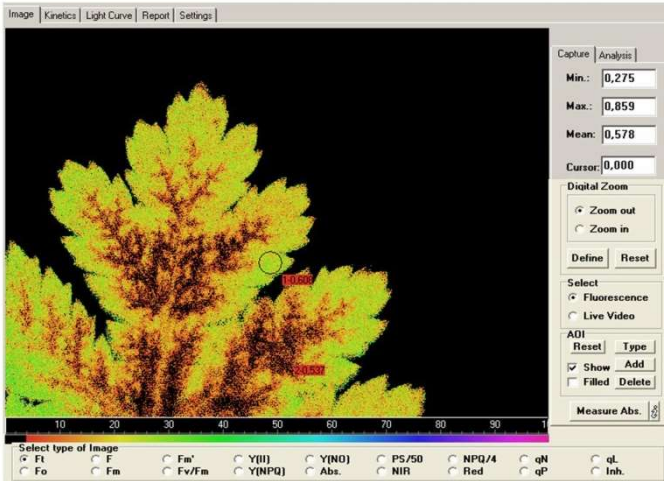


Fig. 68: Expanded Color display (under Capture)

10.2 Kinetics window

On the **Kinetics window** the changes of fluorescence parameters are plotted versus time. Following every F_o , F_m -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.

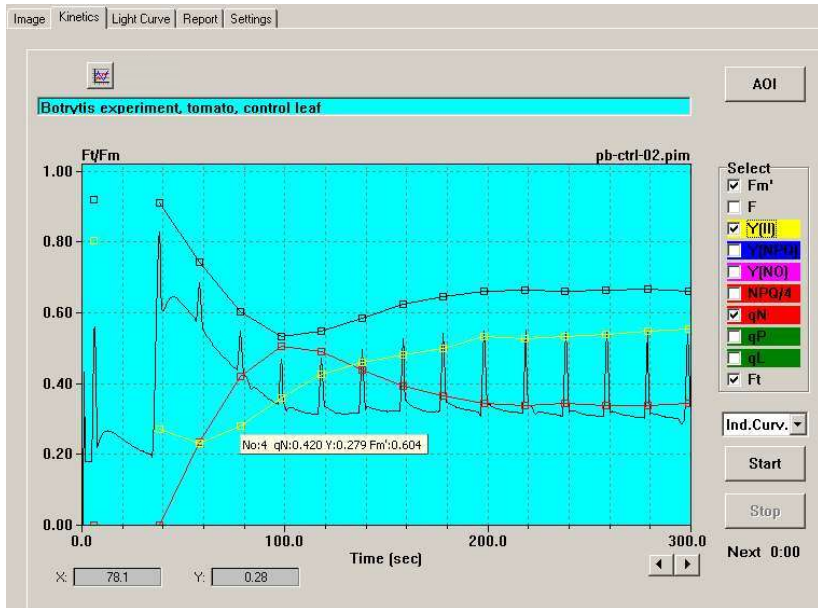
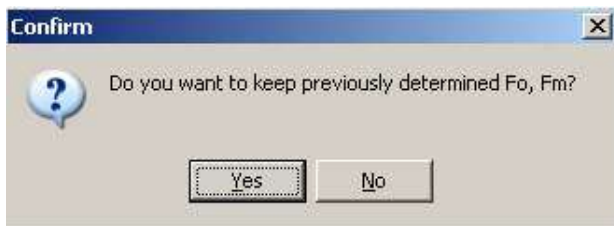


Fig. 69: Kinetics window with display of standard Induction Curve

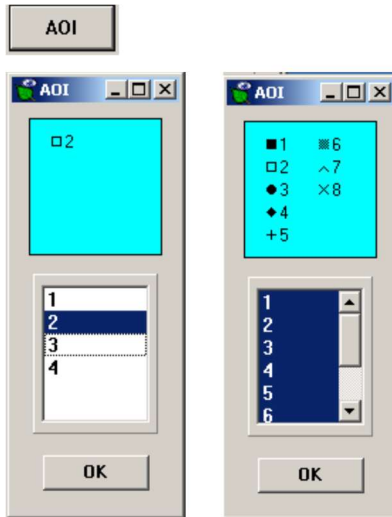
A typical Record of a standard dark-light Induction Curve is displayed in Fig. 69. For the sake of clarity only part of the available fluorescence parameters is displayed (F_m' , $Y(II)$, q_N and F_t) and

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 keep the previously recorded F_o, F_m , when an Induction Curve is measured after a defined preillumination. In this case, the F_o, F_m can be measured before the preillumination and F_o, F_m determination preceding the recording of the Induction Curve can be omitted. Then F_t -



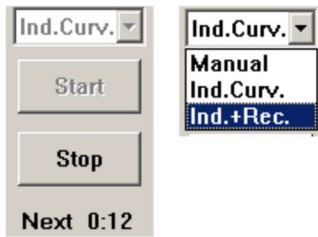
normalization as well as calculation of quenching parameters and of F_v/F_m will be based on the previously determined F_o and F_m values.

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.

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 F_t 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. 70 shows a typical recording of an Induction Curve+Recovery. In this example the Duration-parameter was set to 600 sec.

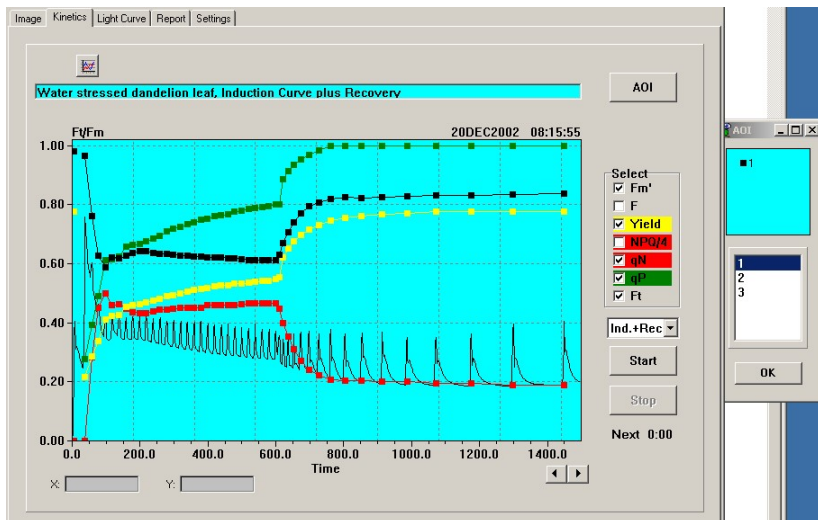
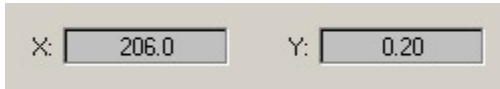


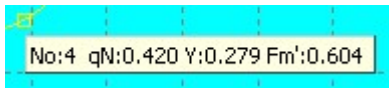
Fig. 70: Typical Induction Curve+Recovery



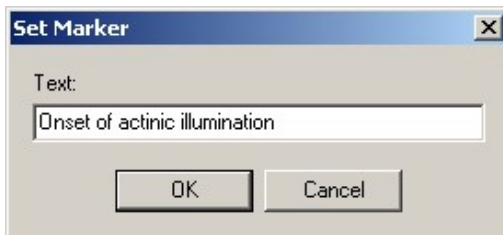
When the **Autoscale** icon is clicked the time scale automatically is changed such that the data fill the Kinetics screen.



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.

10.3 Light Curve window

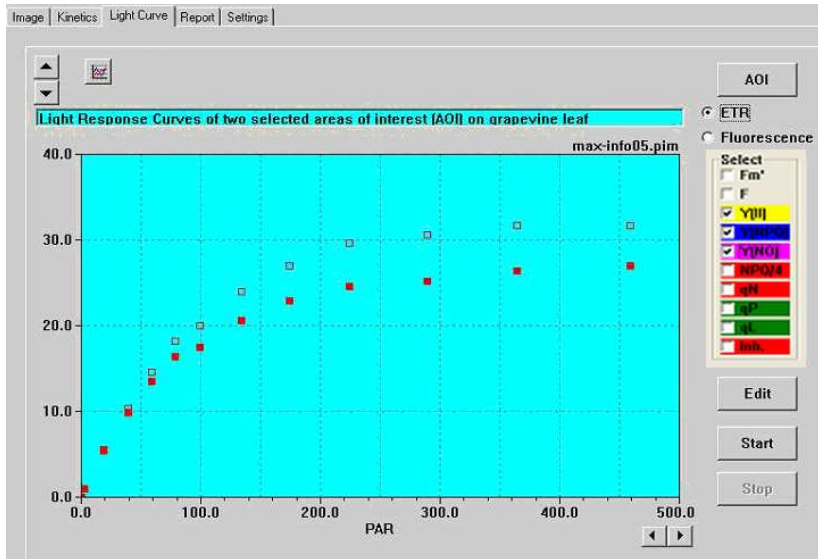


Fig. 71: Light Curve window with display of typical Light Curve of the ETR-parameter

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:

$$\text{ETR} = 0.5 \times \text{Yield} \times \text{PAR} \times 0.84 \mu\text{equivalents m}^{-2} \text{ s}^{-1}$$

(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.

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 F_m' by a few percent will induce a large underestimation of $Y(II)$ and, hence, also of ETR. In this context, the F_m factor is important see 10.5.13), which allows to correct for underestimation of F_m and F_m' 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 F_m Normalization Factor has a strong influence on correct assessment of F_m and F_m' .

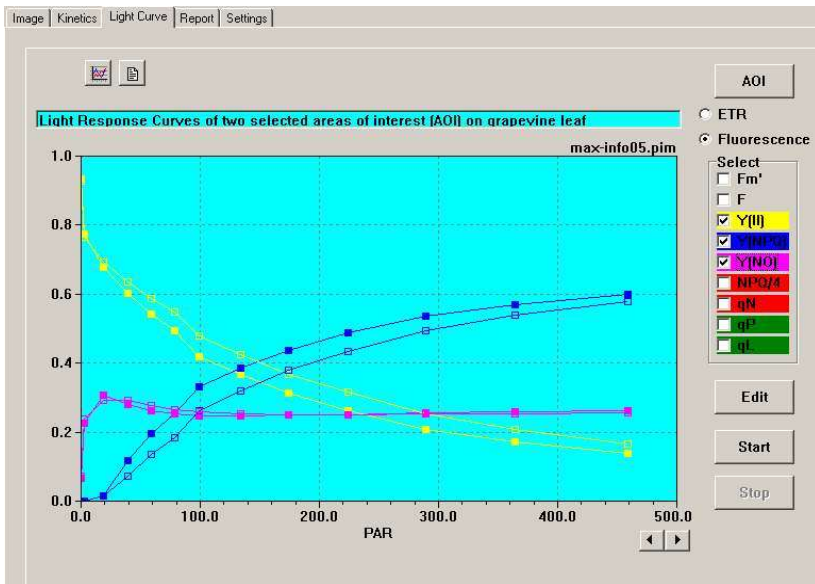


Fig. 72: Light Curve window showing the Light Curves of two AOIs, for which the averaged values of the PS II quantum yield parameters $Y(II)$, $Y(NPQ)$ and $Y(NO)$ are displayed.

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. 72, 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. 73: 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).

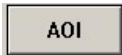


Upon Start automatically the ETR scale limit is set to 50, which can be either changed manually or using the **Autoscale** icon.

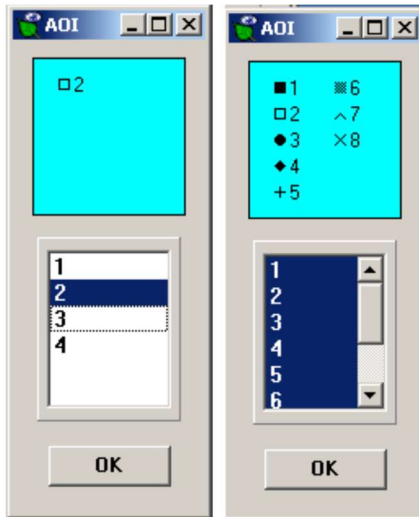


Above the Light Curve the time and date of the recording are documented. The user may also write a short comment into a "**text field**", which is saved together with the Light Curve Record. The text also may be entered or modified in the View-mode. The same text is automatically also written into a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

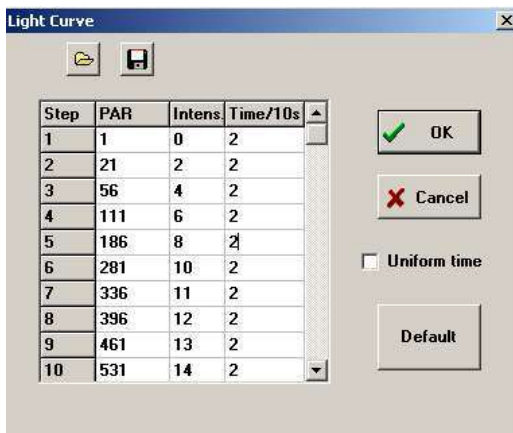


In the View-mode an extensive analysis of the vast information stored during a Light Curve can be carried out.



There is an infinity of possible AOIs (at the Image level) and, hence, in principle also an infinity of Light Curves may be created and analyzed.

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.

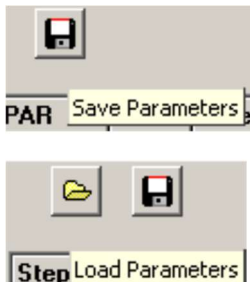


When the **Edit-**button is clicked, a separate window is opened, in which the user may **define the Light Curve parameters**, i.e. the number of illumination steps, the Intensity-setting at each step and the time between

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 $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ 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 losing much time. The lcp-files are saved in the Data-directories of the various Measuring-Heads.

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(III)	Y(III)2	Y(III)
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	0.2868	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

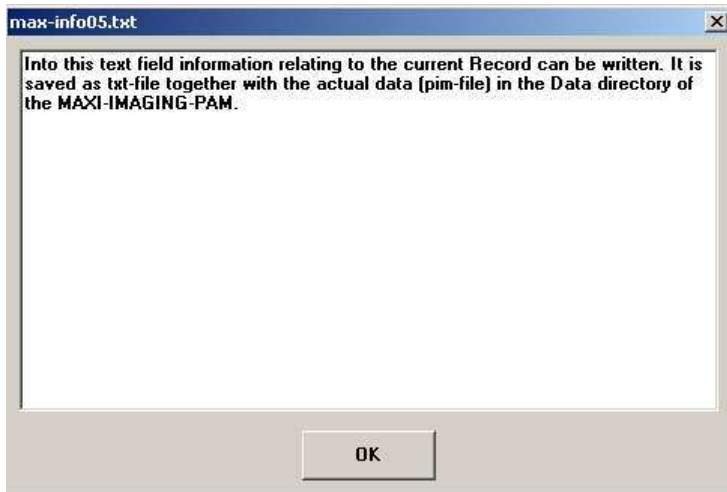
Fig. 73: Report window showing the Record of the Light Curve recording displayed in Fig. 61

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.

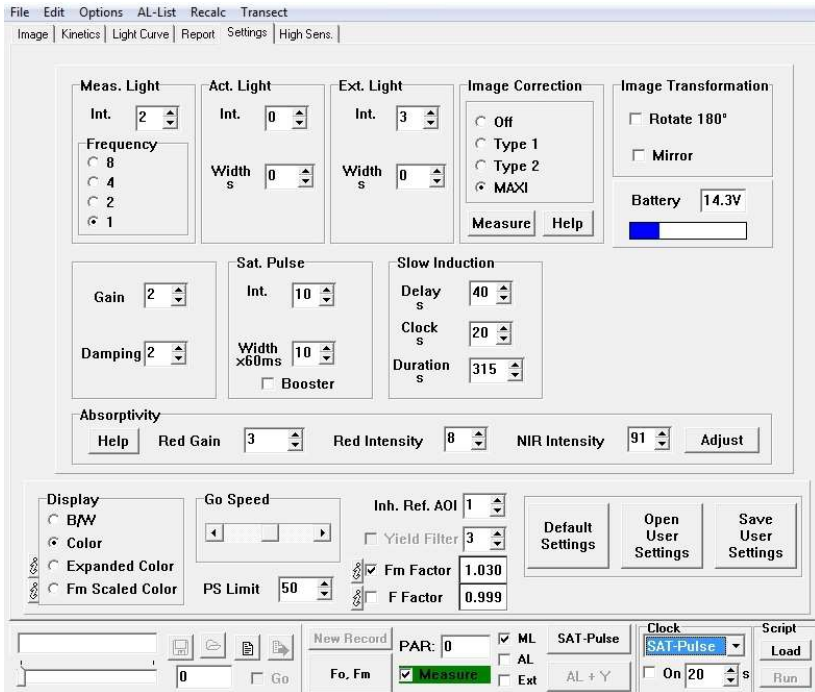
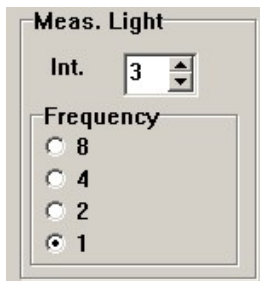


Fig. 74: ImagingWin user surface with Settings window being selected

In Fig. 74 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).

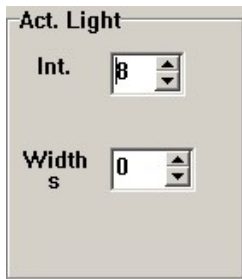
10.5.1 Light parameters

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 μ sec) but very short LED pulses (in the order of 100 μ sec). 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

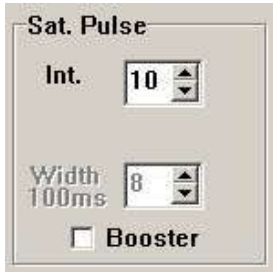
Gain-setting. In this way, the Signal/Noise of F_m , F_m' , F_v/F_m and $Y(II)$ measurements can be considerably enhanced. At a given Meas. Light Int. setting the amplitude of the fluorescence signal can be adjusted by the Gain (see 10.5.2). Meas. Light Int. up to setting 20 can be selected. In this 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, F_v/F_m 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 (F_m or F_m'). Also the fluorescence yield, F_t , 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. As a consequence, the intensity of the emitted light is transiently lowered by about 5%. Unavoidably 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).

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).

When the **Booster**-Checkbox is activated, the maximal possible LED-current is applied during the chosen Saturation Pulse width, which results in a 10 - 15 % increase in Saturation Pulse intensity beyond the normal intensity at setting 10. The additional LED heating effect in the Booster-mode is automatically compensated by a corresponding increase in Measuring Light Intensity for the 800 ms time period of the Saturation Pulse. While in some application the Booster may give somewhat higher Fm' values, in the long run the higher currents will enhance ageing of the LEDs. Therefore application of the Booster is recommended for special applications only. The Booster is not implemented for the MICROSCOPY-versions.

10.5.2 Gain and Damping

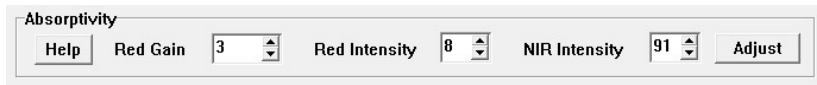


The **Gain** determines the amplitude of the **fluorescence signal, F_t** , at a given setting of Measuring Light Intensity (see 10.5.1). Twenty settings are available, with standard setting 2 for MAXI- and MINI-versions. In the case of the MICROSCOPY-versions, when dealing with relatively low signal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



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.

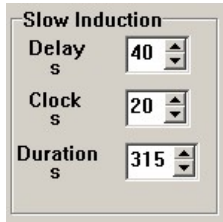
The changes in NIR and Red Settings are recorded in connection with the Absorptivity-measurement (**Measure-Abs.** function, see 10.1.2.1 and 10.1.1.10). The **Red Gain** setting allows **fine adjustment** of the intensity of the Red image.

For each instrument suitable NIR and Red Intensities are determined at the factory and documented together with the corresponding Red Gain on the LED-Array Illumination Unit. 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

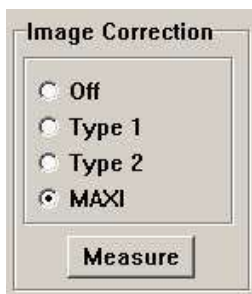
The **Slow Induction Parameters** apply to recordings of preprogrammed dark-light **Induction Curves** in the **Kinetics**



window. After Start and automatic F_0 , F_m determination (see 10.2) some time is given for fluorescence yield to decline back close to the original F_0 -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 F_m' 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 F_0 , F_m 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

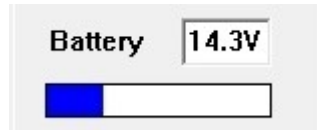


The display of images can be changed by the **Image Transformation** function in order to account for different positions of the camera with respect to the user. Images can be rotated by 180° or mirrored along the vertical midline. Default settings (**Rotate 180°** and **Mirror** boxes not checked) apply 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 month.



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 10.1.2.6). 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).

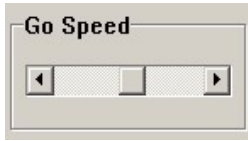
Fm Scaled Color: The Fm Scaled Color can be activated in the **view mode** and serves for emphasizing structures with high fluorescence yield and suppressing structures with low fluorescence yield. This can be particularly useful in Microscopy applications with focused objects displaying high fluorescence 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:

$$PS = 0.5 \times Y(II) \times PAR \times Abs. \mu\text{equivalents m}^{-2} \text{ s}^{-1}$$

(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):

$$\mathbf{Inh.} = (\mathbf{Y}_{\text{control}} - \mathbf{Y}_{\text{sample}}) / \mathbf{Y}_{\text{control}}$$

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



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 F_o, F_m 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 F_o, F_m determination, there is a corresponding warning: "**For changing Fm factor start new record**".

The importance of the Fm Factor increases with PAR, as $\Delta F = F_m' - F$ as well as $Y(II) = \Delta F / F_m'$ decrease and eventually approach zero. For example, when at high PAR values the true ΔF approaches 5.5 % of F_m' , 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 F_m' determination. A systematic underestimation of F_m' and $Y(II)$, however, should be avoided in view of its pronounced effect on Light Response Curves.

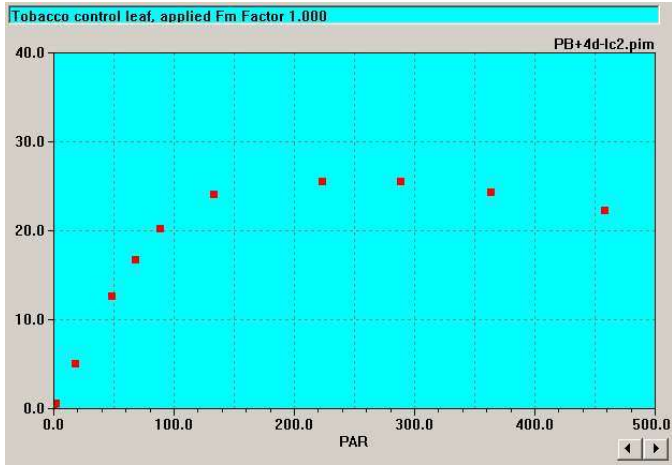


Fig. 75: ETR Light Curve recalculated with Fm Factor 1.000

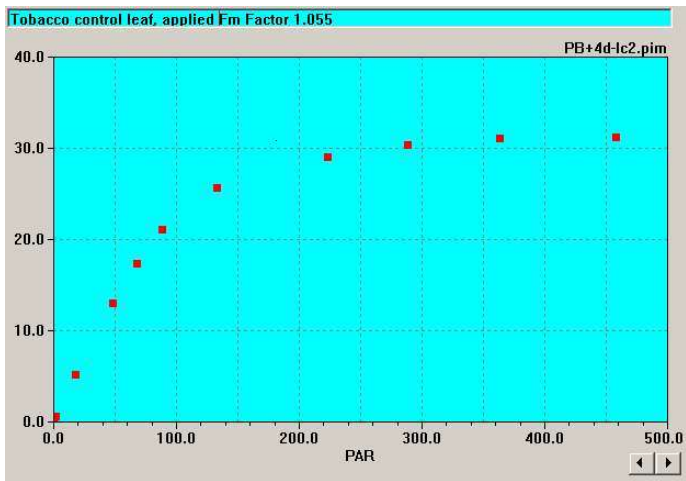


Fig. 76: ETR Light Curve based on the same original data as in Fig. 75, recalculated with Fm Factor 1.055

In Fig. 75 - Fig. 76 the same original Light Curve recording is displayed after recalculation with Fm Factor = 1.000 (Fig. 75) and Fm Factor = 1.055 (Fig. 76). 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 $250 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$, 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 $350 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$, 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.

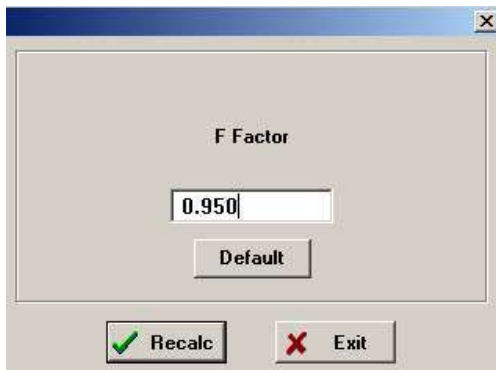
The actual extent of Measuring Light lowering during a Saturation Pulse can be estimated with the help of the plastic **fluorescence standard** delivered with the instrument. As the fluorescence yield of this standard in contrast to that of a living leaf does not change upon illumination with a Saturation 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 can not 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, however, this definition has to be done before the first measurement (normally F_0, F_m deter-

mination). 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 F_0, F_m -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 F_0) 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 F_0 , 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 F_0) (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 F_m' (or F_m). 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 F_0) 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.

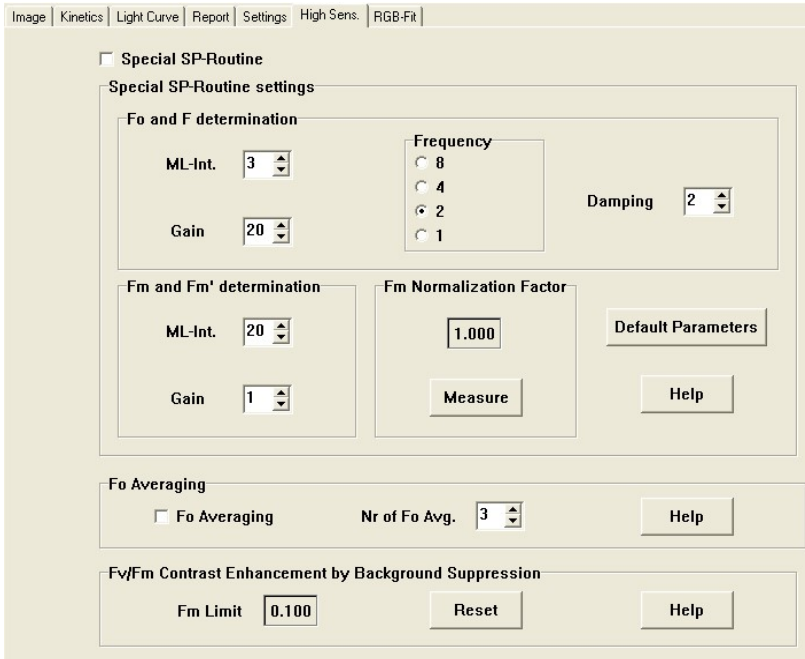


Fig. 77: ImagingWin user surface with High Sens. window being selected

Fig. 77 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 F_0 or F measurements can be disturbed by high measuring light (ML) intensities (unintended closure of PS II centers), F_m or F_m' 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 F_m or F_m' 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 F_m and F_m' 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 F_t) can be selected. Default values are ML3 G9 for MICROSCOPY- and ML2Gain10 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 F_m' determination: These ML-Int. and Gain settings apply during the course of 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

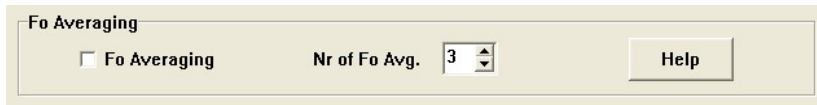
satisfactory images and at minimal Gain setting the noise is quite low.

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 $F_m = F_o$ 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



The screenshot shows a control panel titled "Fo Averaging". It contains a checkbox labeled "Fo Averaging" which is currently unchecked. To the right of the checkbox is the text "Nr of Fo Avg." followed by a numeric spinner box containing the value "3". On the far right of the panel is a "Help" button.

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. $3 \times 12 = 36$ 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.

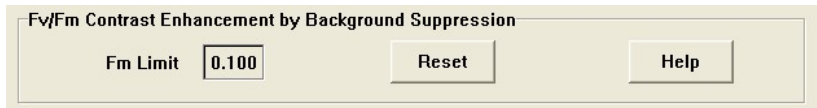
10.6.3 Fv/Fm Contrast Enhancement by Background Suppression

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 it is unavoidable that images show some background from **scattered / reflected fluorescence** as well as fluorescence of non-focused areas. While such “unwanted” fluorescence normally is relatively weak and only marginally disturbs Fo and Fm images, it can strongly affect **Fv/Fm** and **Y(II) images**, which are calculated from ratios of fluorescence

values and, hence, have lost the information on the original signal amplitudes. This leads to a loss of contrast, so that structures that can be well discerned in F_o or F_m images may not show in F_v/F_m and Y(II) images. F_v/F_m pixel associated with low F_m pixel values can be eliminated by setting an appropriate F_m Limit. In this way the disturbing effect of a weakly fluorescing background can be suppressed and the contrast of F_v/F_m images is greatly enhanced. The same applies to Y(II) images.

Definition F_m Limit: For all pixel displaying F_m values below this limit, F_v/F_m will be displayed black. The default F_m Limit value is 0.100, which can be reinstalled via Reset.



A new F_m Limit value can be entered after a left mouse click on the F_m Limit box, which opens a dialog window into which the new value can be typed and confirmed by O.K.

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.

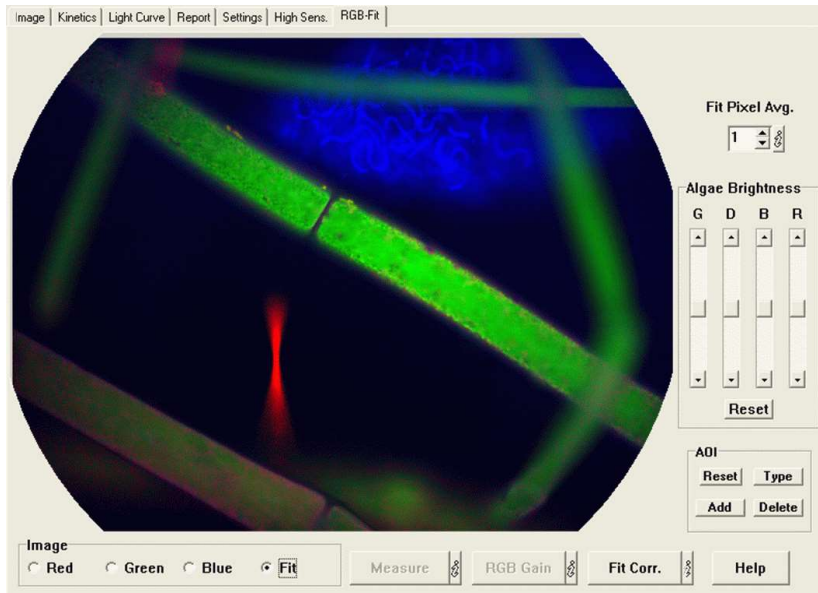


Fig. 78: RGB-Fit window showing RGB-Fit image with deconvoluted diatoms, chlorophytes and cyanobacteria

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

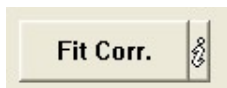
fixed to 1.000, the Red and Blue Gains can be modified either manually or via a special Measure routine (see below). Upon instrument delivery Red and Blue Gains are set to 1.000, as the Red and Blue ML intensities are adjusted to give appropriate R/G and B/G ratios for optimal fitting using the standard 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 is in contrast to the RGB Gain factors.

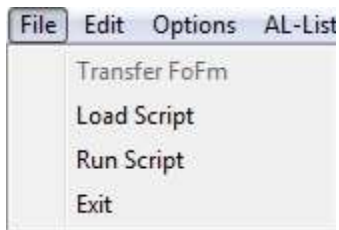
Definition of AOIs is fully equivalent on RGB-Fit and Image-windows (see also 10.1.2.2). With the help of the Fit-image it is possible to define various AOIs that specifically represent particular organisms (e.g. a diatom attached to a filamentous green alga). Consequently the photosynthetic performance 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

File Edit Options AL-List Recalc Transect

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

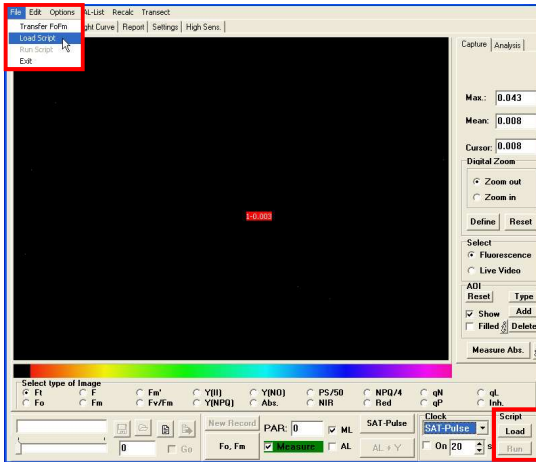


11.1.1 Transfer FoFm

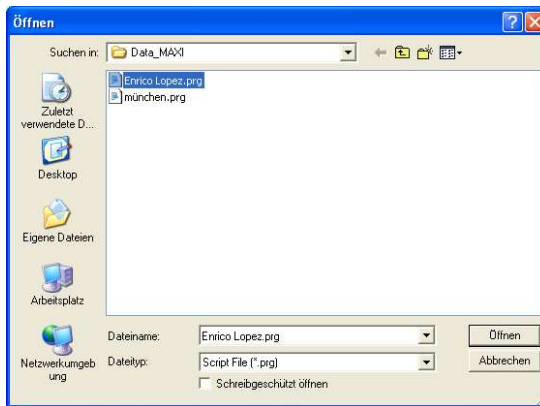
In the view mode a FoFm measurement can be transferred to another .pim file by enabling Transfer FoFm in the file menu. Data will be recalculated with the given parameter.

11.1.2 Using Skript files - Load Script/Run Script

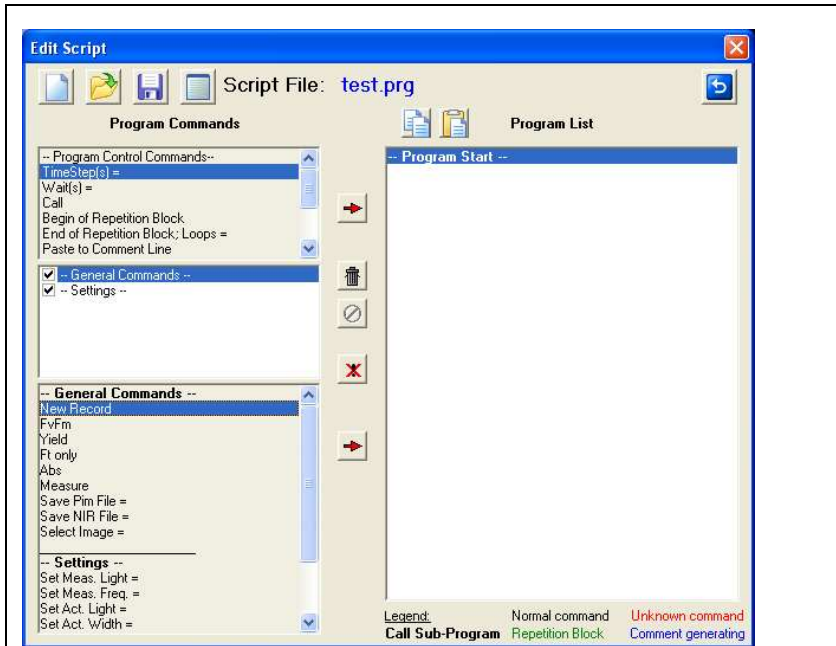
There are two ways to load an existing or to create a new script file. The load script command in the file menu or push the load script file button in the bottom right corner:



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 has to 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

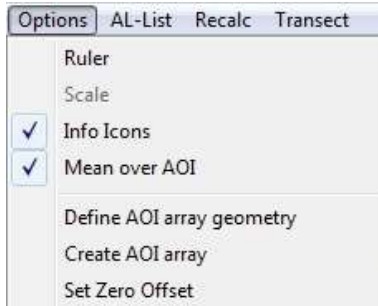
Following the whole procedure can be copied by marking the respective command lines in the listing window and copying with the  button. The button  transfers the copied lines to the lines under the cursor position.

The diskette symbol button saves the script file with the given name and the ending “.prg” in the data folder of the PAM instrument.

11.1.3 Exit

Exit quits ImagingWin.

11.2 Options

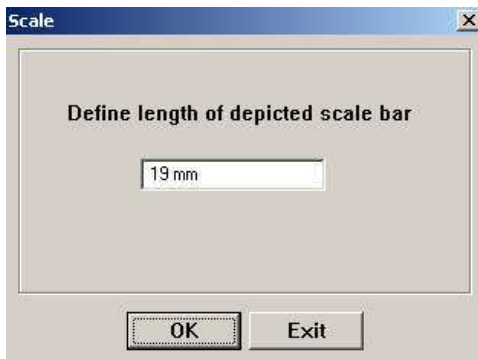


11.2.1 Ruler



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 $F_v/F_m > 0$. 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.

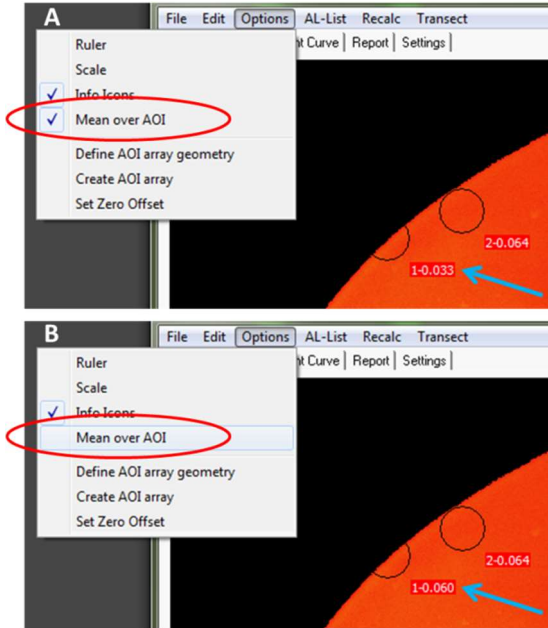
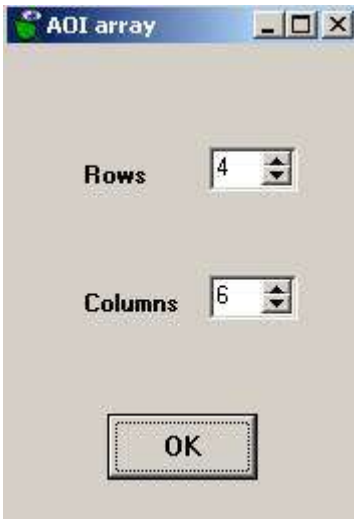


Fig. 79: Mean over AOI enabled (A) and disabled (B)

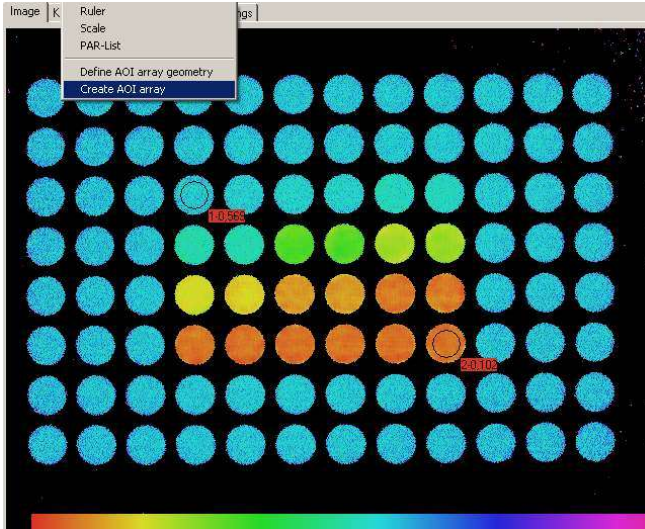
11.2.5 Define AOI-array geometry



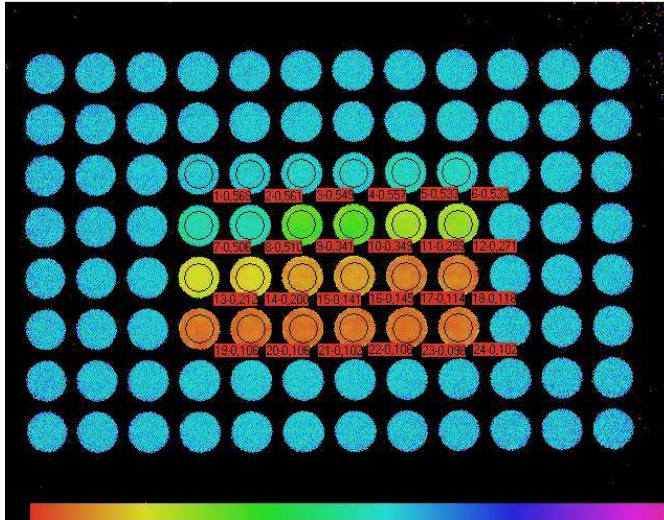
After clicking "Define AOI-array geometry" a window is opened in which the number of rows and lines of the array grid can be defined. After confirmation by O.K. this definition will remain valid until a new definition is carried out. Before an AOI array can be created, the position of this array within the overall image has to be defined. This is done by definition of the AOIs in

the **upper left** and **lower right** corners of the array.

11.2.6 Create AOI array:

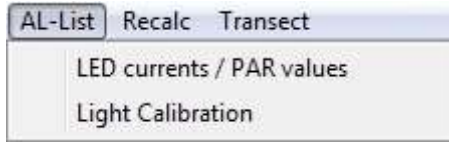


After the two corner AOIs are defined, the **Create AOI-array Option** can be carried out and the corresponding array will be displayed after some delay (calculation time).



In the above example an array of 24 AOIs with 4 rows and 6 lines in the center of a 96 well microtiter plate was defined. While all wells were filled with aliquots of algae suspension, only the 24 wells covered by the array contained increasing concentrations of the PSII inhibitor diuron, which is reflected by decreasing values of $Y(II)$.

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.

11.3.1 LED currents / PAR values

AL	Current	PAR
0	0	1
1	4	10
2	8	20
3	14	35
4	22	55
5	32	80
6	44	110
7	58	145
8	74	185
9	92	230
10	112	280
11	134	335
12	158	395
13	184	460
14	210	530
15	242	610
16	279	700
17	320	800
18	372	925
19	434	1075
20	508	1250

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 $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$. 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 microquantum 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.

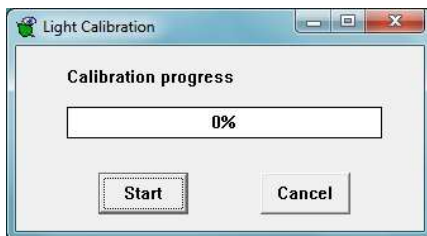
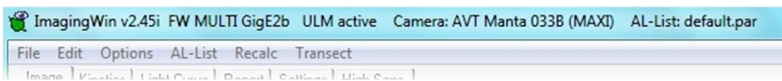
When dealing with leaves, it has to be considered that as a consequence of pronounced light gradients within the samples, the effective PAR at a particular setting does not correspond to one

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.



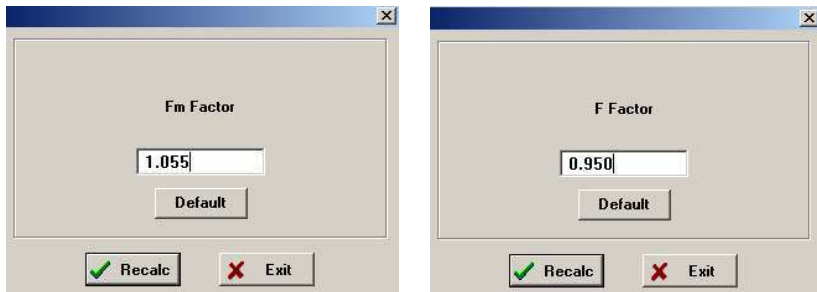
Clicking Light Calibration in the AL-List menu opens up a Light Calibration window. Please place the micro quantum sensor at the object's position and start the automated calibration

routine for the 20 light intensities by pressing Start.

11.4 Recalc

Fm Factor
F Factor

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 on the basis of 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

Define Endpoints
Plot Profile

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.

InputDlg2

Line width (1..5)

3

Endpoints

X1: 234 Y1: 238

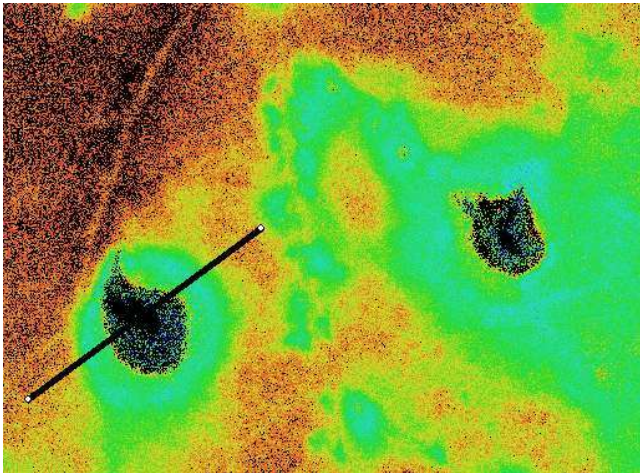
X2: 38 Y2: 331

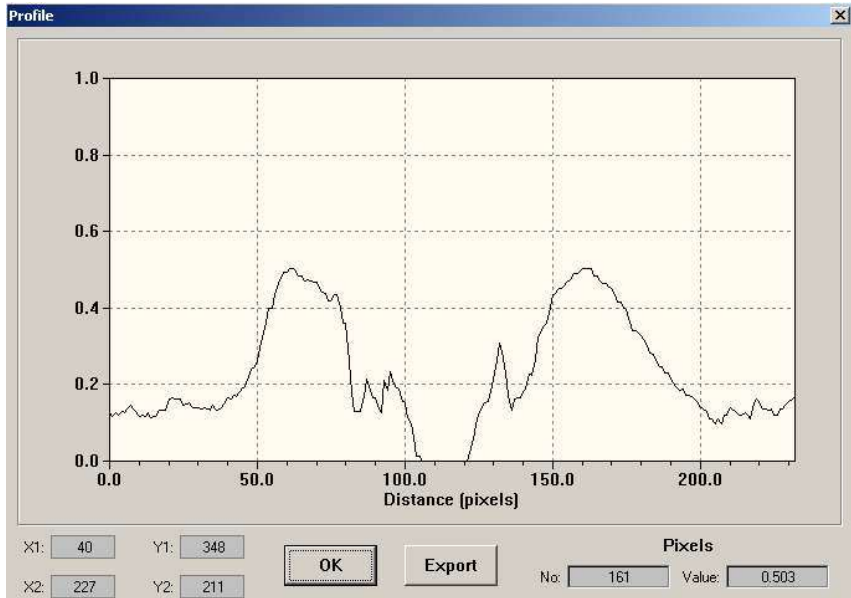
Graphic

OK

Exit

Upon clicking **Define Endpoints** a dialog window is opened in which the user may choose one out of 5 **line widths** and define the **coordinates** 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 at a later time. 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:	Pentium PC with ImagingWinGigE Software; connection via GigE Ethernet; keyboard operation; monitor screen display
Power supply:	Internal rechargeable Li-ion battery 14.4 V/6 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 30 frames/sec featuring
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 objective lens)
Weight:	<200g

13.1.3 IMAG-K6

Design:	Black and white C-mount camera operated in 10-bit-mode at 30 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 objective lens)
Weight:	<200g

13.1.4 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)

13.1.5 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 $\mu\text{mol}/\text{m}^2\text{s}$ PAR, modulation frequency 1-8 Hz; max. actinic intensity 2300 $\mu\text{mol}/\text{m}^2\text{s}$ PAR; max. saturation pulse intensity 5000 $\mu\text{mol}/\text{m}^2\text{s}$ 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 $0.5 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ PAR, modulation frequency 1-8 Hz; max. actinic intensity $1900 \mu\text{mol/m}^2\text{s}$ PAR; max. saturation pulse intensity $3700 \mu\text{mol/m}^2\text{s}$ 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 aluminium 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 objective lens (F1.0/f=8-48 mm) and close-up lens, extension ring, detector filter (RG645, 3 mm) and short pass interference filter ($\lambda < 770$ nm); adapter-frame for mounting CCD-camera MAG K7 at increased working distance.
Dimensions:	57 x 95 mm
Weight:	430 g (without adapter-frame)
Filter Screw Size:	$\phi 55$ 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 objective lens (F1.2/f=12 mm), extension ring and detector filter (RG645, 3 mm) and short-pass interference filter ($\lambda < 770$ 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 objective lens (F1.4/f=12.5 mm), detector filter (RG665, 3 mm) and short-pass interference filter ($\lambda < 770$ 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 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.

13.2.9 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

13.2.10 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

13.2.11 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 70 cm (L x W x H),

Weight: 455 g

13.2.12 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

13.2.13 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 x 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 ccm (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 NVIDIA 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
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intensity, 3700 $\mu\text{mol}/\text{m}^2\text{s}$; max. Saturation Pulse intensity, 8200 $\mu\text{mol}/\text{m}^2\text{s}$; 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 $\mu\text{mol}/\text{m}^2\text{s}$; max. Saturation Pulse intensity, 7700 $\mu\text{mol}/\text{m}^2\text{s}$; 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: Cosmincar-Pentax objective lens (F1.4/f=16 mm) detector filter (RG645, 3 mm) and short-pass interference filter ($\lambda < 770$ 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: Cosmincar-Pentax objective lens (F1.4/f=25 mm) detector filter (RG645, 3 mm) and short-pass interference filter ($\lambda < 770$ 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.A1 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 Fluor 20x are already mounted.

13.5.2 IMAG-L470M

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-L625M

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 specifications are subject to change without prior notice.

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