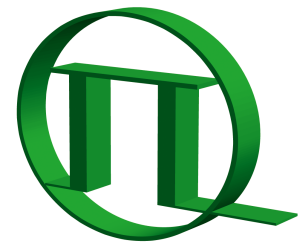


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# FLIMbee

Galvo scanner for Micro Time 200



PICOQUANT



User Manual  
Version 1.2

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## **How to use this manual**

This manual describes the hardware, maintenance, and operation of the FLIMBee add-on for the MicroTime 200 time-resolved fluorescence microscopy platform.

It assumes that you are familiar with the basic functionality and operation of your MicroTime 200 system as described in the separate MicroTime 200 user's manual.

Furthermore, it assumes that you are also familiar with the basic functionality and operation of the SymPhoTime 64 software, which is used to control the MicroTime 200. Please refer to the online help of the SymPhoTime 64 for additional details.

Detailed tutorials for different analysis methods are available on our PicoQuant TCSPC Wiki: <http://www.tcspc.com>.

General information and additional information, as well as tutorials and technical notes related to the MicroTime 200 system can be found at the following places:

- PicoQuant Homepage  
[www.picoquant.com/scientific/technical-and-application-notes#](http://www.picoquant.com/scientific/technical-and-application-notes#)
- PicoQuant Support Online Form  
[support.picoquant.com](http://support.picoquant.com)
- PicoQuant Forum  
[forum.picoquant.com](http://forum.picoquant.com)
- The TCSPC wiki  
[www.tcspc.com](http://www.tcspc.com)
- PicoQuant YouTube channel  
[www.youtube.com/user/PicoQuant](http://www.youtube.com/user/PicoQuant)

## **Support and Serial Number**

When asking for support, please always mention the serial number of your system. Since most of components also have individual serial numbers, finding the systems serial number can sometimes be a bit tricky.

**The serial number is an 8 digit number in the form of 010XXXXX.**

If your system has a "Need support?" card, then this card contains its serial number:



If you cannot find the "Need support?" card then the serial number of your MicroTime 200 can be found on a sticker located on the back side of the Main Optical Unit (MOU).



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# 1. General safety information



## CAUTION!

Before using this device, make sure that you have read and understood the content of this user manual. Store this documentation in a safe and easily accessible place for future reference.

Incorrect handling of this product may result in personal injury or physical damage. The manufacturer assumes no responsibility and cannot be held liable for any injury / damages resulting from operating the device outside of the normal usage defined in this manual.

## 1.1 Warning Symbols and Conventions

The following symbols and conventions will be used throughout this manual. Please take time to familiarize yourself with their meaning before proceeding.

	The <b>general safety alert symbol</b> is used to alert you to hazards that may lead to personal injury or physical damage. Follow all associated safety instructions to avoid possible injury or death.
	A <b>high voltage warning symbol</b> is used to indicate the presence of un-insulated, dangerous voltage inside the enclosure. Note that this voltage may be sufficient to constitute a risk of shock.
	The <b>laser radiation warning symbol</b> alerts you that the device can generate laser radiation. Follow all applicable laser safety instructions to avoid injury or damages.
	The device's susceptibility to electrostatic discharge (ESD) is indicated by the <b>ESD warning symbol</b> . Ensure that you follow proper ESD protection rules to avoid damaging the device.
<b>CAUTION!</b>	Make sure to follow any instructions prefaced with " <b>CAUTION!</b> " to avoid personal injury or damaging the device.
<b>WARNING!</b>	The " <b>WARNING!</b> " label prefaced any instructions that have to be followed to avoid severe injury or death.
<b>NOTICE</b>	Important tips and information for device operation that do not include a risk of injury or damage are prefaced with the " <b>NOTICE</b> " label.
	This symbol indicates that an earth terminal needs to be connected to the ground (to avoid risks of electrical shock).

## 1.2 Electrical power

The delivered instruments are preset by PicoQuant to be operated with either 115V or 230 V AC. The system has a single IEC 320-C14 type male power inlet. The customer is responsible to provide a proper power supply cord (one end with female IEC 320-C13 plug). The total current consumption of a standard system is 4 A at 230 V AC.

## 1.3 Laser Safety Instructions



### **WARNING!** Visible and invisible laser radiation

The FLIMbee comes with one or more laser diode heads that can emit visible, infrared, or UV light. Infrared light is not visible to the eye! **These diode lasers can emit laser light of up to class 3b / IIIb.** Please refer to the labels affixed to the laser head for information on classification.

Lasers for two photon excitation deliver infrared emission which is in most cases not visible for the eye. These lasers emit light of laser class 4 / IV. Laser class 3B / IIIb and 4 / IV require that special laser safety glasses are worn. The installation room of the MT200 must be labeled as laser area. For class 4 / IV lasers, a laser safety officer has to be announced to meet the laser safety regulations.

The lasers in the MicroTime 200 STED can emit near infrared and visible laser light. Near infrared light is only visible to the eye at high laser power levels. **The lasers included in the MicroTime 200 STED can emit light of up to laser class 4 / IV.**

Lasers can be hazardous and have unique safety requirements. Permanent eye injury and blindness is possible if lasers are used incorrectly. Pay close attention to each safety related CAUTION and WARNING statement in the user manual. Read all instructions carefully BEFORE operating this device.

The laser diode heads of the LDH Series are manufactured according to the International Laser Safety Standard IEC 60825-1:2007 and comply with the US law 21 CFR §1040.10 and §1040.11.

### Required Laser Safety Measures

Please observe the laser safety measures for class 3b / IIIb lasers in accordance with applicable national and federal regulations. The owner / operator is responsible for observing the laser safety regulations.

### What does the owner / operator have to observe?

- The owner / operator of this product is responsible for proper and safe operation and for following all applicable safety regulations.
- The owner / operator is fully liable for all consequences resulting from the use of the laser for any purposes other than those listed in the operating manual. The laser may be operated only by persons who have been instructed in the use of this laser and the potential hazards of laser radiation.
- The owner / operator is responsible for performing and monitoring suitable safety measures (according to IEC/EN 60825-1 and the corresponding national regulations).
- The owner / operator is also responsible for naming a laser safety officer or a laser protection adviser (according to the standard IEC/EN 60825-1: "Safety of laser products, Part 1: Classification of systems, requirements and user guidelines" and the respective national regulations).
- When using lasers of **class 4 / IV**, it is required to wear special eye protection (laser safety goggles).
- The room in which the MicroTime 200 STED is installed must be labeled as a laser area.

**The following security instructions must be followed at all times.**

## General Safety Instructions for Operation

- Never look directly into a laser beam or a reflection of the laser beam. Avoid all contact with the laser beam.
- Do not introduce any reflective objects into the laser beam path.
- Every person involved with the installation and operation of this device has to:
  - Be qualified
  - Follow the instructions of this manual
- As it is impossible to anticipate every potential hazard, please be careful and apply common sense when operating the laser diode heads and associated driver unit. Observe all safety precautions relevant to Class 3b / IIIb and Class 4 / IV lasers
- Use of controls or adjustments or performance of procedures other than those specified herein may result in hazardous radiation exposure.
- The laser power levels accessible if the unit is opened can cause instant blindness, skin burns and fires. Class 3B / IIIb or 4 / IV lasers can present a major hazard through exposure to the direct (intra-beam) or reflected (specular or diffuse) laser beams when the laser is inadvertently “on” and there is a direct line-of-sight path to the laser beam or its reflection. If your instrument uses another excitation system, follow the safety instructions of the relevant manual.
- Do not open the filter turret cover when the lasers are powered on and the lids on the MOU and LCUs as well as the protecting cover are closed. Make sure that no light of laser class 4 / IV can reach the microscope body before opening the filter turret cover.
- Never remove the optical fiber from the MOU when the lasers are powered.
- Do not disable the interlocks on the LCUs and MOU or the AUX / STED shutter.
- The delivered instruments are pre-set by PicoQuant to operate on the power outlet line voltage for the country of delivery. Nevertheless, please check that the actual line voltage corresponds to the value set on these instruments!
- If pulsed lasers from PicoQuant are present at the system, please check that the actual line voltage corresponds to the value set on the PDL-800-D laser driver!
- Never connect or disconnect any cable while the data acquisition and control electronics are ON. Charged signal cables can destroy the devices!
- Protect the photon detectors (SPAD, PMA, PMA Hybrid) as much as possible, particularly from excessive light intensities, e.g. room light, a microscope illumination lamp, unattenuated backscattered excitation light, etc. The SPAD detector(s) must be switched off using the remote control unit BEFORE switching off the system by the main power button.

### 1.4 Safety Labels on the Laser Device

The safety labels are affixed to the laser head housings, with an arrow pointing towards the laser emission aperture (see Fig. 1.1 for some examples). The label states the laser product classification, certification, power, and wavelength range relevant for the classification.

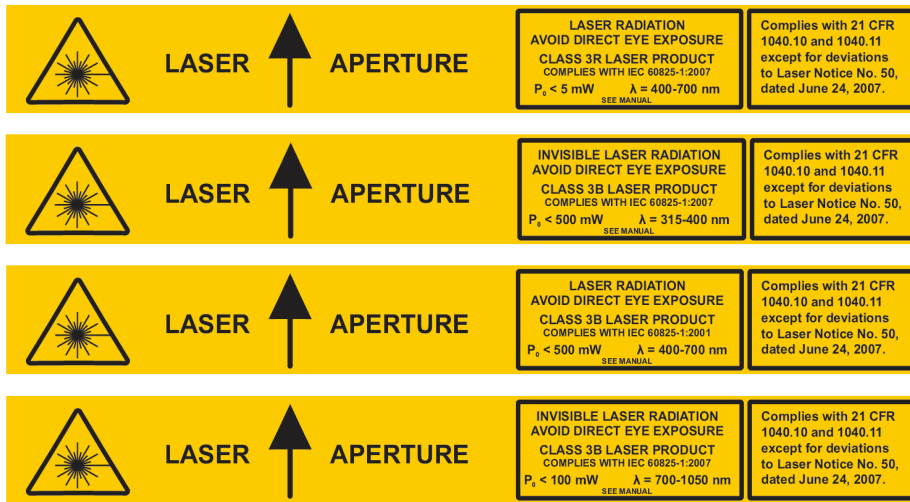


Fig. 1.1: Example of laser warning, certification, and classification label on laser heads of the LDH Series. Top: labels for cylindrical laser heads; bottom: location of such a label on a laser head from PicoQuant's LDH Series.

Please refer to the *laser delivery report* in the Appendix for information on the central emission wavelength, maximum achievable optical output power, and pulse shape of the delivered LDH laser heads.

### 1.5 Safety Labels on the device

Safety labels are located on different parts of the MicroTime 200 as shown below. It also shows the different safety labels for systems equipped with the STED and FLIMbee option. (see Fig. 1.2 for their location).

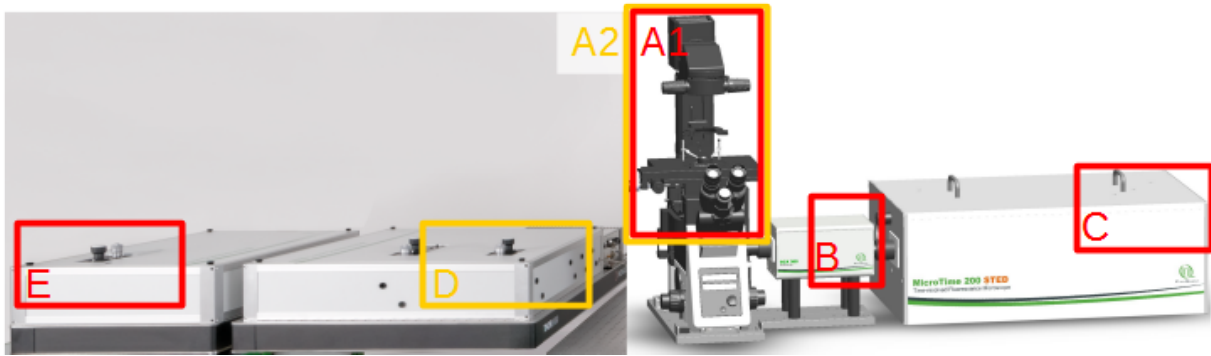


Fig. 1.2: MicroTime 200 overview with the locations of the laser safety labels. **Red:** Standard MT200 with FLIMbee, **Orange:** STED add on

<p>Fig. 1.3: “Danger – Class IIIB” warning label on the lid of the MOU and the Main LCU <b>(C, E)</b></p>	<p>Fig. 1.4: Laser warning label used in combination with other laser safety label (on the lid and the side of the Main- and STED-LCU, on the lid and the backside of the MOU, the lid of FLIMbee, on the XY table of the microscope body) and on any possible laser radiation exit port and on all Mirrors inside the MOU. <b>(A1, A2, B, C, D, E)</b></p>
<p>Fig. 1.5: “Avoid exposure” warning label: marking the objective on the microscope body as open aperture for laser radiation. <b>(A1, A2,)</b></p>	<p>Fig. 1.6: “Laser Aperture” warning label: marking the objective and the condenser on the microscope body as well as all fiber coupling exit ports of MOU as open aperture for laser radiation. <b>(A1, A2,C)</b></p>
<p>Fig. 1.7: Classification &amp; certification label for laser class 4 on the front of the microscope body, the side of the STED LCU and on the backside of FLIMbee. <b>(A2, B, D)</b></p>	<p>Fig. 1.8: Classification &amp; certification label for laser class 3B next to the laser input ports on the backside of the MOU, the side of the Main LCU and the front of the Microscope body. <b>(A1, C, E)</b></p>
<p>Fig. 1.9: “Danger – Class 4” warning label on the lid of FLIMbee and the STED-LCU <b>(B, D)</b></p>	<p>Fig. 1.10: “Caution – Class 4 Laser when open” warning inside FLIMbee when lid is open <b>(B)</b></p>



## 1.6 Remote Interlock Connector

A hardware lock as well as a remote interlock connector are part of the laser driver that is included with your FLIMbee. Removing the connector or breaking the interlock circuit will immediately deactivate the power supply of the laser.

In order to meet laser safety regulations, you may need to install a remote interlock, e.g., a door switch, to deactivate the power to the laser when the door to the laser area is opened.

Please refer to the corresponding laser driver manual for further details on the remote interlock connector.

## 1.7 Third-party lasers

PicoQuant does **NOT** endorse the use of non-approved third-party laser devices with the FLIMbee system. The end-user should never connect laser devices from other manufacturers to or operate the FLIMbee with them.

PicoQuant declines any responsibility and can not be held liable for any direct or indirect damages to the users and/or instrumentation resulting from the use of non-approved third-party laser devices by the end-user.

<b>CAUTION!</b> Use of controls or adjustments or performance of procedures other than those specified herein may result in hazardous radiation exposure!
---

## 1.8 Laser Classification for the Different MicroTime 200 Parts

The complete MicroTime 200 system with FLIMbee add-on is classified as laser **class 3B / IIIB** device. However, some of the subunits of the system exhibit different laser classifications depending on their operating conditions. The table below summarizes these classifications.

### Classification for MicroTime200 with FLIMbee

Component	Status	Laser classification
Main LCU	Lid closed	1 / I
	Lid open	3B / IIIB
MOU	Lid closed	1 / I
	Lid open	3B / IIIB
Microscope body	In all cases	3B / IIIB
FLIMbee	Lid closed	1 / I
	Lid open	3B / IIIB

→ The FLIMbee galvo scanner is classified as a **Class 3B / IIIB** device, when the lid is open. The device itself does not contain any lasers, so its classification depends on the lasers used with the MicroTime 200.

### Classification for MicroTime 200 STED with FLIMbee

Component	Status	Laser classification
STED LCU	Lid closed	1 / I
	Lid open	3B / IIIB
Microscope body	In all cases	4 / IV
FLIMbee	In all cases	4 / IV

As the FLIMbee galvo scanner is an add-on for MicroTime 200 systems, where hazardous radiation exposure of up to laser **class 4 / IV lasers** is possible, the classification of FLIMbee is set to laser class **4 / IV** as well in case of a MicroTime200 STED system.

In general, the user is not supposed to open the FLIMbee housing at any time. Use of controls or adjustments or performance of procedures other than those specified herein may result in hazardous radiation exposure of up to laser **class 4 / IV**. PicoQuant does not endorse the use of controls, adjustments, or performance of procedures other than those specified in this manual and excludes any liability in such cases.

According to the information given in the table for the STED system, two use case scenarios can be distinguished: *operation* and *adjustment*.

#### a.) Operation mode for MicroTime200 STED with FLIMbee

In this mode of operation, all system lids are closed, including the LCUs, MOU, filter turret cover (see Fig. 1.11), and protection cover over the sample. Under these conditions, the LCUs and the MOU can be treated as devices of laser class 1 / I. The microscope body and FLIMbee, however, is classified as laser **class 4 / IV** device, as stray light from the STED laser can escape from it.



Fig. 1.11: Filter turret cover at the inverted microscope body

When the sample protection cover is removed to change the sample or objective, the shutter in front of the STED laser closes automatically. In this situation, the complete system is subjected to the regulations of laser **class 3B/ IIIB**.

Please keep in mind that the MicroTime 200 system has an overall classification of laser **class 4 / IV** when it is running with an active STED laser. Therefore, the appropriate laser safety measures as described in the later parts of this chapter have to be followed.

#### **b.) Adjustment mode for MicroTime200 STED with FLIMbee**

When performing the adjustment procedures described in this manual, the covers of the LCUs and / or MOU are open. Thanks to the interlocks installed in the LCUs and MOU, the shutter in front of the STED laser will automatically close when the respective lids are removed. Thus, the system is subjected to the regulations of laser **class 3B / IIIB** during adjustment procedures

#### **Other Modes of Operation**

Use of controls or adjustments or performance of procedures other than those specified herein may result in hazardous radiation exposure of up to laser **class 4 / IV**. PicoQuant does not endorse the use of controls, adjustments, or performance of procedures other than those specified in this manual and excludes any liability in such cases.

## 2. System Layout for MicroTime 200 with FLIMbee Galvo Scanner

The FLIMbee Galvo Scanner for the MicroTime 200 provides additional components making it possible to perform beam scanning with your MicroTime 200 system. The diagrams in this section present these components along with the relevant conventional confocal microscope parts.

### 2.1 Overall System Layout

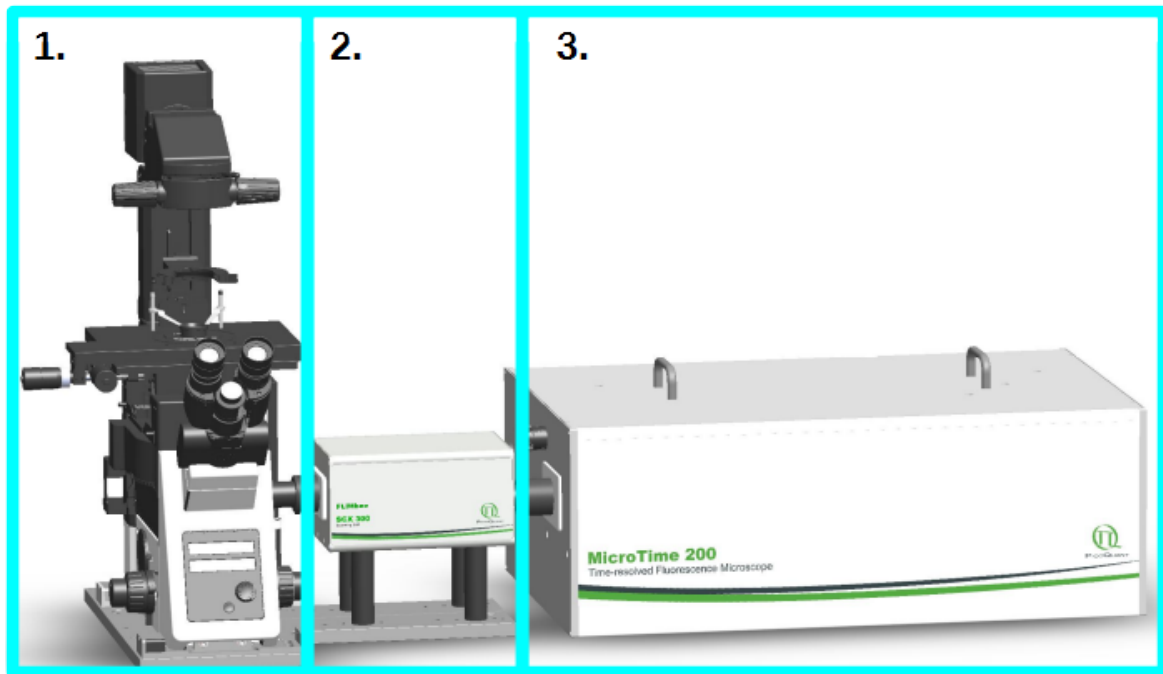


Fig. 2.1: MicroTime200 STED system with FLIMbee upgrade

#### System components:

1. Microscope body
2. Laser scanning Unit (FLIMbee)
3. Main Optical Unit (MOU)

## 2.2 Main Optical Unit (MOU) Layout

The MOU contains all necessary optics required to perform confocal microscopy, including a second beam path used for STED excitation. The MOU can accommodate up to 2 detectors and 3 beam splitter towers, which can be used to route the fluorescence signal to either detector, to a fiber exit port, or into a Detector Extension Unit (DEU) should further detectors be required.

As the MicroTime 200 is a customizable microscopy platform, the layout of your MOU may be slightly different from the one shown in Fig. 2.2. Further details on customization options can be found in the Appendix for your system.

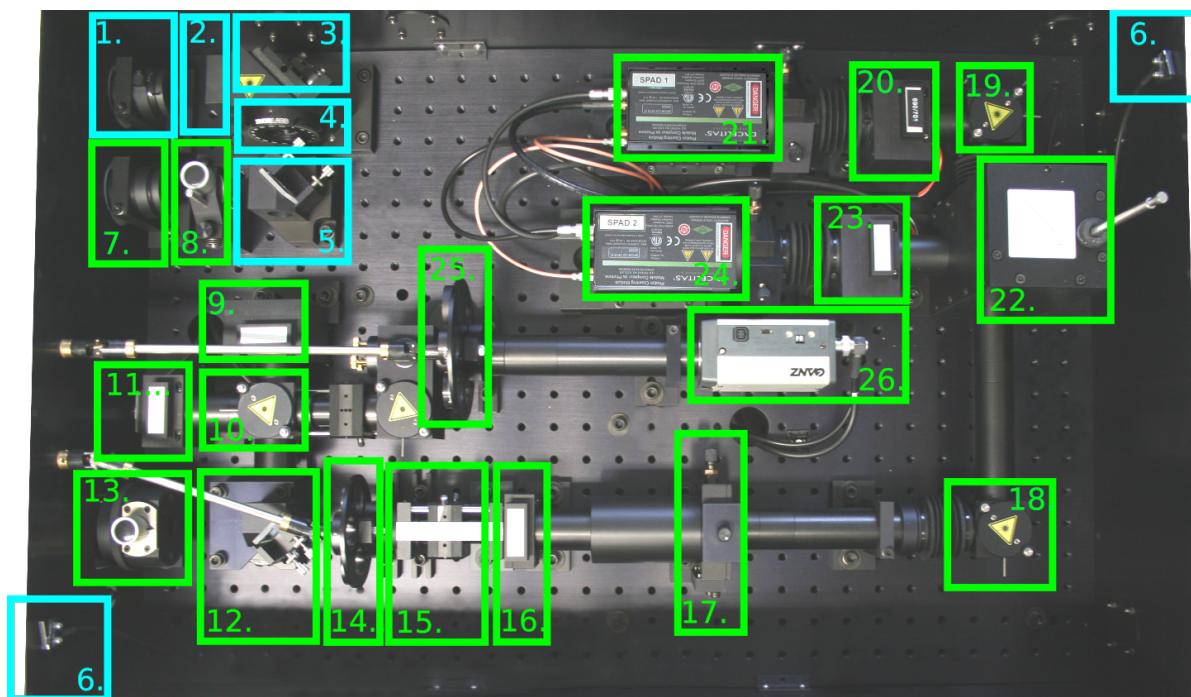


Fig. 2.2: Layout of the main optical unit: Units 1-6 are part of the STED Galvo Scanner, while units 9-26 belong to the standard confocal configuration.

### MOU STED Components:

1. STED fiber out coupler
2. Polarizer
3. Mirror 0 (M0)
4. Achromatic quarter wave plate
5. Beam combiner (M1)
6. STED laser interlocks

### MOU Standard Components:

7. Confocal fiber out coupler
8. Beam displacer for confocal beam path
9. Excitation filter holder and shutter
10. Beam splitter (M2)
11. Photo diode with filter holder
12. Major dichroic
13. Beam displacer → for STED the Beam displacer is on the outside of the MOU
14. Detection filter wheel

15. Detection tube lens
16. Detection filter holder
17. Pinhole adjuster
18. Mirror 5 (M5)
19. Mirror 6 (M6)
20. Detector 1 filter holder and shutter
21. Detector 1
22. Beam splitter tower
23. Detector 2 filter holder and shutter
24. Detector 2
25. Diagnostic filter wheel
26. Diagnostic camera

Detection filters acting on both detectors can be placed at positions 12 and 14. Alternatively, such filters can also be placed in front of each single detector at positions 18 and 21, where they will affect only the respective detector.

## 2.3 Microscope Body

The microscope body used for the MicroTime 200 is either a standard Olympus IX-73 or IX-83 with some custom built parts installed. Additional FLIMbee specific components are also incorporated.

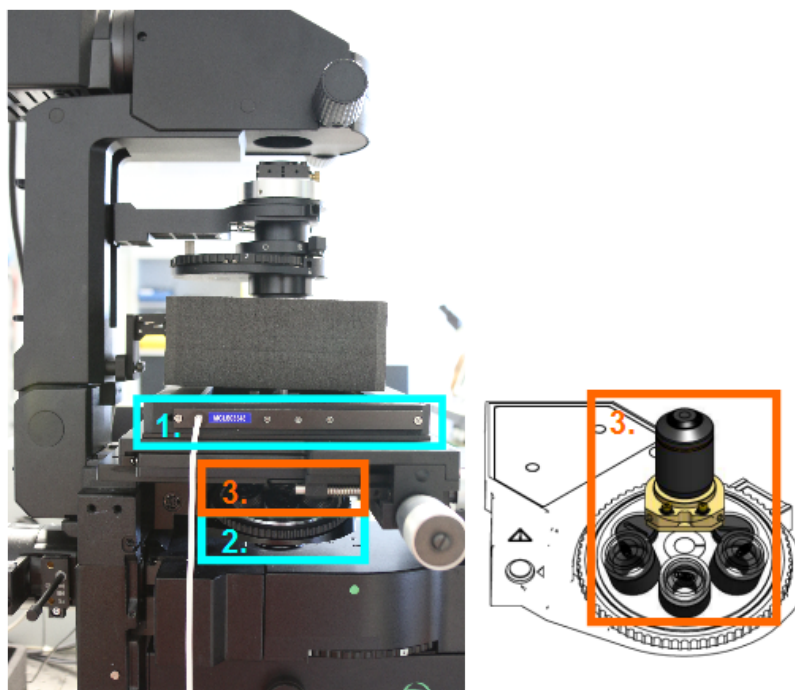


Fig. 2.3: Microscope body showing the components installed as part of the FLIMbee Galvo Scanner

### Microscope body FLIMbee Galvo Scanner Components:

1. Z-table NanoDrive
2. Objective turret

### Additional microscope body FLIMbee Galvo Scanner Components for STED:

3. STED-phase plate and extensions for objectives when used without phase plate

## 2.4 FLIMbee Galvo Scanner



Fig. 2.4: FLIMbee Galvo Scanner

The FLIMbee is a galvo beam-scanning system using three galvanometer driven mirrors. Due to the design of the scanner optics, the laser beam is not moving at the entrance lens of the objective.

The FLIMbee Galvo Scanner unit contains both the scan lens and scanner block.

**NOTE:** The housing of the FLIMbee Galvo Scanner (see Fig. 2.4) should remain closed at all times and not be opened by the user!



## 2.5 Data Acquisition and Control Electronics

All electronics controlling the MicroTime 200 are contained within a wheel mounted 19" rack mounted. The exact configuration will vary depending on your particular MicroTime 200 system.



Fig. 2.5: Microscope controller 19" rack front side (left) and back side (right) showing the control electronics for the MicroTime 200 and FLIMbee Galvo Scanner

### Microscope Controller Standard Control units (locations of the different units can differ):

1. Laser driver (see separate manual for details)
2. MCL NanoDrive (for z-stage)
3. TCSPC electronics (slot empty if a TimeHarp 260 is used)
4. FLIMbee scan control (+ inside the Controller: FLIMbee power supply (not visible from outside))
5. Power supply

### Microscope Controller STED Galvo Scanner Control Units:

6. Laser Interlock



## 2.6 Computer Controlled Oscillator Module with Electronic Delay (SOM-D)

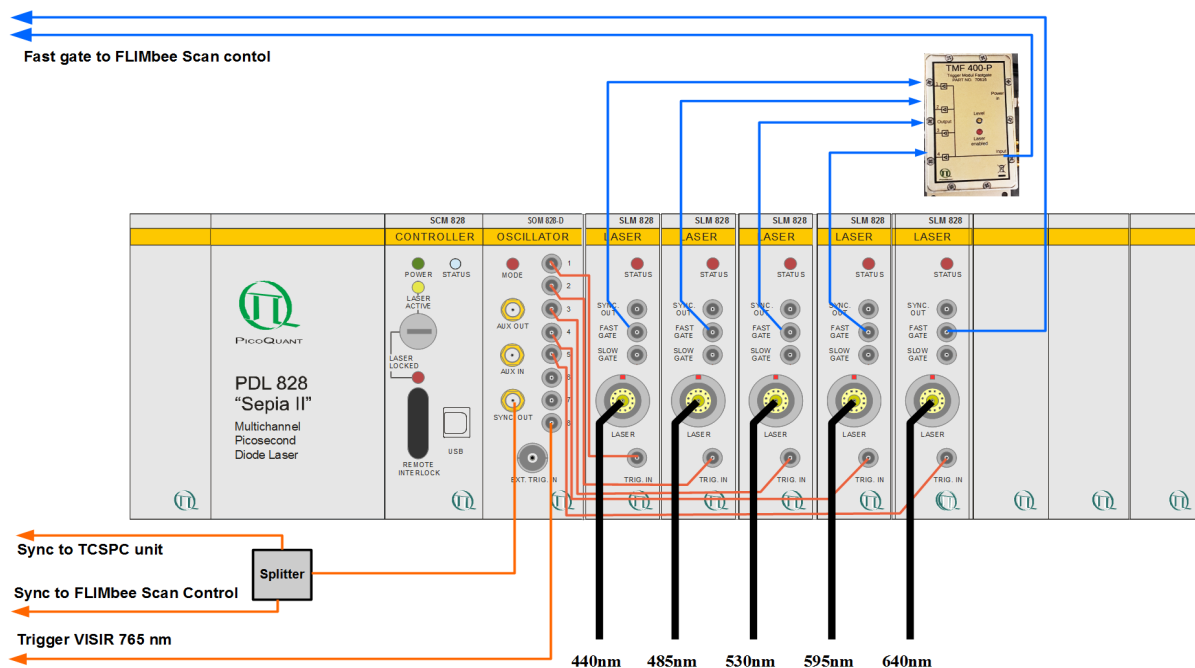


Fig. 2.6: Front panel of the PDL 828 “Sepia II” laser driver equipped with a SOM-D module in the center and 5 single channel laser modules (SLM) on the right hand side. The wiring scheme depicts a case where 3 laser heads (440 nm, 485nm, and 530nm) are present in the VIS-LCU and 2 excitation lasers (595nm and 640nm) together with the STED laser (VisIR 765nm, if available) are located in the STED-LCU. The fast gate connections for laser beam blanking are connected to a TMF-400-P module (top right, connections shown as blue arrows) and from there to the FLIMbee Scan Control (connector “laser blanking”). Fiber amplified lasers (e.g., from PicoQuant’s LDH-FA Series) can not be used with beam blanking.

The SOM-D (Sepia Oscillator Module with electronic Delay) allows generating arbitrary synchronized or alternating pulse patterns for both excitation and STED lasers through an easy to use software interface as well as introducing electronic delays between respective laser pulses. The oscillator module also generates the reference signal to synchronize with the TCSPC unit (see Fig. 2.6).

A detailed description of the features and operation of the SOM-D module is provided in the PDL 828 “Sepia II” Manual.

In order to use beam blanking with the FLIMbee Galvo Scanner, the appropriate cables have to be plugged in.

Beam blanking is best suited for mono-directional scanning. It switches laser emission off during the fly-back phase of the beam during the image scanning process, leading to a reduction in bleaching.

**Caution:** Fiber amplified lasers from PicoQuant’s -TDA and -FA Series can not be beam blanked as their laser emission turn-on time is too long. The STED laser can also not be used for beam blanking for the same reason.

When beam blanking is used, all excitation lasers are switched on only when a measurement is started (e.g., point measurement). The laser power reading in the SymPhoTime 64 software is only accurate during point measurements. Do not use the laser power reading while performing an image scan.

## 2.7 PC

The PC delivered with the MicroTime 200 microscope is configured for demanding TTTR data acquisition. The user should avoid installing additional PCI expansion cards or connecting unnecessary USB devices.

The FLIMbee Scan Control is connected to the PC using an USB 2.0 connection.

### 3. Everyday Alignment for MicroTime 200 with FLIMbee Add-on

In this chapter, reference measurements for assessing the alignment quality of the MicroTime 200 with FLIMbee add-on will be presented along with respective alignment procedures. We distinguish between the **Everyday Alignment** and the more thorough **Fundamental Alignment**. The everyday alignment is a quick check of the optical hardware and takes typically less than 10 minutes to complete (without the optional crimson bead imaging). In case you notice any changes in system performance, this procedure gives you a simple way to check the alignment of your system.

**We recommend to do the Everyday Alignment at least once a week, prior to a new measurement session, or after changing a key element of the optical layout, for example the major dichroic (e.g., changing the excitation wavelength), microscope objective, or switching from confocal to STED measurements.**

The Fundamental Alignment procedure is described in the subsequent chapter and should only be carried out if the measurement performance is significantly worse than expected or after a major change to the setup of your system (e.g., removal of the objective turret, adding a PiFoc, etc.)

The basic idea of the **Everyday Alignment** procedure is to follow the excitation beam path step by step from the excitation laser in the LCU via the MOU and inverted microscope body to the sample and back to the video camera as well as to the detectors in the MOU.

The everyday alignment starts with the beam displacer in the MOU because the LCU itself do not require any everyday alignment. Only the excitation lasers should be used for the following alignment procedures.

Note: this manual is for the alignment of the FLIMbee add-on only. The detailed alignment procedure for the MicroTime200 and STED add-on can be found in the corresponding manuals. All alignment procedures described here are meant as a short summary.

Note for alignment with STED: Depending on the configuration of the STED laser, up to 2 W of light at 765 nm can be emitted from the laser at a repetition rate of 10 MHz or up to 1.7 W after the exit of the fiber attached to the LCU. For this reason the STED laser should never be operated when the cover of the LCU or MOU is removed. No alignment procedure inside the MOU requires the STED laser shutter to be open while the cover of the MOU is removed. As such, the AUX shutter should remain closed whenever the MOU is open. The STED laser beam path in the LCU can be aligned without removing the STED LCU-cover. For this reason interlock switches have been installed that will close the STED laser shutter if the cover is removed from the STED LCU. Any alignment procedures requiring access to the STED laser beam path are performed under controlled conditions by a PicoQuant technician before delivery.

**Please note that if your MicoTime 200 system has both a FLIMbee scanner and a STED add-on installed, all turret positions (except the one featuring the phase plate) are equipped with an objective extension. These extensions should never be removed**

### 3.1 Everyday Alignment of the Main Optical Unit (MOU)

This is a short summary of all steps required for the Everyday Alignment procedure. A detailed version can be found in the Standard MicroTime200 Manual

1. **This alignment has to be performed without phase plate. Please turn the objective turret to a position without phase plate before proceeding.**
2. Clean the objective and, if required, put immersion medium on top of the objective lens
3. Mount a clean glass cover slip of standard thickness #1 or #1.5. It should be mounted in a holder (see Fig. 3.1) Instead, you can also use the PicoQuant Fluorescence Microscopy Standard Slide or a mirror. (can be found in the toolbox)

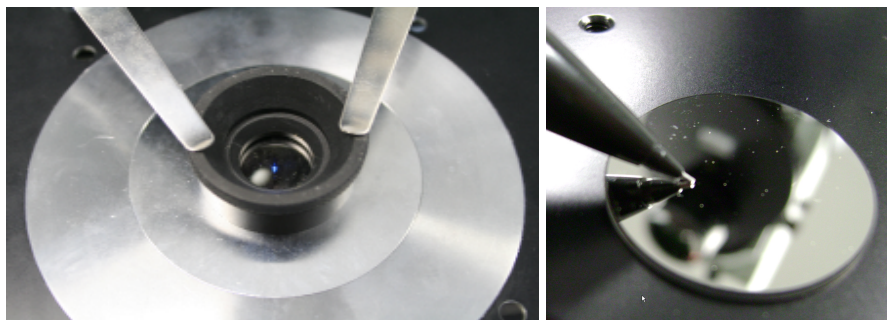


Fig. 3.1: Cover slide mounted as a test sample (left) and mirror with coated side facing upwards (right)

4. When using a mirror select the OD3 filter in the MOU detection filter wheel.
5. Do not use the STED laser, instead choose one excitation laser (wavelength depending on the major dichroic)
6. Set to the excitation laser to maximum power in pulsed mode. Make sure that the detectors are switched off and their shutters are closed before carrying this step out!!
7. Start a point measurement in the center of the scan field ( $x = y = 0 \mu\text{m}$ ) using the test measurement mode in the SPT64
8. Slowly raise the objective towards the sample (using the microscope focusing knob) until a back reflection is visible on the diagnostic video camera
9. Adjust the image brightness with the MOU diagnostic filter wheel  
Reduce laser intensity if the camera image is over-illuminated
10. Check the beam alignment (see Fig. 3.2)
  - **Well aligned beam:** Changing the focus Z-position will affect the image but should not influence the symmetry (in 2D) of the intensity distribution.
  - **Misaligned beam:** changing the focus results in an asymmetric change in the image

#### Alignment:

1. Adjust the position of the beam displacer until the intensity distribution is symmetric
2. Adjust the focus to determine if the image changes in a symmetric manner; if not, readjust the beam displacer and try again

**If the working range of the beam displacer is not sufficient to recover a symmetric back reflection, a full Fundamental Alignment of the MOU may be necessary (see chapter 4)**

3. If the working range of the beam displacer was sufficient and you were able to generate a symmetric distribution of the back reflection, check the position of the back reflection in front of the pinhole by placing a target in front of it

If the position of the back reflection is not centered on the target in front of your pinhole, a full Fundamental Alignment of the MOU should be performed as well. **(see chapter 4)**

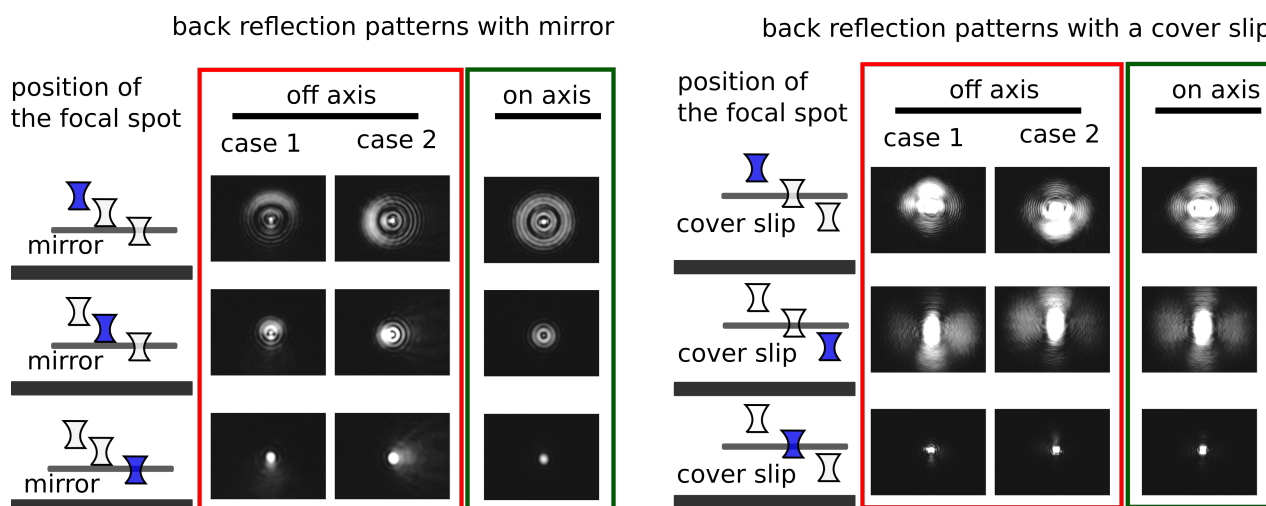


Fig. 3.2: Beam spot images of slightly defocused back reflected excitation light. The exact pattern depends on the excitation wavelength as well as on its polarization. However, the pattern should be symmetric.

## 3.2 Alignment of FLIMbee optics

The FLIMbee optical parts are very stable and therefore no user adjustments are necessary. Please leave the housing of the FLIMbee Galvo Scanner closed at all times.

## 3.3 Pinhole Alignment

### Alignment Test

The best method for determining the pinhole alignment quality is to maximize the intensity of the fluorescence signal. The procedure is outlined below. The position of the XY lens in front of the SPAD detectors should be optimized at the same time.

### Alignment:

**General Remark: If you moved one adjustment element and lost the signal, NEVER move to a different adjustment element. Always readjust the element you just moved until you get the signal back.**

1. Prepare a  $\sim 20 \mu\text{M}$  solution of a fluorescent dye suitable for the selected laser wavelength (the color of the solution should be clearly visible) or use the PicoQuant Fluorescence Microscopy Standard Slide
2. Remove the STED phase plate by turning the objective turret
3. Clean the objective and apply immersion medium
4. Mount either a cover glass in the sample holder and put a drop of the dye solution on it, or mount the PicoQuant Fluorescence Microscopy Standard Slide
5. Place a suitable fluorescence filter in front of one detector (and make sure that the OD3 filter is not in the detection beam-path)
6. Reduce the laser power (select OD2 or OD3 on the LCU) to prevent over-illumination of the detector
7. Start a point measurement in the center of the scanfield ( $x = y = 0 \mu\text{m}$ ), using the test measurement mode in the SPT64 and switch to the time trace view
8. Raise the objective so that the focus is  $1 \mu\text{m}$  inside the solution
9. Set the count rate to 2 Mcps by adjusting the laser intensity
10. Move the pinhole iteratively in both directions ( $x$  and  $y$ ) using the knobs and adjust for maximum count rate in each direction

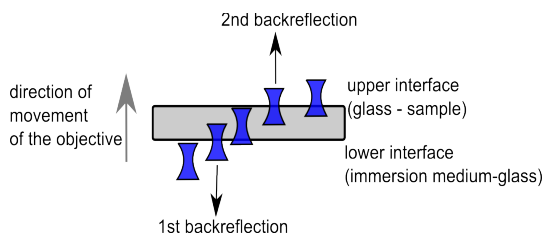


Fig. 3.3: Focus positions when moving the objective towards the cover slip

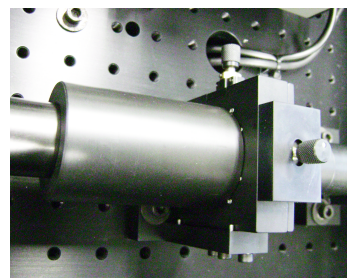


Fig. 3.4: Adjustment knobs for pinhole alignment.

### Adjusting the XY lens position in front of the SPAD detectors

1. Set the excitation intensity so that the signal count rate is approximately 2 Mcps
2. Start a point measurement in test-mode (center of the scan field:  $x = y = 0 \mu\text{m}$ ) and switch to the time trace view
3. Turn one of the XY SPAD lens position knobs back and forth. You should observe an intensity plateau with steep edges (for SPCM-AQRH, AQR- or  $\tau$ -SPAD modules with a large active area) or a single sharp maximum (for MPD SPAD modules).
4. Adjust the XY SPAD lens position until the signal is in the middle of the plateau range or on the maximum (depending on SPAD type) by iteratively turning both knobs
5. Only for SPCM-AQRH, AQR- or  $\tau$ -SPAD modules: Switch to the TCSPC histogram view. Set the y – axis to linear and magnify the rising edge of the time response curve
6. Only for SPCM-AQRH, AQR- or  $\tau$ -SPAD modules: Adjust both XY SPAD lens position knobs so that the rising edge of the TCSPC curve moves as much as possible to shorter times (refer to Fig. 3.6)
7. repeat the lens position optimization procedure for every SPAD installed in the MOU

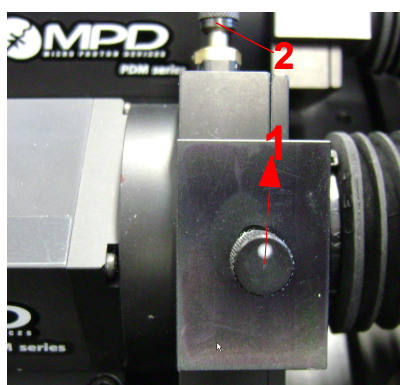


Fig. 3.5: XY SPAD lens position adjustment 1 and 2

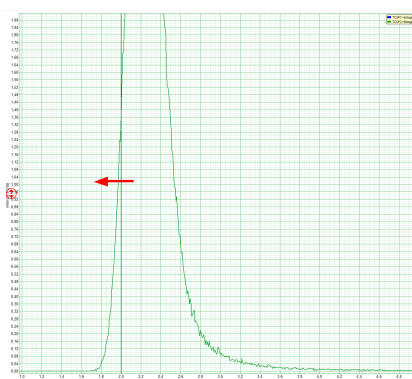


Fig. 3.6: Zoomed in view: Fine tuning the SPAD lens alignment for a SPAD. Move the rising edge of the TCSPC curve as much as possible to the left. This corresponds to the sharpest IRF. For your convenience click on the “Search Edges” button on the top of the graph.

### 3.4 Phase Plate Alignment with the FLIMbee Add-on (for STED only)

#### Alignment Test:

1. Screw the STED Objective onto the phase plate in the objective turret
2. Clean the objective and, if required, put immersion medium on top of the objective lens
3. Mount a gold-bead sample
4. Select the OD3 filter in the MOU detection filter wheel and remove any other detection filter in the beampath
5. Select the PIE Measurement mode in the TCSPC settings of the SPT64 and tick the “add STED Laser” box.
6. Select the correct detector and time gates in the online-view of the test measurement so that you see the confocal and STED images simultaneously. (e.g., Pulse Rate: 20 MHz, TimeGate1: 0 - 50 ns, TimeGate2: 50 - 100 ns) (refer to Fig. 3.7)
7. Use the 640 nm laser and insert the correct major dichroic for 640 + 766 nm excitation.
8. Start a point measurement in the center of the scan field ( $x = y = 0 \mu\text{m}$ ), using the test measurement mode in the SPT64 (detector shutters closed) and raise the objective so that you see the back reflection of your sample (the gold-beads can be found close to the back reflection)
9. Make a quick overview Image of  $dX = 20 \mu\text{m}$ , ( $X = -10 \mu\text{m}$ ,  $Y = -10 \mu\text{m}$ ,  $\text{Img. Size } 300 \text{ px}$ ,  $\text{Dwell time } 5 \mu\text{s / px}$ ) to find the gold-beads. Adjust the z-Position until the gold-beads are in focus. As soon as you focused the beads, bring one bead (or more) into the center of the scan field ( $x = y = 0$ ) by using the x and y drive of your microscope stage. (refer to Fig. 3.7)

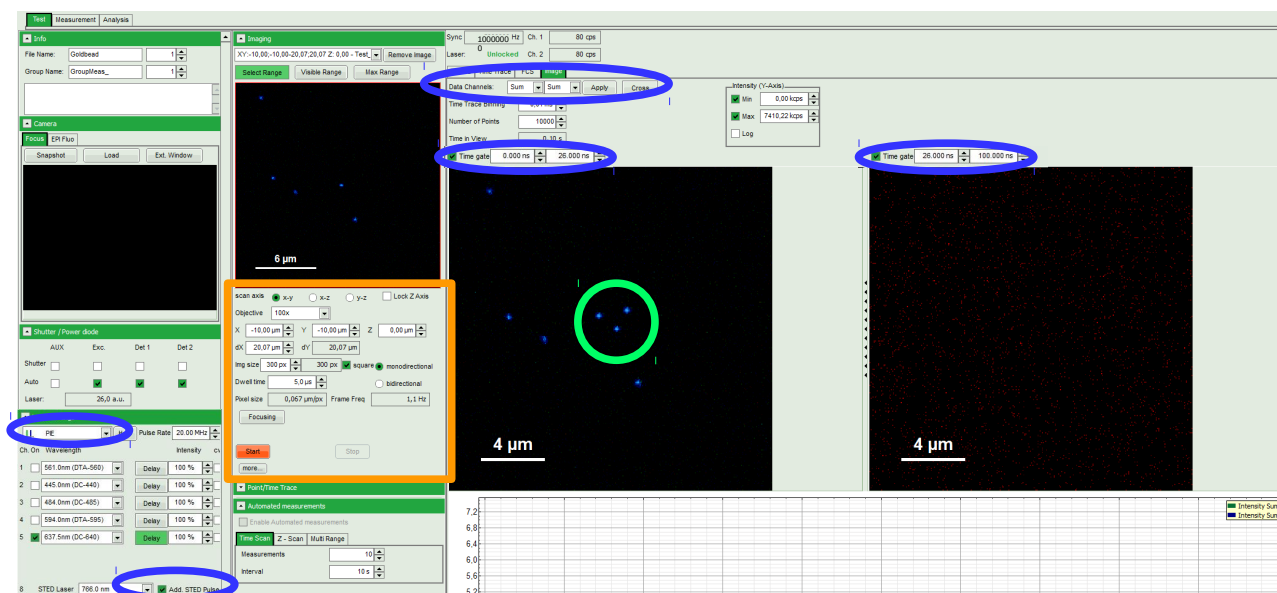


Fig. 3.7: **blue:** Select PIE-Measurement Mode and add STED pulse, select data channels and use time gates to see the bead excited by 640 nm and STED laser simultaneously. **orange:** Start a test measurement with an image size of approximately 20 μm; change z focus until you see the gold beads. **green:** Bring one gold bead (or more) into the center using the micrometer screws of your microscope stage.

10. Reduce the scan range ( $dX = 2\text{-}3 \mu\text{m}$ ) to only scan the gold beads located in the center of the scan field. Switch on the STED laser, reduce the intensity for both STED and 640 nm laser in order to prevent over-illumination of your detector. Please be aware that a too high STED laser power can melt the gold bead under investigation. Start a test measurement and increase the intensity of both lasers slowly during the test measurement until you can clearly see the bead on the left side and the STED donut on the right side (refer to Fig. 3.8).



- You can now optimize the STED donut shape by adjusting the beam displacer. Start by loosening the locking screws of the adjustable beam displacer outside of the MOU. Then slightly turn the adjustment knob and rotate the beam displacer until you obtain a symmetric STED donut (refer to Fig. 3.8). To ensure that the minimum of the donut approximately overlaps with the maximum of the bead, press the “cross” button and place the orange cross onto the center of your bead

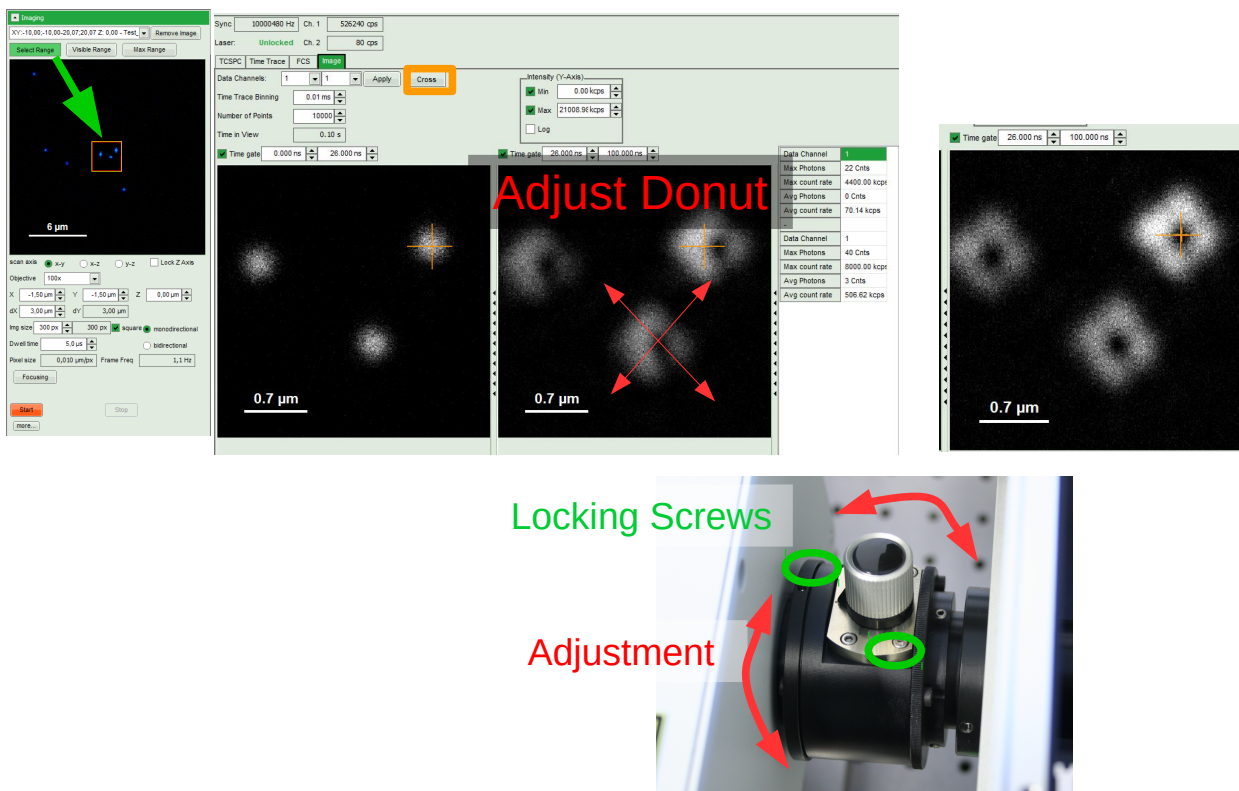


Fig. 3.8: Reduce the scan range to measure a 2-3µm area. Adjust the STED donut shape by slightly turning the adjustment knob and rotating the beam displacer.

- Once you obtained a symmetrically shaped STED donut, run a test measurement and vary the maximum value in the event count display until the whole STED donut has an homogeneous brightness with a clearly visible center. The optimum position for the phase plate is reached when the STED donut shape is simultaneously as symmetrical as possible with a center as dark as possible (same counts as background or less) (refer to Fig. 3.9).

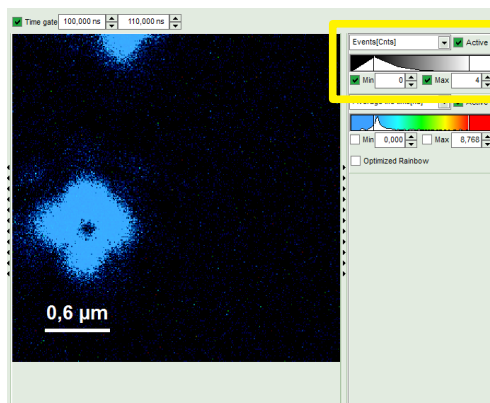


Fig. 3.9: Increase brightness of the donut and evaluate the number of counts in its center. The central area should ideally have a similar amount of counts as the background.

- For a more detailed evaluation of the donut shape quality, perform a measurement in “measurement mode” (stop after 1 frame, dwell time: 150 µs/px), see Fig. 3.10. Select the obtained file and start a Gated STED-Image analysis. Two images are visible in this type of analysis. The left image shows the whole image without time gates, while the right image displays only data from the selected time gate (first peak 640nm (beads), second peak 765nm (donut)). Select the time gate corresponding to

the peak you want to evaluate and press calculate. In case of gold beads, it might happen that several reflection peaks from the STED Laser (765nm) are visible. This, however, will not affect the results. Activate the “data reader” by right clicking inside the image and select “show data reader”

- Evaluate the background counts (estimated average) and the minimum counts of the center of the donut → the donut center should be as dark as the background or even darker (in the example given in Fig. 3.10: average of background = 5 counts, center of donut = 4 counts)

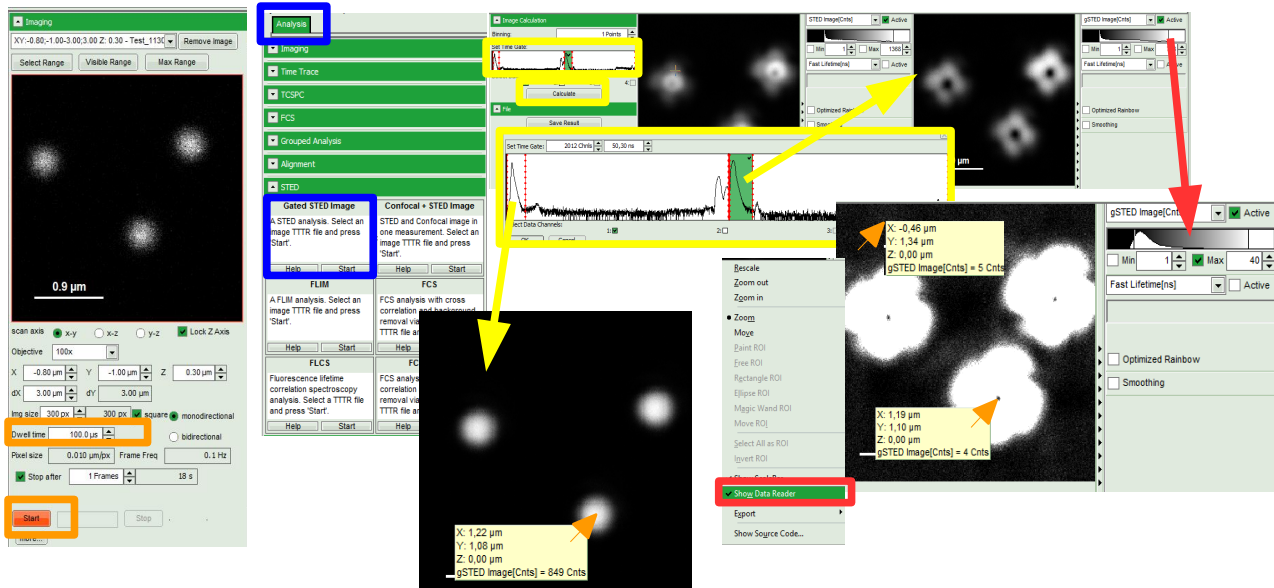
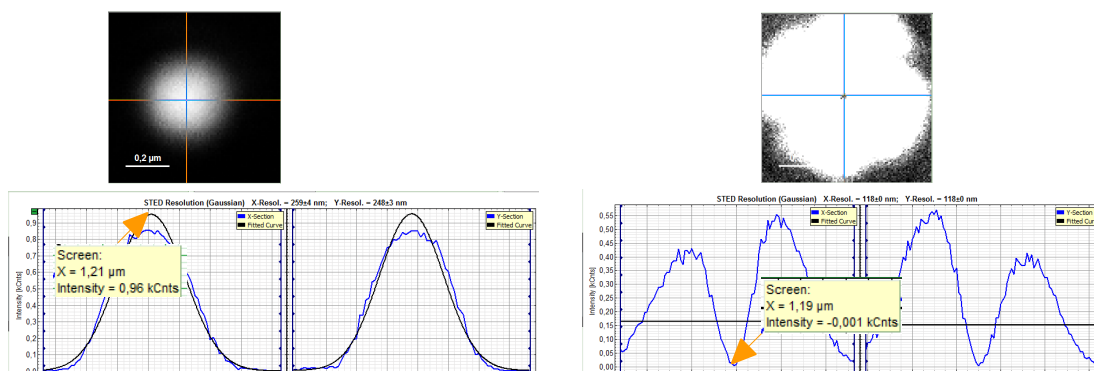


Fig. 3.10: more detailed analysis of the donut quality:

- Step 1 (orange): start a measurement in “Measurement-Mode” with a dwell time of 100-150 µm and stop after 1 frame.
- Step 2 (blue): select the measured file, go to analysis and press the “start” button under the “Gated STED Image”
- Step 3 (yellow): define time gates for both beads and donut, then press calculate when the corresponding time gate is selected.
- Step 4 (red): activate the data reader and evaluate the coordinates as well as the number of counts

- Evaluate the overlap between the bead maximum and donut minimum. For a better evaluation you can zoom into the area of interest and select the respective center. Use the screen reader to evaluate the coordinates from the graph (see Fig. 3.11). The distance should be smaller than 80nm



• Fig. 3.11: evaluation of the overlap between bead maximum and donut minimum

14. Fasten the two locking screws carefully to make sure that the adjustment is not lost
15. The phase plate located in the objective turret is pre-aligned. **This alignment should never be changed by the user!** Please do not remove the phase plate from the objective turret or tamper with any of its adjustment screws.

Please contact our support if you cannot achieve a symmetrical donut shape (via [support@picoquant.com](mailto:support@picoquant.com)).



## 4. Fundamental Alignment for MicroTime200 with FLIMbee Add-on

The MicroTime200 including the FLIMbee add-on is a stable instrument as long as it is regularly operated and the Everyday Alignment procedures are carried out. Performing a Fundamental Alignment should only be necessary in rare cases. The procedures described in this chapter should only be carried out if the system performance is significantly worse than expected and the Everyday Alignment procedure is not able to recover it.

Should you need to change the system layout, especially with regards to the microscope body and the distance between objective and FLIMbee (e.g. by removing the objective turret or adding a PiFoc), the whole system needs to be realigned by PicoQuants support. Please contact our Support (via [support@picoquant.com](mailto:support@picoquant.com)) for further information regarding this topic.

### 4.1 Laser Combining Unit (LCU)

The positions of the mirrors and beam displacers inside the LCU and STED LCU will shift slightly over long periods of time and the coupling efficiency of the various lasers will drop. When the laser power available in the MOU is no longer sufficient, a realignment of the LCU can resolve this issue. In order to perform the alignment of the LCU, please refer to the main MicroTime 200 manual as well as the STED manual for the STED-LCU.

**Before performing any alignment, read carefully the laser safety instructions provided in the separate FLIMbee, MicroTime 200, and STED -LCU manuals.**

### 4.2 Main Optical Unit (MOU)

The description provided here is intended for the alignment of a MicroTime 200 system with STED and FLIMbee add-on. If you have a confocal MicroTime 200 with FLIMbee add-on, you only need to perform the fundamental alignment as described in the main MicroTime 200 manual.

In order to achieve the best possible STED performance, the STED beam path inside the MOU of a MicroTime 200 needs to be optimized. All alignments described here are done using the excitation lasers only in the STED LCU (and not in the VIS LCU). The STED laser should never be used during this alignment procedure.

Minor misalignments can be corrected by using the beam displacer, which is placed directly after the major dichroic in the MOU.

The full alignment procedure is outlined in the MicroTime 200 manual (section "Alignment and Benchmark Measurements"), but instead of using mirror 1 (M1), mirror 0 (M0) in the STED beam path is used. The STED beam path should always be aligned before aligning the VIS / confocal beam path as the latter will be affected by the former.

A simplified and adapted version of the alignment procedure including the STED beam path is provided here as a reminder:

**This alignment has to be performed without phase plate. Please turn the objective turret to a position without phase plate before proceeding.**

**Please note that if your MicroTime 200 system has both a FLIMbee scanner and a STED add-on installed, all turret positions (except the one featuring the phase plate) are equipped with an objective extension. These extensions should never be removed**

1. Mount a flat mirror with the coated side facing the objective (see Fig. 3.1)
2. Switch off the detectors and keep their shutters closed!
3. Set the beam displacer located after the major dichroic outside of the MOU into a position that is just off from neutral.

4. Start a point measurement in test-mode in the center of the scan field ( $x = y = 0 \mu\text{m}$ ) and open the excitation shutter
5. Adjust the position of the major dichroic until a symmetric intensity distribution can be seen on the diagnostic camera.
6. Check the position of the back reflection just before the pinhole by placing the alignment target just before it. If the back reflection is centered, the alignment procedure is complete. If not, continue to step 7
7. Adjust mirror 0 (M0) of the STED beam path until the displacement from the center is twice as large as previously observed, then return to step 5. (see Fig. 4.1)

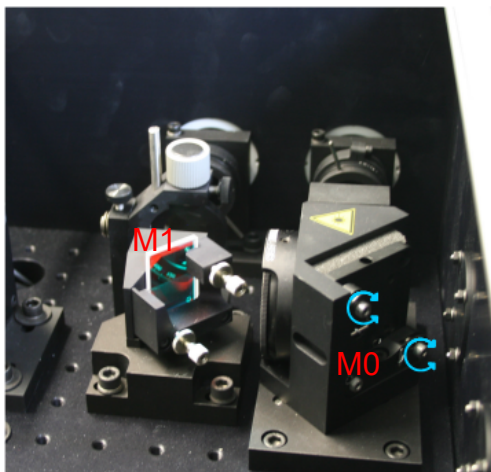


Fig. 4.1: Mirror 0 (M0) in the STED beam path with its adjustment screws marked (blue arrows)

### 4.3 Confocal beam path

Before aligning the confocal beam path, the STED beam path should be aligned as described above. Once this has been done, the back reflection of both beam paths can be used to overlap them on the sample. The following procedure should be performed with the phase plate removed from the beam path.

1. Apply an appropriate immersion medium to the objective, if required
2. Mount a reflective OD3 filter or mirror into the sample stage
3. Turn on the 640 nm laser in the STED LCU, start a point measurement in test-mode in the center of the scan field ( $x = y = 0 \mu\text{m}$ ), and open the excitation shutter
4. Focus the laser light on to the sample using the diagnostic camera
5. Turn on a laser in the VIS LCU and observe the location of the new diffraction pattern on the camera relative to the original diffraction pattern from the 640 nm laser in the STED LCU
6. Adjust the power of both lasers until both diffraction patterns have a similar intensity (see Fig. 4.2)

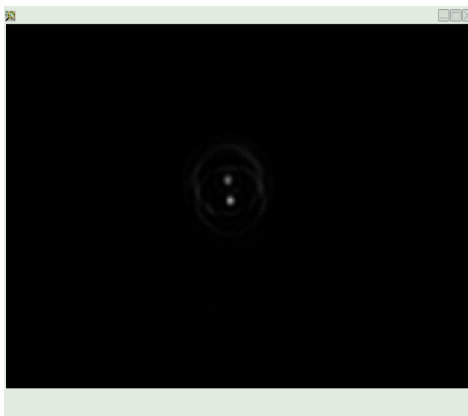


Fig. 4.2: After the laser power is adjusted, the two diffraction patterns have a similar intensity. Here they are almost overlaid

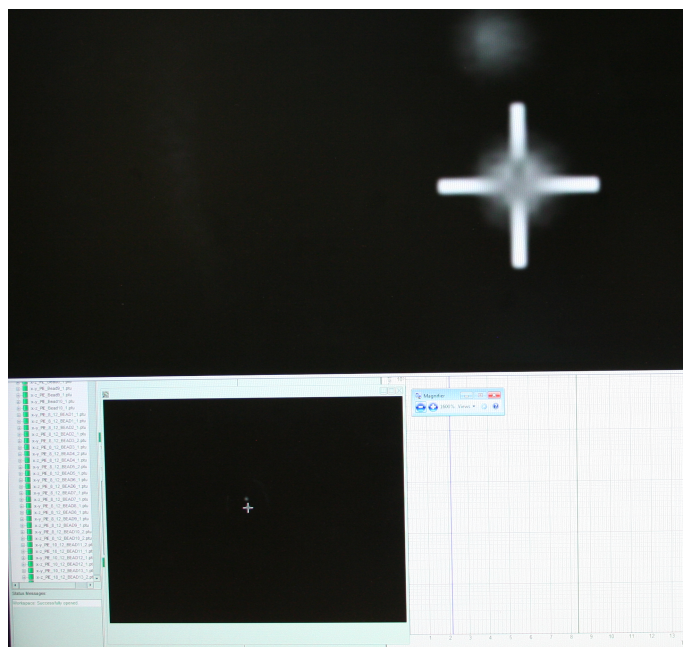


Fig. 4.3: Cross hair showing the position of the diffraction pattern: lower left in the diagnostic camera window of the SPT64 software; top part: magnified view obtained with the Windows app "Magnify".

7. Open the Windows app "Magnifier", set it to maximum zoom (1600%), and set the view to "Docked"
8. Place the cross hair in the center of the diffraction pattern originating from the 640 nm laser located in STED LCU, as shown in Fig. 4.3 and switch off the 640 nm laser afterward
9. Adjust the position of the diffraction pattern originating from the confocal beam path by adjusting the x and y screws of the combining dichroic M1 until the two diffraction patterns are in exactly the same spot

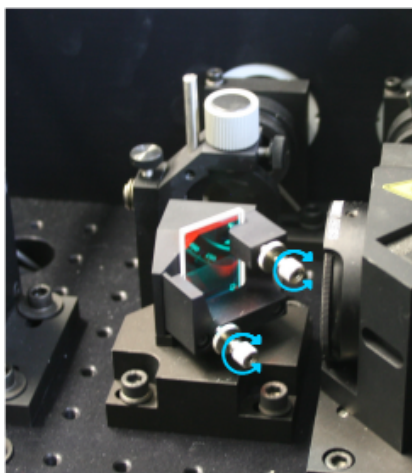


Fig. 4.4: Combining dichroic M1 with adjustment screws.

10. Tighten the fastening screws of the combining dichroic M1. It is possible that during this process the two diffraction patterns will no longer be overlapping. In this case, loosen the fastening screws and attempt to compensate for this effect before re-tightening them.
11. Check the intensity distribution of the confocal beam's diffraction pattern. If it is asymmetric, adjust the beam displacer directly after the confocal fiber collimator until the pattern is symmetric.

## 4.4 Detection Beam path

After the excitation beam path for STED, confocal, and FLIMbee is aligned, the detection beam path can be aligned following the standard procedure described in the MicroTime 200 manual. A summary of this procedure is provided below:

1. Screw in an objective
2. Apply an appropriate immersion medium to the objective, if required
3. Mount a reflective OD3 filter or mirror into the sample stage.
4. Switch off the detectors, turn on the 640 nm laser in the STED LCU, start a point measurement in test-mode in the center of the scan field ( $x = y = 0 \mu\text{m}$ ), and open the excitation shutter
5. Focus the laser light on to the sample using the diagnostic camera.
6. Check the position of the back reflection just before the pinhole with the alignment target. It should still be centered on the target.
7. Adjust the pinhole with the back reflection
8. Adjust mirror 5 in order to get the back reflection centered in front of detector 2
9. Adjust mirror 6 in order to get the back reflection centered in front of detector 1
10. Align pinhole with fluorescent solution as described above in section 3.3
11. Insert the phase plate if you are going to perform STED measurements and align the phase plate as described in section 3.4

## 4.5 Software Settings

Finally, the Software Settings can be optimized using the Hardware Settings tab in the SymPhoTime 64. A detailed description can be found in the next chapters “Hardware Settings” and “Software”.

## 4.6 FLIMbee Optics

In general, the optics in the FLIMbee device are very stable and it should not be opened by the user. should you notice any problems with your measurements, that cannot be solved by performing the full fundamental alignment, please contact our support at [support@picoquant.com](mailto:support@picoquant.com).

## 5. Hardware Settings and Software

The FLIMbee can be configured in the SymPhoTime 64 *Settings* → *Hardware Setup* menu. In the dialog window, you will find a section titled “FLIMBee/ScanBox”.

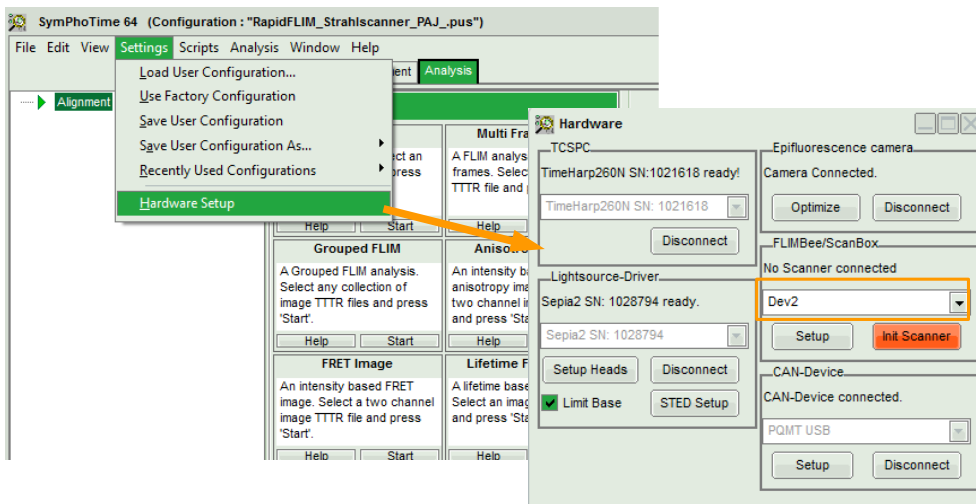


Fig. 5.1: Hardware Settings of SymPhoTime 64

The drop down box lists all National Instruments NI-DAQ boards connected to the PC. In most cases there is only one NI-DAQ card present and the device “Dev1” has to be selected. Press the “init Scanner” button to connect the FLIMbee to your system. This FLIMbee NI-DAQ USB device will then be used by SymPhoTime 64.

### 5.1 FLIMbee Hardware Setup

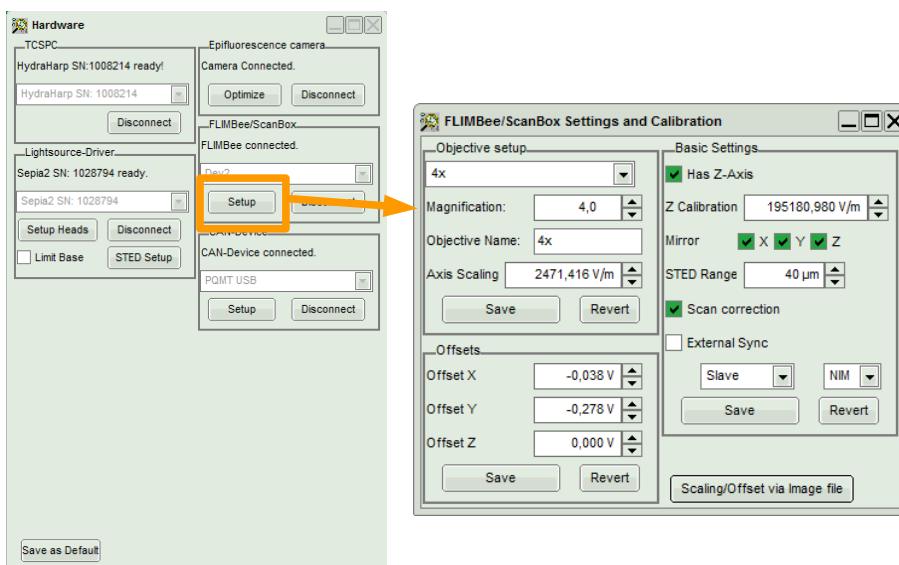


Fig. 5.2: Hardware setup menu for FLIMbee scanning system

The following options are available in the FLIMbee hardware setup menu:

- Basic Settings
  - If a z-stage is attached to the system that enables movement of either the objective or sample, the check-box “Has Z-Axis” has to be ticked. The *Z Calibration* value is calculated in the following way:
    - Mad City Labs provides the maximum displacement height in microns, e.g., 102.469  $\mu\text{m}$ . This maximum is reached at end of a voltage ramp going from  $-10\text{ V}$  up to  $+10\text{ V}$ . Therefore, a voltage change of  $20\text{ V}$  is needed to reach the full displacement of  $102.469\text{ }\mu\text{m}$  (in our example).  
The *Z Calibration* value is then calculated by dividing these  $20\text{ V}$  by the maximum height displacement (in our example  $20\text{ V} / 102.469 \times 10^{-6}\text{ m} = 195180,981\text{ V} / \text{m}$ ).
  - Mirror:
    - Ticking the “Mirror” check-box mirrors the respective axis around the zero (center) point of the image.
    - Default settings:
      - The X and Y check-boxes should be ticked, so that the recorded image has the same orientation as the one seen through the oculars.
      - The Z check-box should not be ticked. In an image recorded as XZ – Scan, the cover slip will be facing downwards and the sample placed on the upward facing side of the cover slip.
  - Scan correction
    - Default setting: checked. Enabling *Scan correction* leads to a better positioning of point measurements in the recorded image. Additionally, ROI (region of interest) scans in an acquired image will be located precisely where they should be. Lastly, bi-directional scans require only a slight correction through the “Bidirectional shift” setting.
  - External Sync
    - Default setting: not checked. With this setting, the timing of the laser pulses are not synchronized with the timing of the pixel clock.
    - Synchronizing laser pulses with the pixel clock is necessary when performing fast scans in combination with low laser repetition rates, for example when studying slowly decaying fluorescence. Without this synchronization, vertical stripes (Moiré pattern) can appear in the recorded image. In order to switch the synchronization on, tick the “External Sync” check-box in the scanner hardware setup. Two methods, called “Master” and “Slave”, can be selected from the drop-down menu:
      - FLIMbee Scan Control is set as “Master”:  
With this setting, the FLIMbee Scan Control generates either a NIM or TTL pulse sequence (can be selected from the NIM/TTL drop-down menu) for controlling an external laser connected to the “Laser Sync Out” port.
        - The signal is emitted from the “Laser Out” connector located in the right upper part of the FLIMbee Scan Control panel.
        - The laser repetition rate can be selected in the “Laser Settings” window of the SymPhoTime 64 software. This window is only available when no PDL 828 “Sepia II” laser driver is connected.
        - This setting should be selected when a single-channel laser driver (e.g., PDL-800-D) is used.

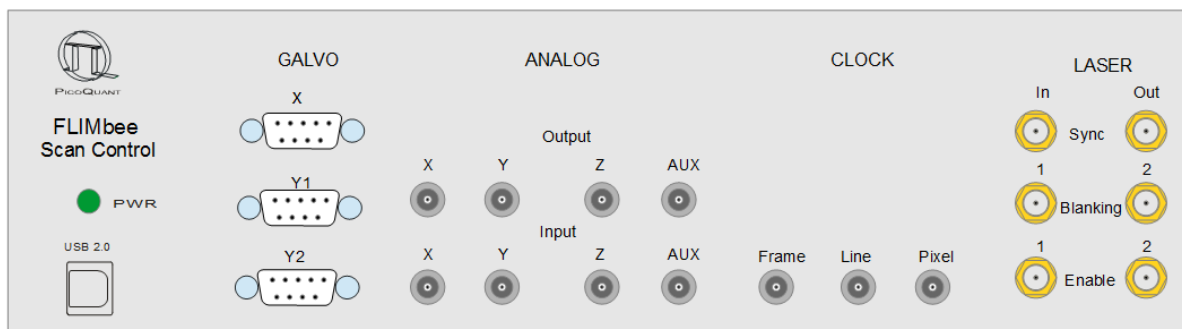


Fig. 5.3: Front panel of FLIMbee Scan Control  
Laser Synchronization and blanking connectors are on the right side.

- FLIMbee Scan Control set as “Slave”:
  - This setting requires either a NIM (pulse height  $-0.8\text{ V}$  at  $50\ \Omega$ ) or TTL signal at the “Laser Sync In” connector (signal type selectable from the NIM/TTL drop-down menu). The signal should follow the laser pulse sequence of the applied laser. The cable is connected to the “Laser Sync in” port located at the right side of the FLIMbee Scan Control panel.
  - The pixel clock will be generated in correlation with the detected pulse sequence. The electronic setup ensures that a minimum of 8 laser pulses are generated for each pixel, which can lead to a limitation of the maximum scan speed.
  - When connecting a PicoQuant laser driver to the “Laser Sync In” port of the FLIMbee Scan Control, the “Sync out” signal of the laser driver should be spitted by an appropriate  $50\ \Omega$  50/50 power splitter. One output signal of the splitter has to be connected to the “Sync in” of the TCSPC device, while the other has to be plugged into the “Laser Sync In” connector located in the right part of the FLIMbee Scan Control front panel.
  - This setting should be selected when using a multi-channel laser driver (e.g., PDL 828 “Sepia II”).
- Laser Blanking
  - The front panel of the FLIMbee Scan Control has two “Laser Blanking” connectors. They produce a TTL pulse which is “high” as long as data acquisition takes place. For mono-directional scans the signal is “low” during the fly back phase after each line. Connecting this output to the “fast gate” input (if available) of a PicoQuant PDL laser driver will switch the laser off during the fly back phase and also as long as no measurement acquisition is running. Should this blanking be needed for more than 2 lasers, the PicoQuant TMF 400-P module can be used. Each TMF 400-P provides 4 outputs.
    - Caution: The fast gate works only when the laser is operated in the pulsed mode; lasers set to cw mode are not blanked. Laser blanking can not be used in conjunction with fiber amplified lasers (such as those from the LDH-FA and LDH-TDA Series) since they may not switch “on” fast enough after being switched “off” by the fast gate input connector, which would lead to dark parts in the image.
- Laser Enable
  - The front panel of the FLIMbee Scan Control has two “Laser Enable” connectors. They deliver a TTL pulse which is “high” as long as data acquisition takes place. For mono-directional scans the signal is also “high” during the fly back phase after each line. Connecting this output to the “slow gate” input (if available) of a PicoQuant PDL laser driver will switch the laser on only during the acquisition of a measurement. Should more than 2 lasers need to be switched in this manner, the PicoQuant TMF 400-P module can be used. Each TMF 400-P provides 4 outputs.
- Clock
  - Three clock BNC connectors are present on the front panel of the FLIMbee Scan Control, namely “Pixel”, “Line” and “Frame”. All signals are of TTL type and need a high impedance input.
    - The rising flank of the “Pixel” connector output indicates the start of a new pixel.



- During the acquisition of each line, the “Line” output is “high”, otherwise it is “low”.
  - During the acquisition of each frame, the “Frame” output is “high”, otherwise it is “low”.
  - The SymPhoTime 64 software uses only the “Line” and “Frame” markers.
- Analog Output
    - The “X” and “Y” BNC analog output connectors of the FLIMbee Scan Control unit are only wired internally and not connected to the BNC ports at the front panel.
    - The “Z” Output connector is used to control an axial scanning device such as a PIFOC or z-stage. It delivers an analog signal ranging from -10 V (highest negative z-range setting) to +10 V (highest positive z-range setting).
    - The “AUX” port is wired to the “AO3” output connector on the NI-DAQ board. It is currently not used by the SymPhoTime 64.
  - Analog Input
    - The “X” and “Y” BNC analog output connectors of the FLIMbee Scan Control unit are only wired internally and not connected to the BNC ports at the front panel.
    - The “Z” input port is wired to the “Ai5” input connector on the NI-DAQ board. It is currently not used by the SymPhoTime 64.
    - The “AUX” input port is wired to the “Ai6” input connector on the NI-DAQ board. It is currently not used by the SymPhoTime 64.

## 5.2 Scanner Calibration

Scanner calibration can be performed within the SymPhoTime 64 Hardware Setup.

Generally the alignment is pre-set when your system is installed. The calibration for standard objectives is done automatically with respect to their magnification value. In case you want to calibrate any further objectives, you can use the following procedure.

- Screw in the objective you want to calibrate
- Clean the objective and, if required, put immersion medium on top of the objective lens
- Mount a micro-meter scale bar as a sample
- Place a prominent point of the scale bar (e.g., the point of 1.0) into the center of the view field by using the cross-hair of your left ocular as a reference point and shifting the sample using the xy microscope cross stage
- Align the scale bar so that it is not tilted. Focus onto the sample using the binocular of your microscope body (make sure that the binocular is adapted for your eyes (dioptries)) (see Fig. 5.4)

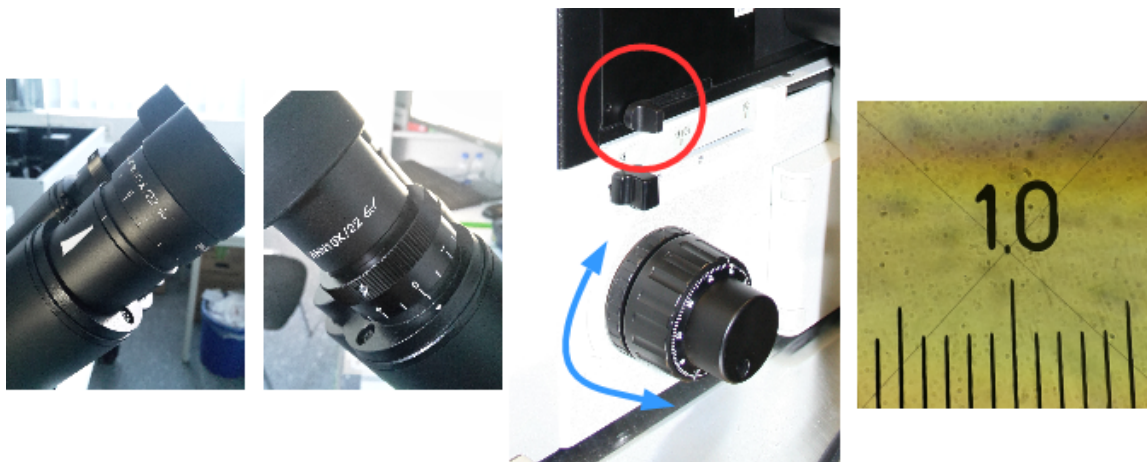
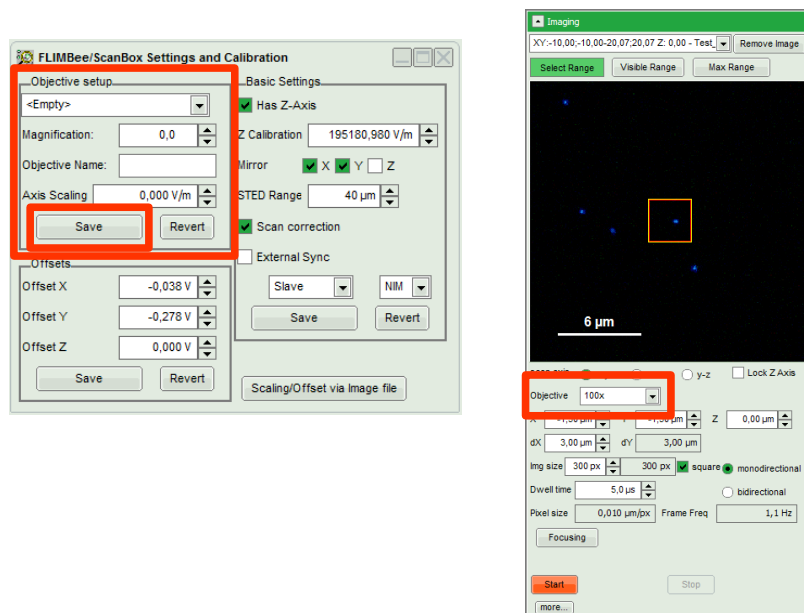


Fig. 5.4: Side Port Slider for binocular observation



- Add a new objective entry in the hardware settings of FLIMbee (see Fig. 5.5):
  - Click on “Setup” in the FLIMbee/ScanBox field in Hardware Settings to open the Calibration Window
  - Select an <Empty> Objective in the drop-down menu of the objective setup window
  - Type in the magnification (e.g., 50) and a appropriate name (e.g., 50x)
  - Click save and close the hardware setup window
  - Select the new objective in the imaging tab before starting a measurement



- Fig. 5.5: Adding a new objective in the SymPhoTime 64 FLIMbee Hardware Setup
- Select the OD3 filter from the MOU detection filter wheel. Do not use the STED laser; instead choose one of the excitation laser (depending on the installed major dichroic)
- Decrease the laser power to prevent over-illumination of the detectors
- Change the side port slider for imaging and start a test measurement with maximum scan range (300 x 300 px, 5 µs / px)
- Adjust the laser power to get a bright image of the scale bar
- Make a measurement of the scale bar using the measurement mode
- Go back to the hardware setup and start the calibration of your new objective (see Fig. 5.6)
  - Click on “Setup” in the FLIMbee/ScanBox field in Hardware Settings to open the Calibration Window
  - Click on “scaling / offset via image file”
  - Chose the recorded image containing the scale bar
  - Determine the scaling setting:
    - Select the objective you want to calibrate
    - Zoom in, if necessary, by dragging with your mouse a box around the desired area
    - Draw a line between between two prominent marks of the scale bar (e.g., 100 µm)
    - Type in the correct length of your line
    - Select the objective you where using (“set this calibration Value for: ...x”)
    - Press “set”
  - Perform the offset setting:
    - Zoom into the prominent point of the scale bar that is located in the center of your image

(e.g., the point of “1.0”)

- Set this point as the center
- Press the “set” button
- Close the scaling and offset window
- The calibration process has now changed the values for both axis scaling and offset
  - Press the “save” button in the “Offset” area
  - Select the correct objective in “Objective Setup” and press the “Save” button

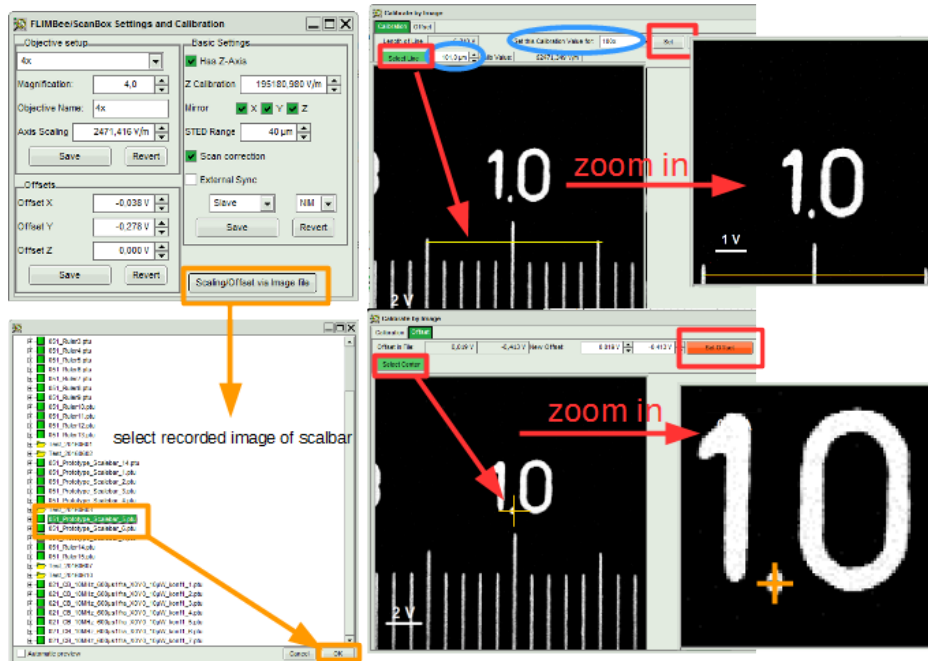


Fig. 5.6: Calibration of the objective in SymPhoTime 64 Hardware Setup

### 5.3 Software Control of the FLIMbee in SymPhoTime 64

The operation of the FLIMbee Scanner is fully integrated completely into the SymPhoTime 64, which makes handling straight forward.

Only the SymPhoTime 64 parts relevant to the operation of the FLIMbee will be described in this section.

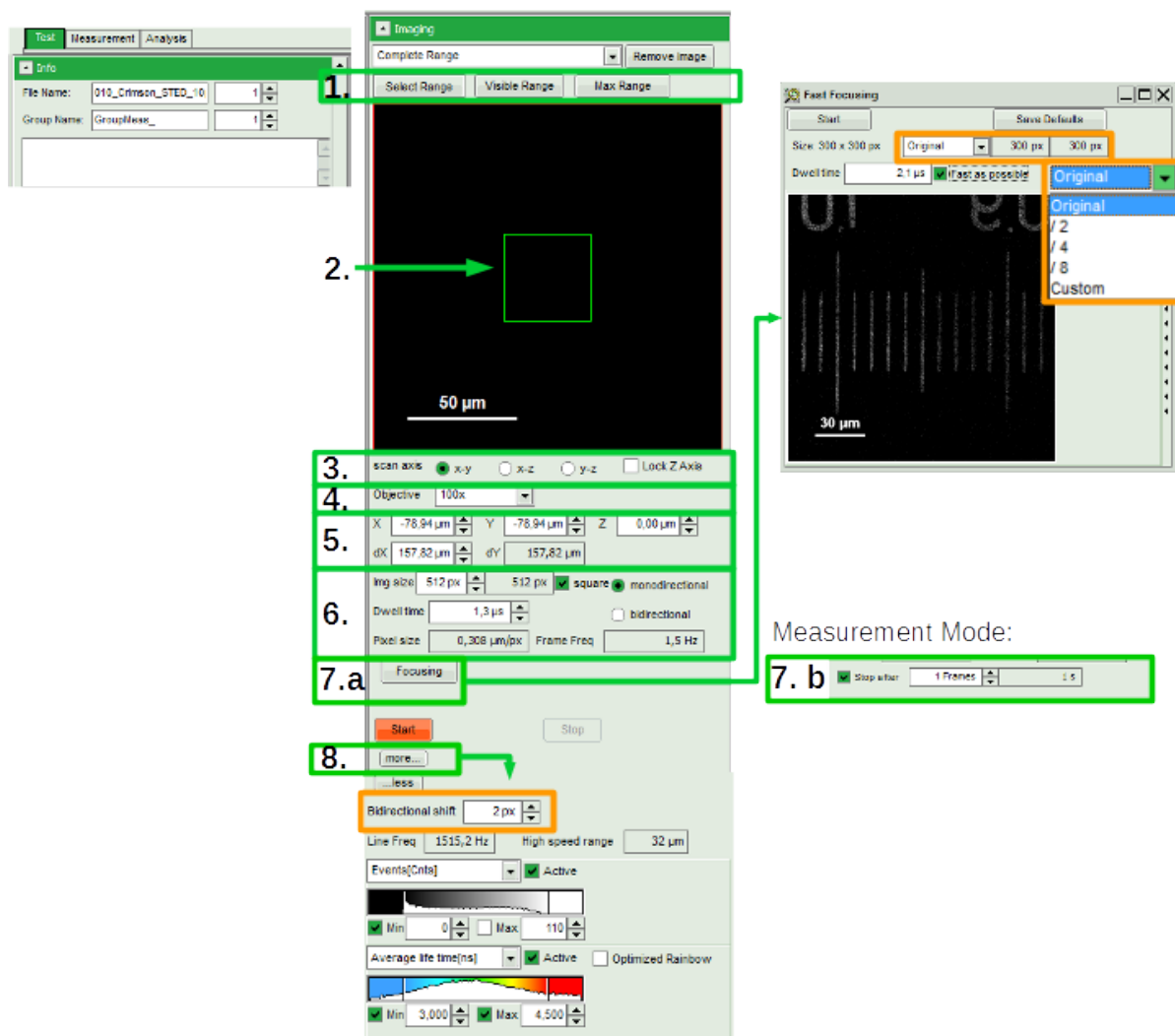


Fig. 5.7: Scan parameters FLIMbee

The FLIMbee software control panel is shown in Fig. 5.7 and the different parts are described below.

You can switch between Test and Measurement mode by clicking on their respective tabs. The main difference between both modes is the way in which measurements are saved and displayed.

When performing a scan in Test mode, the number of frames to be recorded is unlimited and the measurement will only stop after the user presses the “Stop” button. The online view will always display only one frame at a time. These single frames are not summed up during the measurement and only the image of the current frame will be saved. No raw data file (\*.ptu) is saved in test mode.

When performing a scan in measurement mode, it is possible to specify a number of frames and the measurement will stop exactly after the chosen number of frames. All frames are summed up and saved in the image. Should no number of frames be specified, the measurement will continue to sum up all frames until the user presses the “Stop” button. Single frames can be evaluated separately by using the multi-frame FLIM analysis.

There are several settings that can be used to optimize the measurement.

1. **Scan range:** Select the area you want to measure. There are different ways to do so:
  - By clicking on “Max Range”, the scan area is automatically set to the maximum. The exact value depends on the objective in use
  - After performing, for example, an overview scan, you can select the recorded image from the upper drop-down menu. You can also scroll through the captured images by clicking into the drop-down menu and using the arrow keys. Zoom into the region of the sample that should be measured by drawing a square with your mouse. When you now click on “Visible Range”, only the visible area will be used for the next measurement
  - After clicking the “Select range” button, you can draw a square around the area to be measured

- It is of course also possible to select the area you want to measure by typing the desired values in the “X”, “Y”, “dX”, and “dY” fields of the control panel (area number 5 in Fig.5.7). The origin of the coordinate system lies in the upper left corner of the image. The “X” and “Y” values define the upper left corner of the area to be scanned. The “dX” and “dY” values represent the width and height of the scan area. If the check-box “square” in area 6 of the control panel is ticked, input into the “dY” spin-box will be disabled and mirror the value of the “dX” spin box
2. **Best performance:** The green square in the center of the full range field is a 40 x 40  $\mu\text{m}$  scan area, where best performance for STED, PIE, and FCCS can be achieved. When measuring inside this 40 x 40  $\mu\text{m}$  field, the limitations due to lateral and axial color aberration effects of the objective are in most cases negligible for high quality (APO) objectives. The exact scan field size within the green square can be set in the FLIMbee hardware menu
  3. **Scan axis:** You can select here which kind of measurement you want to perform. If your system is equipped with a z-stage, you can choose between a normal x-y scan or one including the z-axis (y-z or x-z).  
x-y scans: By clicking on “Lock Z Axis”, the current value of the z position will be fixed, even when you select older images from the drop-down menu where different z-values have been used
  4. **Objective:** select the objective you are using for your measurement here
  5. **Scan area:** The exact position and size of your scan area is displayed here. The center of the full field of view is located at  $X = Y = Z = 0 \mu\text{m}$ . When selecting an area, X and Y values indicate the coordinates of the upper left corner, while dX and dY specify the size of the scan area in  $\mu\text{m}$
  6. **Scan parameters:** The scan parameters can be set here, including the number of pixels, shape of your image (square or non square), scanner movement (mono – or bidirectional scanning), and scan speed (dwell-time per pixel in  $\mu\text{s}$ )

After inserting all the parameters, the SymPhoTime 64 software automatically calculates the Pixel Size and the Frame Frequency

**Dwell-Time:** In order to select the dwell-time per pixel you can either type in the desired time or use the arrows next to the dwell-time field to adjust the displayed value in steps of 0.2  $\mu\text{s}$ . The software will automatically use the closest possible dwell-time for the selected value. In some cases when trying to adjust the displayed dwell-time by clicking on the arrow fields will result in it always jumping back to the previous value. This is due to the step size being too small for reaching the next valid dwell-time. Should this happen, you can click and hold down the arrow field until the desired dwell-time is reached. The shortest possible dwell-time depends on both image size and number of pixels.

**Bi-directional scanning:** The software tries to correct the offset between the lines scanned from left to right and the lines scanned from right to left. However, in most cases, there is a slight mismatch of a few pixels (most visible if horizontal stripes are present in your image). When clicking on the “More” button, the bi-directional offset can be adjusted to get rid of this mismatch. At very high scan speeds, the offset might be different in selected regions in the image. This artifact cannot be corrected for.

7. **Test and Measurement mode:**
  - (a) **Focusing in Test mode:** When clicking on the “Focusing” button a new window for fast focusing opens. This gives you the opportunity to quickly find a good measurement area or the correct z-focus of your sample. The number of pixels (original, 1/2, 1/4, 1/8, or custom) and dwell-time can be selected here. The less pixels used here, the faster you can scan during the focusing process.  
This mode is for focusing only and no data will be saved.
  - (b) **Number of frames:** No “Focusing” button is available in Measurement mode. However, it is possible to selected the number of frames that should be recorded for the image
8. **More:** by clicking on the “more...” button, it is possible to get detailed information about the current settings such as line frequency. It also provides information about the high speed range, which describes the area in which the fastest scan-speed can be used.

For bidirectional scanning, the offset between the two scan directions can be set here in order to avoid the formation of horizontal stripes in the image.

For more information about the SymPhoTime 64 software, please refer to the standard MicroTime 200 manual.

## 6. Technical Data

### 6.1 Specifications

#### Connectors Controller

AC Out Switched / Direct	IEC-60320/ C13 female (10A out)
AC In	IEC-60320/ C14 male (16A in max.)
Power Supply MOU / DEU1 / DEU 2	Binder 7pol. female
USB HUB 4x	USB-A
USB	USB-B
Laser Interlock LOOP1+2	Lemo.00 4pol.
Door Interlock	4mm Connector

#### Connectors MOU / DEU

CAN (1, 2, 3 )	SUB-D 9pol. male
Interlock	Lemo.00 4pol.
Detector NIM/ TTL	SMA female
USB	USB-B
Power	Binder 7pol. female
Video	BNC female (75Ω )
Fiber Coupling	FC-APC / FP-XPC

#### Connectors FLIMbee

Controll Cables	SubD 9pol. male
Connectors for Watercooling	Ø6x1 Tube

#### Connectors FLIMbee Controller / Power Supply

USB	USB-B
GALVO	SubD 9-pol. female
Analog Output / Input	BNC female
Clock Frame / Line / Pixel	BNC female
Laser Sync In+Out	SMA female
Laser Blanking 1+2	SMA female
Laser Enable 1+2	SMA female
Power	IEC-60320/ C14 male (10A in)

#### Connectors FLIMbee Power Supply

Power	IEC-60320/ C14 male (10A in)
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#### Operating Environment

Operating temperature	15 to 25°C
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#### Power Supply

Supply Line 100..240VAC 50/60Hz	max. 700VA
Fuse	B16A/ 250V

## Dimensions

Contoller.....	550 x 600 x 898mm
MicroscopeBody.....	310 x 235 x 140mm
MOU.....	770 x 506 x 283mm
DEU.....	255 x 506 x 283mm
FLIMbee.....	400 x 160 x 235mm
MOU with Microscope Body.....	~1250 x 765 x 750mm
MOU with MicroscopeBody and DEU.....	~1500 x 765 x 750mm
MOU with MicroscopeBody, DEU and FLIMbee.....	~1800 x 765 x 750mm

## Weight

Contoller.....	~ 40 kg
MicroscopeBody IX 73 / IX 83.....	~ 35 kg / ~ 47 kg
MOU.....	~ 35 kg
DEU.....	~ 15 kg
FLIMbee.....	~ 5 kg

## Retraction of old Devices

Waste electrical products must not be disposed of with household waste. This equipment should be taken to your local recycling centre for safe treatment.

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All information given here is reliable to the best of our knowledge. However, no responsibility is assumed for possible inaccuracies or omissions. Specifications and external appearance are subject to change without notice.

## 7. Abbreviations

AUX	auxiliary
BNC	British Naval Connector or Bayonet Nut Connector or Bayonet Neill Concelman
CCD	Charge-Coupled Device
CFD	Constant Fraction Discriminator
cps	Counts per Second (the natural intensity unit in photon counting applications)
DEU	Detector Extension Unit
e.g.	for example
FA	(MOFA) Master Oscillator Fiber Amplifier
FCS	Fluorescence Correlation Spectroscopy
FIFO	First In, First Out (buffer type)
Fig.	Figure
FLCS	Fluorescence Lifetime Correlation Spectroscopy
FLIM	Fluorescence Lifetime Imaging
FPGA	Field Programmable Gate Array
FRET	Förster Resonance Energy Transfer
FWHM	Full-Width at Half-Maximum
IEC	International Electrotechnical Commission
IO	Input-Output
IRF	Instrument Response Function
LCU	Laser Combining Unit
LDH	Laser Diode Head
LED	Light Emitting Diode
LSM	Laser Scanning Microscope
MCP	MicroChannel Plate (photomultiplier)
MCS	Multichannel Scaling
MCA	Multichannel Analyser
MOU	Main Optical Unit
MPD	Micro Photon Devices
NA	Numerical Aperture
NIM	Nuclear Instrument Module (electrical signal type)
OD	Optical Density
PC	Personal Computer
PCI	Peripheral Component Interconnect (industry standard bus)
PCIe	PCI Express (newer, faster version of PCI)
PDL	Picosecond Diode Laser Driver
PIE	Pulsed Interleaved Excitation
PiFoc	piezo-actuated Z-scanner
PMT	Photomultiplier Tube
RGB	Red-Green-Blue (color scheme)
ROI	Region of Interest
SMA	Sub-Miniature version A (a connector type)

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SMD	Single Molecule Detection
SOM-D	Sepia Oscillator Module with electronic Delay
SPAD	Single Photon Avalanche Diode
SPCM	Single Photon Counting Module
STED	Stimulated Emission Depletion
SYNC	Synchronization (signal)
TAC	Time-to-Amplitude Converter
TCSPC	Time-Correlated Single Photon Counting
TDA	Trigger Diode Assembly
TDC	Time-to-Digital Converter
TTL	Transistor-Transistor Logic (electrical signal type)
TTTR	Time-Tagged Time-Resolved
USB	Universal Serial Bus
UV	Ultraviolet light
VIS	Visible light
WD	Working Distance (of objective)



## 8. Support

If you observe any errors or bugs, please try to find a reproducible error situation. E-mail a detailed description of the problem and relevant circumstances to [support@picoquant.com](mailto:support@picoquant.com) or fill in PicoQuant's online support form at [support.picoquant.com](http://support.picoquant.com). Please attach the log file of the measurement. Your feedback will help us to improve the product and documentation.

In any case, we would like to offer you our complete support. Please do not hesitate to contact PicoQuant if you would like assistance with your system.

Of course, we also appreciate good news: If you have obtained exciting results with the MicroTime 200 or published scientific papers, we would also like to know! Please send us an e-mail containing the appropriate citation. Gain additional publicity! We maintain a database of publications mentioning PicoQuant devices and/or written by us. It can be found at our website at <http://www.picoquant.com/biblio.php> and it is a valuable source if you want to know which laboratories are using PicoQuant products or how broad is the field of various applications.

Thank you very much in advance for your kind co-operation!

### Retraction of old devices

Waste electrical products must not be disposed of with household waste. This equipment should be taken to your local recycling centre for safe treatment.



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