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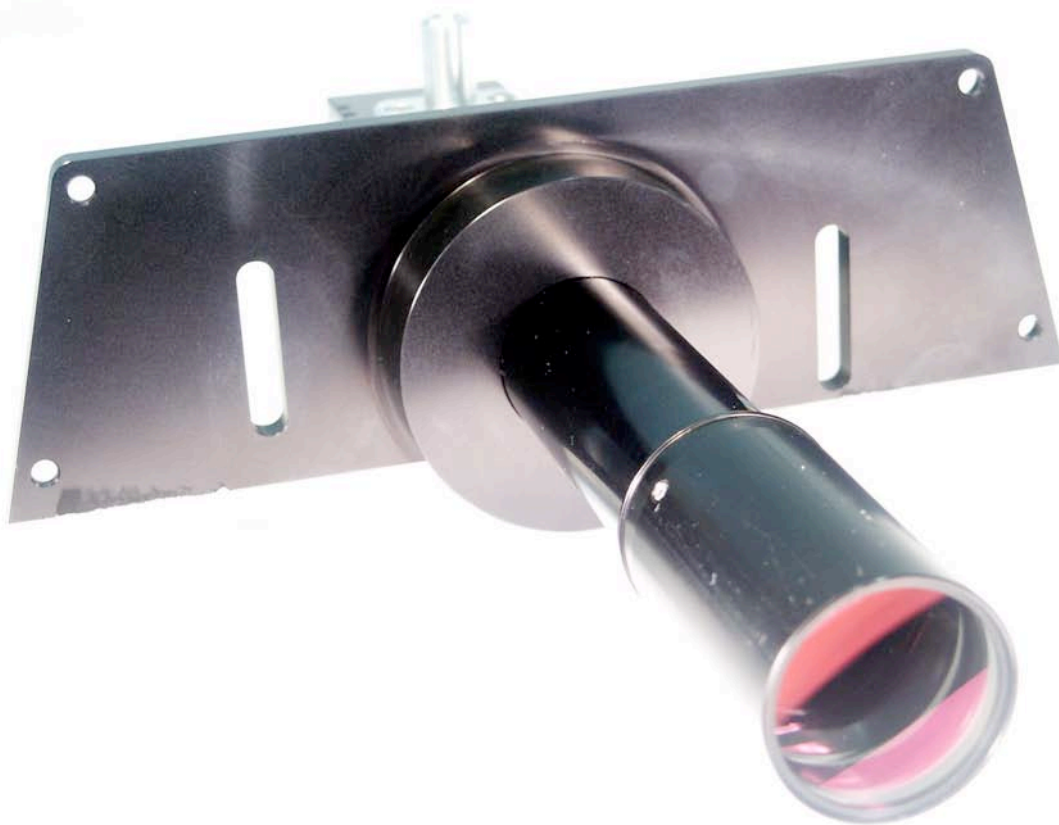
# TILL TIRF Quick Start

Features, Installation and Alignment of the TILL TIRF-condenser

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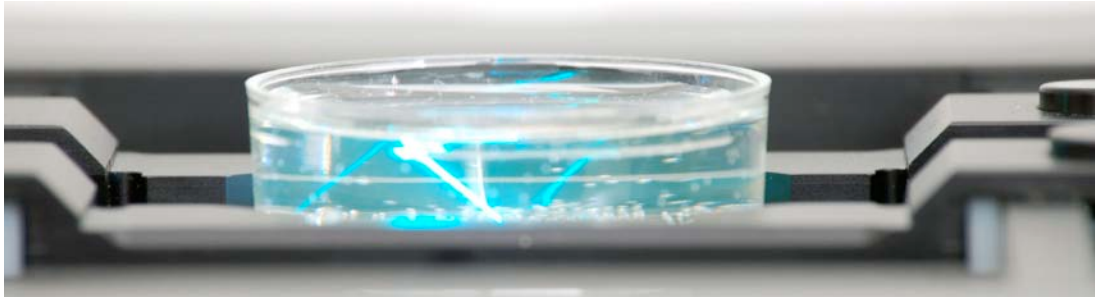


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# TIRF Microscopy

## TOTAL INTERNAL REFLECTION FLUORESCENCE MICROSCOPY

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### What does TIRF stand for?

TIRF stands for total internal reflectance fluorescence microscopy. It is a powerful method to observe and measure membrane associated processes in living cells, biological or chemical events at liquid-solid interfaces and even single molecule dynamics.

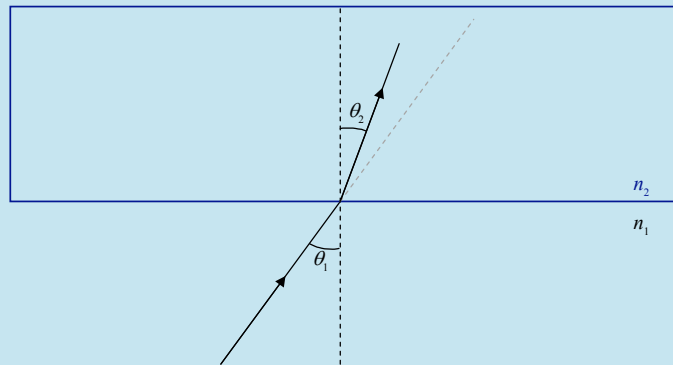
### What are the advantages of TILL's objective based TIRF condenser?

In objective based TIRF illumination of the probe is achieved through high numerical aperture objectives ( $NA \geq 1.45$ ). The optical design of the TILL-TIRF system provides the possibility to vary the TIR angle by almost  $8^\circ$  depending on the NA of the objective lens. The fluorescence signal is collected through the full numerical aperture (no central annulus). With this design, all advantages of an objective TIRF setup – full access to the specimen, easy handling and adjustment – are combined with the advantages of a classical prism TIRF setup – variable angle, high signal collection efficiency. The TILL-TIRF is designed to be fully integrated into the TILL-Imaging System. Within milliseconds, TIRF-, wide field fluorescence- and trans illumination can be alternated and fast time-lapse experiments can be performed.

### How does TIRF work?

Total reflectance occurs when light which travels from a medium with a low refractive index ( $n_1$ ) into a medium with a higher refractive index ( $n_2$ ) hits the denser medium under an angle  $\Theta$

### Box 1: Snell's Law



$$n_1 \sin \Theta_1 = n_2 \sin \Theta_2$$

$n_1$  is the refractive index of the first medium, the medium the light is traveling before it gets to the interface

$\Theta_1$  is the angle which makes the incident ray with the surface normal (dotted line)

$n_2$  is the refractive index of the second medium, the medium the light is traveling after it went through the interface

$\Theta_2$  is the angle which makes the refracted ray with the surface normal (dotted line)

In case that the angle of the refracted ray ( $\Theta_2$ ) reaches an angle  $90^\circ$  (for  $n_1 > n_2$ ), the **critical angle** has been achieved and the incident ray is completely reflected. To calculate the critical angle for TIRF applications we need the refractive index for  $n_1$ , which in microscopy is the refractive index of the cover slip ( $n = 1,518$ ) and  $n_2$  which we assume to be about  $n_2 = 1,33$ . Give the equation (for  $\Theta_2 = 90^\circ$ )

$$\theta = \sin^{-1} \frac{n_2}{n_1}$$

the critical angle is for most life science applications is about  $61^\circ$ .

which is higher than that which is given by [Snell's Law \(Box 1\)](#). The angle at which total reflection in life science applications usually occurs is about an incident angle of  $61^\circ$  (given  $n_1 = 1,518$ ;  $n_2 = 1,33$ ). To decrease this so-called critical angle you need to increase either  $n_1$  (e.g. using a sapphire cover slip) or decrease  $n_2$  (which in most cases is not influenceable).

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# Installation

## INSTALLATION OF TILL DUAL PORT TIRF CONDENSERS

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The following pictures show the installation of the TILL TIRF condensers in an ZEISS AxioVert 200/200M. The main steps shown here are the same for all supported microscope types (NIKON TE2000, OLYMPUS IX71/81 & 50/70 & ZEISS Axiovert 200/200M) but are mostly less invasive than the installation steps described next.

### Installation of the TIRF fluorescence filter sets



**Step 1:** Installation of the TIRF laser filter set.

To install the laser filter bring the installed xy-stage in its opposing position and unfasten the filter turret fixation screw.



**Step 2:** Installation of the TIRF laser filter set.

Gently pull the filter turret out of its position.



**Step 3:** Installation of the TIRF laser filter set.

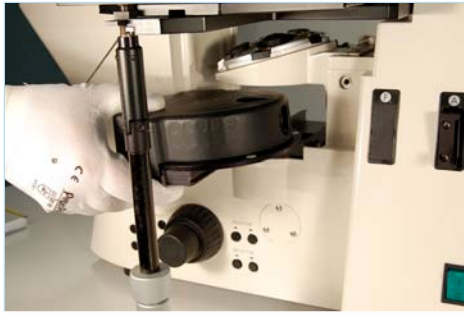
Pull the fixing bolts aside and remove the cover of the filter turret to install the filter set.



**Step 1:** Installation of the TIRF laser filter set.

Click the filter set in. To prevent the pollution of the filter please wear clean gloves. In case the filter surface had been polluted rinse with ethanol and clean with soft, not fuzz free paper.

**CAUTION: Please make sure that the dichroit filter reflects excitation to the objective lens (up) and the emission filter points down!**



**Step 1:** Installation of the TIRF laser filter set.

Insert the filter turret into the microscope and fasten the fixation. To prevent the installed filter sets from pollution make sure that the turret cover is installed the and properly closed to guarantee laser safety.

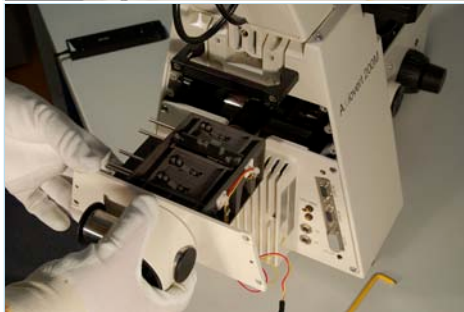
Please make sure that you have read and understood the installation guide

## Installation of the TIRF condenser



**Step 1:** Installation of the Axiovert 200/200M TIRF condenser

Before the start, if attached, remove lamp housing, power plug and data connections from the microscope (see microscope manual for details). Then remove the four screws which fixate the epi fluorescent unit of AxioVert 200/200M. Keep the screws, you will need them later on.



**Step 2:** Installation of the Axiovert 200/200M TIRF condenser

Gently pull out the epifluorescent unit. Disconnect attached cables and put them back into the open microscope body.



**Step 3:** Installation of the Axiovert 200/200M TIRF condenser

Insert the TIRF condenser and fasten the screw to ensure tight positioning of the TIRF condenser.



**Step 4:** Installation of the Axiovert 200/200M TIRF condenser

Remove the fibre protection from the laser fiber and insert it in to the FC coupler of the condenser.

**CAUTION:** Do not touch the fibres tip and prevent any other contact to ensure its function.



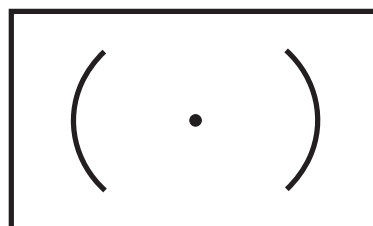
**Step 5:** Installation of the Axiovert 200/200M TIRF condenser

Insert the light guide of the Polychrome V and secure its position by fastening the position screw.

Please make always sure that exposed optical part like lenses or mirrors are not polluted in any sense. Due to the high power laser radiation pollution might not only cause a minor optical performance but may also lead to destruction of lenses or coatings. For detailed information how to clean polluted lenses and other optical part please refer to page XX [☞ Cleaning optical parts \(Box 3\)](#).

### Alignment of the TIRF condenser

To align the TIRF condenser you have to understand its principle. A central element of the TIRF condenser is the so called „mask“. The mask itself is a mirror containing a central pinhole for alignment, two lateral slits and a reflecting surface facing the microscope. Due to the size of the back aperture of the different microscope objective lenses the position and sizes of the slits vary with the magnification of the objective lens. Before you start with alignment, please make sure to install the alignment tool in the objective lens thread and make sure you have all needed tools at hand (see list next page).



*Figure 1: TIRF mask with central pinhole for alignment and lateral slits.*

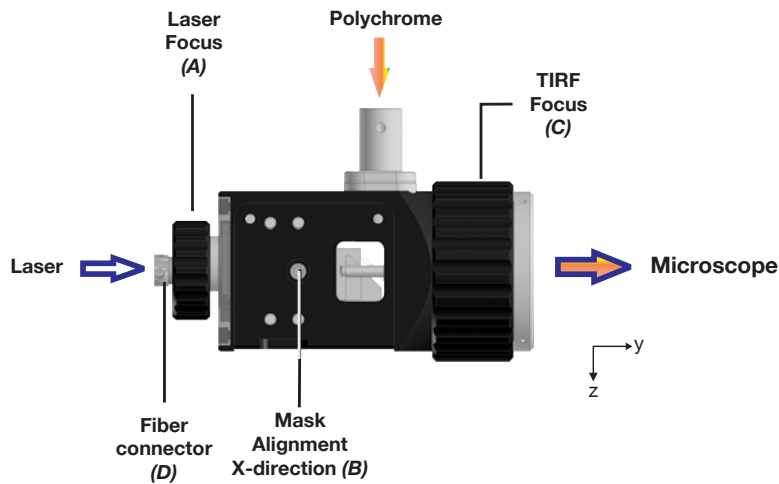
## Box 2: Safety Precautions

The TIRF condenser is a laser class 3B device. The device is fitted for a maximum laser power of 225 mW (continuous wave). While handling the TIRF condenser you should always keep the following points in mind:

- Please follow the laser safety instructions for the installed lasers (see also DIN EN 60825-1 /IEC Publication 825-1) and inform your local laser safety officer! TILL Photonics GmbH is not responsible for any damaged caused by laser radiation or improper use of the TIRF condenser. The user takes the responsibility to follow all laser safety issues appropriate for the installed lasers!
- Shut off the lasers whenever you remove the TIRF condenser from the microscope.
- Wear UV protective eye wear to protect your eyes and skin from hazardous radiation when a UV laser is installed.
- Do not look directly into the light emerging from the microscope objective or the light guide.
- Do not let moisture get into the TIRF condenser or any of its parts. A short-circuit exposing you to voltages or a shutter failure may result. If you accidentally spill any liquid on the TIRF condenser immediately disconnect it from power by unplugging the power supply. If any liquid gets inside the TIRF condenser, do not use it, but notify TILL Photonics GmbH for instructions. Organic solvents or other flammable liquids could be ignited if they come in contact with hot elements of the shutter.
- Never touch the optic elements inside the TIRF condenser or the tips of the light guide as these components cannot be cleaned other than by removing dust with gentle puffs of clean dry air. If the ends of the light guide are touched or contaminated in any way, they must be replaced in order to obtain the specified performance.
- Keep the TIRF condenser and the microscope as clean and as dust free as possible to maintain the maximum output power and beam stability, and to protect the lasers from premature failure.
- Handle light guide gently. It is quite springy and brittle. Bends must have a radius of no less than 15 cm (about 6 inches). Keep the protective covers on the ends of the light guide except when the ends are plugged in. Do not touch the ends of the light guide .

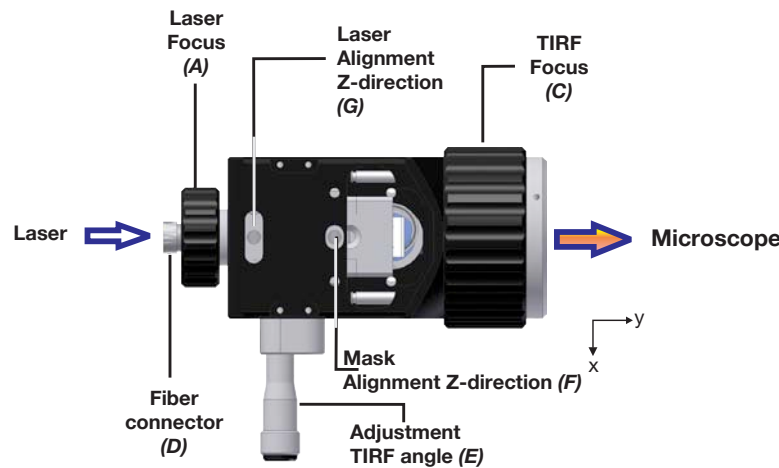
Before you start the alignment procedure please check that you have the following tools at hand:

- 100 nm FluoSpheres (Molecular Probes), with an excitation and emission wavelength according to your fluorescent filter set
- glass bottom petri dish
- Laser safety goggles
- a set of metric allen keys
- TILL TIRF alignment tool



**Figure 2: Side View of the TILL TIRF condenser.**

The dust protection has been removed in this view to reveal all screws necessary for aligning the mask. The screw **A** focusses the laser on to the mask, while **C** focusses the laser onto the back focal plane of the objective lens. By turning the alignment screw **B** (x-direction) in or out you change the x-position of the mask.



**Figure 3: Top View of the TILL TIRF condenser.**

The dust cover has been removed in this view to reveal all screws necessary for aligning the mask. By using the micrometer screw **E** you can easily fine tune the TIRF angle before or during your experiment (x-direction). The screws **G** and **F** are intended for adjustment of the laser position (z-direction) or the mask (z-direction) position and should only be used for initial alignment.



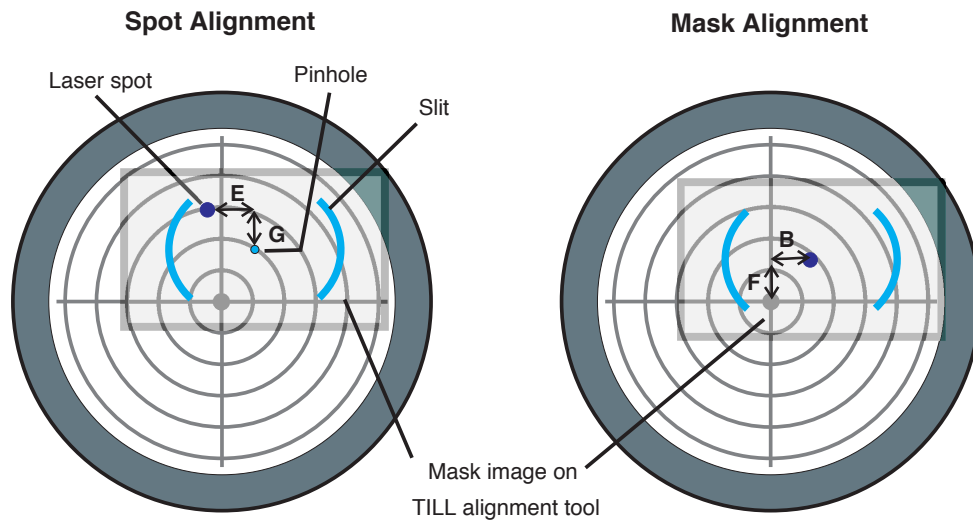
**Before you start aligning the condenser please make sure that you have read and understood the laser safety precautions. The following procedure includes steps in which laser radiation can be emitted and by that endanger the user.**

1. Focus to a probe

Dilute the FluoSphere stock solution (100 nm) 1:1000 in distilled water and fill it into the glass bottom petri dish. Turn the transillumination or the Polychrome on and focus onto the bottom of the petri dish. Install the TILL TIRF alignment tool in an empty objective lens thread and bring it in position, without changing the focal plane.

## 2. Aligning the mask

Turn on the laser and set it to minimal power output. Bring the TIRF alignment tool in position and open the shutter. You should see a picture of the mask including the two slits and the central pinhole as well as a bright spot indicating the actual laser position on the mask (see figure 1). Use the TIRF angle adjustment screw (**E**) and the z-alignment screw of the laser (**G**) to align the laser spot to the central pinhole of the mask. To center the mask with respect to the objective lens use the screws **F** and **B**.



**Figure 4: Alignment of laser spot and mask position.**

The figure shows the TILL alignment tool with projected mask image. To align the laser spot to the central pinhole of the mask use screws **E** and **G** (see also figure 2&3). To align the mask itself to the center of the objective lens use the screws **F** and **B** (see also figure 2&3).

## 3. Focus the laser on to the reflecting mask

To focus the laser beam on to the mask, bring the laser into its middle position, so that light passes through the central pinhole. Use the laser focus screw (**A**) to focus the laser beam to the mask. A maximal power throughput indicates a well focussed laser beam.

## 4. Focus the TIRF condenser to the back focal plane

To focus the TIRF condenser to the back focal plane of the objective lens, change back to your TIRF objective lens and open the shutter and bring the laser on to its middle position. Now focus to your probe using the z-drive of your microscope. In order to focus the TIRF condenser itself, you have to have a look at the laser spot at the ceiling. The TIRF condenser is focussed when you see a spot but **no** (or as less as possible) interference rings.

## 5. Fine tuning of the mask and laser position

soon to come

## 6. Testing your TIRF alignment

To test your TIRF conditions you need to a glass bottom petri dish, fluorescent beads and a fluorescent dye solution, which both can be excited using the installed laser. Dilute your fluorescent dye (for example fluorescein) to a final concentration of 1 mM (about 2 ml) and add 1-2  $\mu$ l of beads stock to the dye solution. Bring the probe on to your microscope and open the shutter and move the laser into the middle position. Now focus to the bottom of you petri dish using your TIRF objective. The picture you see right now should show a enormous background signal due to the dye in the solution. Now change the angle of the incident laser beam until the background disappears and the beads seem to drop in and out of your field of. Now you have reached the TIRF mode.

### Box 3: Cleaning optical parts

#### Cleaning utilities

Optical parts like lenses, mirrors and color filters are coated with so called thin layers. The intactness of these layers is crucial for the functionality and performance of the optical part. Since these layers are often only a view nanometers thick and their adhesion to the underlying substrate is low the microscopist has to take extra precautions not to damage the surface while cleaning it.

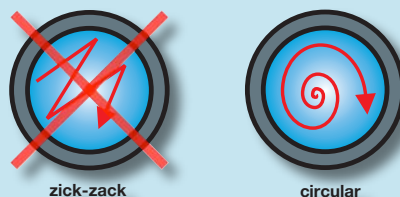
TILL Photonics recommends the following Thorlabs Inc. products for cleaning optical parts:

- Lint-Free kimwipes (part number: KW32)
- Lens tissues (part number: MC-5)
- Cotton tipped applicators (part number: CTA10)
- Canned air (part number: CA2 & CA3)
- Cleaning solution: Gasolin 85%, Isopropanol 15%

or

- Cleaning solution: 60% Aceton, 40% distilled water
- Distilled water

#### Cleaning optical surfaces



**While cleaning an optical surface always follow a circular motion (no zick-zack)**

##### 1. Dusty surfaces

Dust on optical parts leads in most cases to a blurred picture or shadows in the picture. To clean accessible parts of the microscope (tube lens or filter set) or the TIRF condensor from dust use canned air. Since canned air often contains oil or other lubricants never direct the air flow directly to the optical surface but use it in a tangential manner.

##### 2. Oily or fatty pollution

To remove fatty pollution use the described cleaning solution. Dip the cotton tipped applicator shortly into the solution and start cleaning in the middle of the surface (see figure above). Never use applicators with a plastic shaft, since solvated plastic softener will pollute the surface again.

##### 3. Pollution by water soluble substances

Pollution by salt or aqueous solutions are more harmful to mechanic parts than to optics, but occur more often. To prevent early corrosion of mechanical parts make sure that all surfaces which come in contact with salt containing solutions are cleaned on regular base using distilled water. Optical parts which are polluted by salt or other water soluble substances must be rinsed using distilled water. Using cotton tipped applicators will lead to digs and scratches on the surface and should therefore not be used.