Optimization of an optical setup for combined TIRF/confocal fluorescence microscopy

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This work deals with the construction of a microscope for combined total internal reflection fluorescence (TIRF) and confocal microscopy. It is especially designed for single-molecule fluorescence spectroscopy. The design of the microscope body is based on the miCube (Hohlbein lab, Wageningen University, NL). The excitation and detection pathways were adapted to allow both TIRF and confocal illumination as well as camera and point-detection for two color-channels to allow single-molecule Förster resonance transfer measurements.

1. Introduction

Fluorescence microscopes from common manufactures equipped with single-molecule detection capacities are very expensive (> 100 k€). Therefore, such microscopes have been designed as open source concept such as the miCube [1], the liteTIRF [2] and the smfBox [3] which achieve comparable good results for single-molecule FRET experiments [4]. Herein, we adapt the concept of the miCube for combined total internal reflection fluorescence (TIRF) and confocal microscopy with two color alternating laser excitation (ALEX) and two-color detection, i.e., detecting the donor and acceptor fluorescence, to enable single-molecule Förster resonance energy transfer (smFRET) measurements.

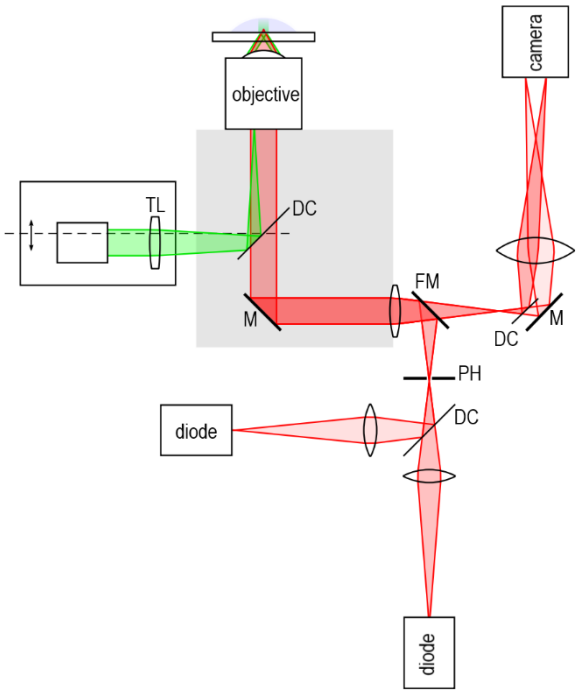

Figure 1. Excitation (green) and detection (red) beam path of the microscope. The excitation beam path includes the beam collimator (BC) with TIRF lens (TL) and *y*-travelling post (↔). To switch from TIRF to confocal mode remove TL and adjust the *y*-stage. The detection beam path includes a flipping mirror (FM) to switch between widefield camera-detection and confocal point-detection including a pinhole (PH) to reduce out-of-focus light. Both color-channels are separated via dichroic mirrors (DC).

Fluorescence microscopy is a widely used technique to study fluorescently labelled biomolecules. Due to the Stokes-shift only fluorescent light is detected while the excitation light is blocked form a dichroic mirror which separates the detection and excitation pathway.

For objective-based TIRF microscopy the parallel excitation beam is focused into the back focal plane of the high-numerical aperture (NA > 1.4) objective and is totally internal reflected at the interface between the sample (usually dissolved in water $n_{water} \approx 1{,}33$) and the cover slip (e.g. BK7 glass $n_{glass} = 1{,}51$). The emerging evanescent field excites the fluorophores only close to the surface due to its low penetration depth (< 100 nm). Therefore, this method is perfectly suited to suppress background fluorescence and to reach a high signal-to-background ratio. The fluorescent light is finally detected with an EMCCD or sCMOS camera.

In confocal microscopy the parallel excitation beam is focused with a high NA objective into the sample. The subsequently emitted fluorescent light is collected with the same objective. Thus, excitation and detection pathway overlay each other according to the confocal principle and build a “cuvette” with a volume in the fL range. Usually, out-of-focus light is blocked with a pinhole in the detection pathway to reduce the background signal, again, to reach a high signal-to-background ratio.

To switch the excitation pathway from TIRF to confocal and *vice versa*, the TIRF lens needs to be removed and the collimator stage is adjusted to reach a perfect symmetric filling of the back focal plan of the objective. The associated detection pathway can be chosen with a flipping or magnetic mirror post (compare Fig. 1).

2. Microscope design

The microscope setup is composed out of commercially available opto(mechanics) complemented with a home-build microscope body, sample holder and collimator stage. A complete list of all commercial parts can be found elsewhere [1]. The self-designed elements are partially based on miCube templates (cube and collimator). All newly designed elements are described herein.

The setup is split into four groups: the excitation beam path, the microscope body, the sample holder, and the detection beam path. The excitation path includes two 150 mW diode lasers (Oxxius) with 515 nm and 638 nm wavelength, respectively, both coupled into one optical single-mode fiber (Thorlabs). The reflective fiber collimator (Thorlabs) is set on a travelling stage and equipped with an iris aperture to allow under- and overfilling of the objective and a removeable (TIRF)lens to switch between TIRF and confocal excitation. The microscope body/cube design ensures that objective, fiber collimator and imaging tube lens are in correct distance to each other (Fig. 2). The central part of the cube is the dichroic mirror (515/638 DC, AHF analytics) which separates the excitation from the detection beam path.

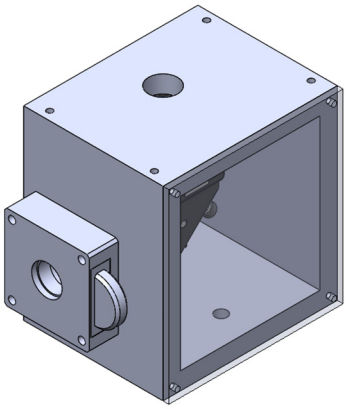

Figure 2: CAD model of the microscope body with the tube lens holder.

To keep costs low the sample holder is built out of commercially available elements with manual *x*-*y*-axis travelling stage and a motorized *z*-axis traveling stage (Fig. 3). The latter is used to easily adjust the focus position. The round insert of the sample holder is made of magnetic steel. This not only allows fast switching of samples but to easily keep the cover slip in place with magnets. A component overview is listed in Tab. 1.

Table 1. Component overview for the sample holder.

title	quantity	Price (excl. VAT)
x-*y*-linear axis	1	€ 793
rail connector	1	€ 35
66 mm rail	1	€ 44
rail slide	1	€ 67
motorized linear *z*-axis	1	€ 810
controller	1	€ 616
sample holder	1	home-build
total		€ 2365

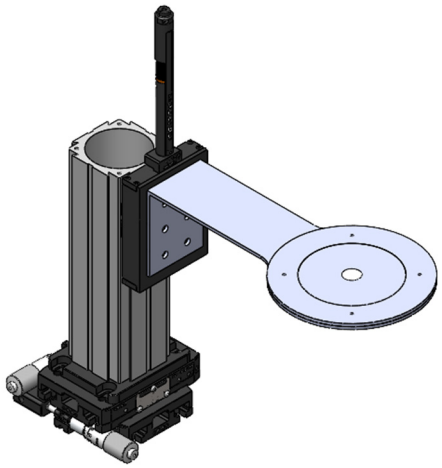

Figure 3: CAD-model of the sample holder with motorized *z*-stage, and manual *x*-*y*-stage.

The detection path consists out of two distinct beam paths for each detection mode. A flipping mirror or a magnetic mirror post allows easy and fast switching.

For TIRF microscopy the image is recorded via the prime BSI express camera from photometrics. The camera is equipped with a scientific CMOS sensor with a very high sensitivity and very low noise characteristics to allow high signal-to-noise ratio (SNR) mandatory for single molecule fluorescence detection. The field of view (FOV) is split into two-color channels via a DC mirror and imaged on the camera.

For confocal microscopy, a telecentric lens system allows easy adjustment of the pinhole. A DC mirror splits the beam into two-color channels. The fluorescence signal is measured with two avalanche photo diodes (APD, Picoquant).

Most of the parts except of the cube and the laser baseplate are printed out of ABS with a custom 3D printer. The cube and the laser baseplate were built out of aluminum due to the numerous tapped holes.

3. Conclusion

We have realized a combined TIRF- and confocal microscope for less than 50 k€. The most expensive parts are the CFI P-Apo 100x 1.45 oil immersion objective (Nikon), the laser (Oxxius), the APDs (Picoquant) and the sCMOS camera (Photometrics). All optical and optomechanical components cover only 20% of the budget. A good way to save money is the additive manufacturing of the cube etc. as the material costs are low and the CAD models are freely available.

The camera and the high NA objective are especially necessary for single-molecule microscopy. Additional three-axis piezo scanner for sample positioning and sample scanning will allow for confocal imaging capacities.

4. Acknowledgement

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5. Literature

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